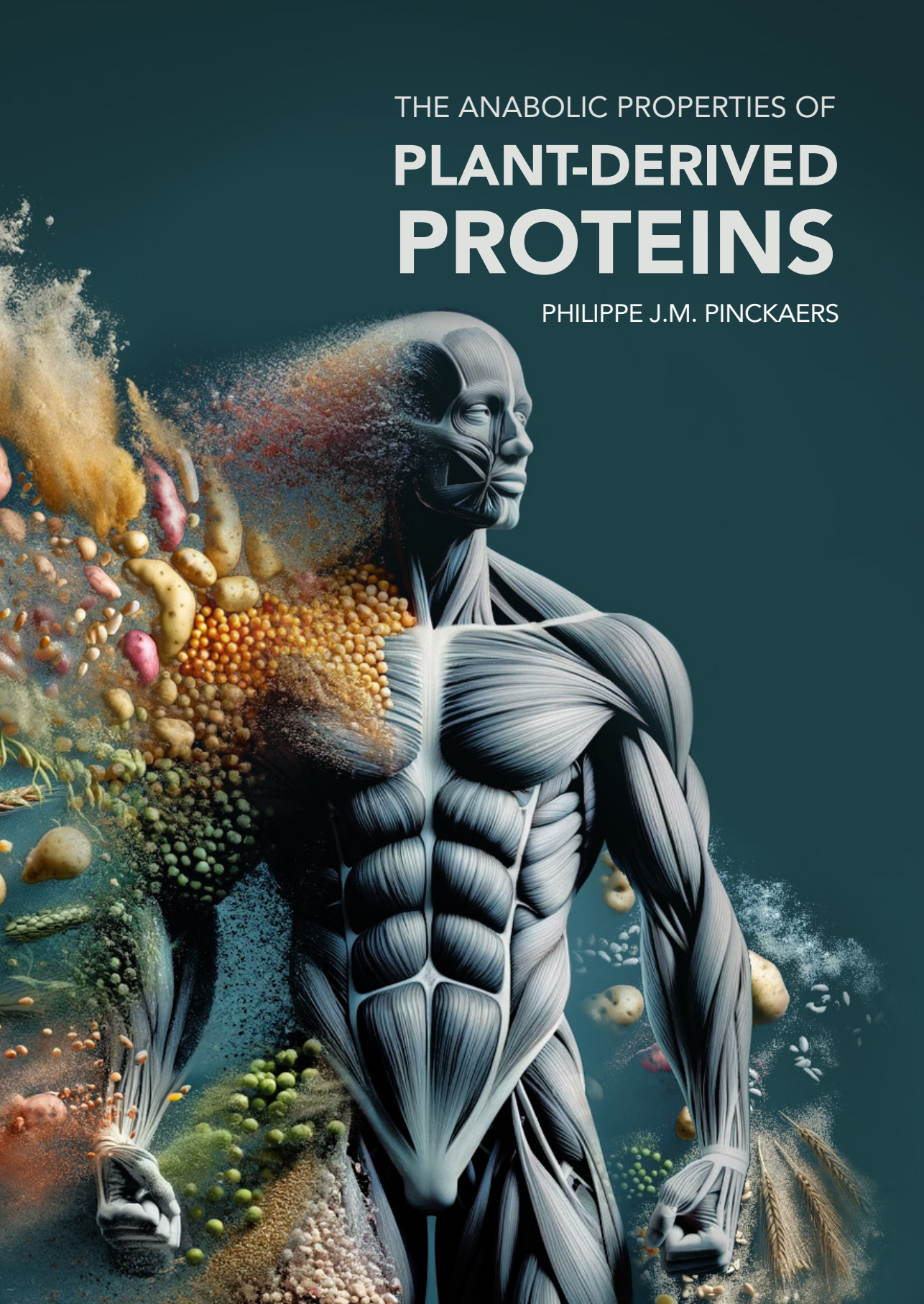
The anabolic properties of plant-derived proteins

THE ANABOLIC PROPERTIES OF

PLANT-DERIVED PROTEINS

PHILIPPE J.M. PINCKAERS



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The studies presented in this thesis were performed within the NUTRIM, School of Nutrition and Translational Research in Metabolism at Maastricht University. Part of the studies presented in this thesis were performed within the framework of TiFN.

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TABLE OF CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

INTRODUCTION

Consumption of sufficient amounts of dietary protein is fundamental to human growth and overall health [1, 2]. Dietary protein plays an important role in improving diet quality, promoting healthy aging [3], supporting bodyweight management [4], improving body composition [5, 6], regulating appetite [7-10], and maintaining/increasing skeletal muscle mass, in healthy [11-13], athletic [14], as well as clinical populations [15]. Therefore, protein consumption also plays an important role in managing current public health issues such as obesity and age-related skeletal muscle loss (i.e., sarcopenia). The regulation of skeletal muscle mass, is a continuously ongoing procces. Even when muscle mass is constant, muscle tissue is continuously turning over, in which the rates of muscle protein synthesis and breakdown are in balance [16, 17]. An imbalance between muscle protein synthesis and breakdown rates results in either a net gain (synthesis > breakdown) or net loss (synthesis < breakdown) in muscle mass and function. Protein ingestion directly increases muscle protein synthesis rates [18]. The dietary protein derived amino acids act as signalling molecules activating different anabolic pathways in muscle tissue. In addition, they provide building blocks for synthesising de novo muscle proteins. Not only protein ingestion, but also physical activity (i.e. exercise) represents an important anabolic stimulus. Physical activity directly stimulates muscle protein synthesis rates, an effect that can persist for up to 48 h after exercise cessation [16, 19, 20]. Besides stimulation of muscle protein synthesis rates, a single bout of exericse also stimulates muscle protein breakdown, albeit to a lesser extent [20]. More importantly, however, net muscle protein balance will remain negative during post-exercise recovery until protein is ingested [21, 22]. Dietary protein intake shortly after exercise, inhibits exercise induced muscle protein breakdown, and further augments the exercise induced increase in muscle protein sythesis rates, resulting in a (more) postive muscle protein balance [22-27]. In addition, performing exercise before protein intake sensitizes the muscle, allowing for a greater use of dietary protein–derived amino acids for de novo muscle protein synthesis [28]. Hence, protein ingestion forms a key component to support muscle tissue health, recovery (i.e. following injury) and reconditioning (i.e. in response to physical activity).

When ingesting protein, a variety of factors affect the muscle protein synthetic response (with or without exercise). The extent to which protein ingestion stimulates muscle protein synthesis rates depends on the amount [29-32], timing [33], and quality [34-36] of the ingested protein. Ingesting 20-30 g of protein, containing ~2.5 g leucine, has been reported to maximally stimulate muscle protein synthesis rates in humans [37, 38]. Timely protein intake ensures a sufficient supply of amino acids serving both as building blocks for muscle proteins, and signaling molecules promoting muscle protein synthesis. Furthermore, the distribution of protein ingested throughout the day can have a major impact on day-to day muscle protein turnover [33]. An even distribution of total protein intake over the main meals results in a more effective stimulation of muscle protein synthesis over the day compared with an unbalanced protein intake distribution [39]. Besides the amount and timing of protein intake, the quality of the ingested protein has been suggested to also represent an important determinant for the stimulation of muscle protein synthesis. Protein quality is determined by the amino

acid composition [40-42], with leucine content being of particular relevance [43-48], and the protein digestion and amino acid absorption kinetics [49]. As these characteristics are unique for each protein and protein source, the muscle protein synthetic response may differ substantially following ingestion of different proteins [40, 42, 50-52]. Over the past two decades, most research on the impact of protein ingestion on the muscle protein synthetic response in humans has been focussed on post-prandial protein handling following the ingestion of animal-derived proteins (i.e. whey, casein, milk, meat). However, a large part (~50 %) of our dietary protein consumption is of plant-derived origin [53]. This thesis provides a literature review on the current insights on the capacity of plant-derived proteins to stimulate muscle protein synthesis, and presents a series of studies that compare the anabolic repsonse following the ingestion of various plant- *vs* animal-derived protein sources.

With the global population being projected to reach approximately 9.6 billion by 2050, the production of sufficient amounts of conventional animal-derived, protein-dense foods to meet global dietary demands, may no longer be desired nor feasible [54]. From a global sustainablity staindpoint, plant-derived foods are proposed to be more sustainable compared with animal-derived foods [55, 56]. The production of plant-derived foods is suggested to require less water, land, and energy, posing less environmental burden and lower financial cost of food production [56]. Hence, there is an increasing clinical and consumer market interest towards the use of plant-derived proteins to meet the growing global protein demands. Despite the potential envionmental advantages of transitioning towards a more plant-derived protein diet, there are also concerns regarding the quality of plant-derived proteins. In comparison to animal-derived proteins, plant-derived proteins often contain an insufficient amount of one or more essential amino acids, with leucine, lysine and methionine in particular [57]. Deficiencies of particular amino acids in plant-derived protein could limit their capacity to stimulate post-prandial muscle protein synthesis rates. Furthermore, it has been suggested that protein digestion and absorption kinetics may also differ between animal- and plant-derived protein, with less protein derived amino acids becoming available for muscle protein synthesis following the ingestion of plant- compared with animal-derived proteins [58, 59]. To date, merely a handful studies have compared the anabolic potential of plant- *vs* animal-derived proteins [36, 40, 42, 51, 60]. Furthermore, the available studies report equivocal results and are limited to soy and wheat protein ingestion [36, 40, 42, 51, 60]. Given the large variability in plant protein sources, it is essential to evaluate the anabolic properties of a greater variety of plant-derived proteins *in vivo* in humans.

THESIS OUTLINE

This thesis describes a series of studies comparing the acute anabolic properties of various plant-derived *vs* conventional animal-derived protein sources in young, recreationally active, individuals, both at rest, and during post-exercise recovery. To start, Chapter 2 provides an overview on factors determining protein quality, and addresses potential challenges, limitations, but also potential solutions to consider when animal-derived proteins are replaced,

in part or entirely, by plant-derived proteins. In Chapter 3 the muscle protein synthetic response following the ingestion of pea-derived is compared with milk-derived protein. Chapter 4 compares the anabolic properties of wheat protein and a blend of wheat+milk protein with milk protein. Similarly, Chapter 5 compares the anabolic properties of corn protein and a blend of corn+milk protein with milk protein. Chapter 6 assesses the muscle protein synthetic response following ingestion of a plant-derived protein blend with milk protein. Chapter 7 assesses the anabolic potential of potato derived protein in comparison to milk protein both at rest, as well as during recovery from a single exercise session. Subsequently, in Chapter 8 the muscle protein synthetic response is determined following ingestion of a plant-based meat substitute and compared with the ingestion of an equivalent amount of chicken breast filet. Finally, the general discussion (Chapter 9) addresses how the current findings can be translated to general dietary practice, and will provide some directions for future nutrition research.

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CHAPTER 2

THE ANABOLIC RESPONSE TO PLANT-BASED PROTEIN INGESTION

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ABSTRACT

There is a global trend of an increased interest in plant-based diets. This includes an increase in the consumption of plant-based proteins at the expense of animal-based proteins. Plant-derived proteins are now also frequently applied in sports nutrition. So far, we have learned that the ingestion of plant-derived proteins, such as soy and wheat protein, result in lower post-prandial muscle protein synthesis responses when compared with the ingestion of an equivalent amount of animal-based protein. The lesser anabolic properties of plant-based versus animal-derived proteins may be attributed to differences in their protein digestion and amino acid absorption kinetics, as well as to differences in amino acid composition between these protein sources. Most plant-based proteins have a low essential amino acid content and are often deficient in one or more specific amino acids, such as lysine and methionine. However, there are large differences in amino acid composition between various plant-derived proteins or plant-based protein sources. So far, only few studies have directly compared the muscle protein synthetic response following the ingestion of a plant-derived protein versus a high(er) quality animal-derived protein. The proposed lower anabolic properties of plant- versus animal-derived proteins may be compensated for by 1) consuming a greater amount of the plant-derived protein or plant-based protein source to compensate for the lesser quality; 2) using specific blends of plant-based proteins to create a more balanced amino acid profile; 3) fortifying the plant-based protein (source) with the specific free amino acid(s) that is (are) deficient. Clinical studies are warranted to assess the anabolic properties of the various plant-derived proteins and their protein sources in vivo in humans and to identify the factors that may or may not compromise the capacity to stimulate post-prandial muscle protein synthesis rates. Such work is needed to determine whether the transition towards a more plant-based diet is accompanied by a transition towards greater dietary protein intake requirements.

1 INTRODUCTION

A single exercise session increases muscle protein synthesis rates, and to a lesser extent, muscle protein breakdown rates [1]. However, net muscle protein balance does not become positive unless exogenous amino acids are provided [2]. Dietary protein ingestion increases muscle protein synthesis rates at rest [3-5] and further increases muscle protein synthesis rates during recovery from exercise. Previous work has shown that besides the amount of protein [6-9], the digestion and absorption kinetics [10] and amino acid composition of a protein (source) [11, 12] largely determine the muscle protein synthetic response to feeding. The muscle protein synthetic response to protein ingestion can, therefore, vary substantially between different dietary protein sources [11-15]. The differential muscle protein synthetic response to feeding is largely dependent on the post-prandial rise in plasma essential amino acid concentrations [5], with plasma leucine concentrations being of particular importance [16-22]. The post-prandial rise in circulating amino acids and the subsequent increase in muscle protein synthesis rate is regulated on various levels, ranging from dietary protein digestion, amino acid absorption, splanchnic amino acid sequestration, post-prandial tissue perfusion, uptake of amino acids by the muscle, and the activation of the muscle protein synthetic machinery [4, 23]. To date, most studies have focused on assessing the post-prandial muscle protein synthetic response to dairy protein [13, 15, 19, 24-29] and meat [8, 30-32] ingestion. The substantial increase in muscle protein synthesis rates observed following ingestion of these proteins or protein sources has been attributed to the rapid post-prandial rise in circulating plasma essential amino acid concentrations.

With the global population projected to reach approximately 9.6 billion by 2050, the production of sufficient amounts of conventional animal-based, protein-dense foods to meet global dietary protein demands may no longer be desired or feasible. Affluent

KEY POINTS

It has been suggested that the muscle protein synthetic response to the ingestion of a single bolus of plant-derived protein is less robust when compared with the response following ingestion of an equivalent amount of animal-derived protein. However, this comparison remains limited to a few plant-derived proteins.

Most plant-derived proteins have a lower essential amino acid content when compared to animal-derived proteins, and many are deficient in specific amino acids such as lysine or methionine. However, there is considerable variation in amino acid composition between various plant-based proteins.

The muscle protein synthetic response to plant-derived protein ingestion may be improved by increasing the amount of protein ingested. In addition, it has been speculated that consuming blends of different plant-derived proteins or consuming plant-derived proteins fortified with the deficient (free) amino acid(s) increases the post-prandial muscle protein synthetic response.

Western societies show a strong trend in the transition towards a more plant-based diet [33]. This includes an increase in the consumption of plant-based proteins at the expense of animal-based proteins. Although the current market already offers a wide selection of plant-derived proteins and plant-based protein sources, there is a paucity of studies that have assessed the bio-availability and anabolic properties of plant-based proteins [11, 12, 14, 34-36]. Some [12, 14, 34], but not all [11, 35, 36] of these studies show that the ingestion of plant-derived proteins, such as soy and wheat protein, result in a lower muscle protein synthetic response when compared with the ingestion of an equivalent amount of animal-derived protein. Consequently, plant-based proteins are typically considered to have lesser anabolic properties. However, this concept is based on a limited number of comparisons and may not translate to all plant-based protein sources. The proposed lesser anabolic properties of plant- versus animal-based proteins have been attributed to differences in their protein digestion and amino acid absorption kinetics, as well as to differences in amino acid composition between these proteins. Previously, we reported substantial differences in amino acid composition between various plant-based protein sources [37]. Although the amino acid composition can be quite variable between different plant-based proteins, most plant-based proteins are relatively low in essential amino acid content and are often deficient in one or more specific amino acid, such as leucine, lysine, and/or methionine [37]. So far, only a few studies have directly compared the muscle protein synthetic response following the ingestion of a plant-derived protein versus a high(er) quality animal-derived protein [11, 12, 14, 34-36]. Furthermore, even less is known about the different strategies that can be applied to improve the anabolic properties of plant-based proteins. The purpose of this review is to provide an updated overview on the bio-availability and anabolic properties of plant-based proteins in vivo in humans. We will discuss different strategies that can be applied to compensate for the lesser quality of plant-based proteins and, as such, to increase post-prandial muscle protein synthesis rates. We will discuss the need to advance nutrition research by extending studies from merely comparing post-prandial muscle protein synthesis rates following the ingestion of plant- versus animal derived protein isolates or concentrates, towards assessing the impact of ingesting whole-foods and mixed meals on post-prandial muscle protein synthesis. Finally, we will discuss the current beliefs regarding the use of plant-based proteins in the field of sports nutrition, and provide examples of other alternative protein sources that can be applied to support muscle conditioning in the future.

2 PROTEIN DIGESTION AND AMINO ACID ABSORPTION

Following food ingestion, dietary protein needs to be digested and absorbed for the amino acids to become available in the circulation where they can modulate muscle tissue protein synthesis and breakdown rates. Protein digestion occurs in the mouth, stomach, and small intestine, where protein undergoes mechanical and chemical breakdown into smaller constituents [38]. When amino acids are subsequently taken up from the gastrointestinal lumen they are considered to be absorbed. A substantial part of the absorbed amino acids

will be retained and metabolised in the splanchnic region, but the majority will be released in the circulation after which they become available for uptake into peripheral tissues. The quantitative assessment of protein digestibility, absorbability, splanchnic extraction, and amino acid release in the circulation is complex and only few studies have tried to quantify post-prandial protein handling *in vivo* in humans [4]. Studies have reported substantial differences in protein digestion and amino absorption kinetics following ingestion of different proteins and protein sources. In general, plant-based whole foods have a lower absorbability when compared to animal-based whole foods. For example, recent data in humans have shown that ~85-95% of the protein in egg whites, whole eggs, and chicken is absorbed, compared to only ~50-75% of the protein in chick peas, mung beans, and yellow peas [39, 40]. The lower absorbability of plant-based proteins may be attributed to anti-nutritional factors in plant-based protein sources, such as fibre and polyphenolic tannins [41]. This seems to be supported by the observation that dehulling mung beans increases their protein absorbability by ~10% [42]. When a plant-based protein is extracted and purified from anti-nutritional factors to produce a plant-derived protein isolate or concentrate the subsequent protein absorbability typically reaches similar levels as those observed for conventional animal-based protein sources [43]. This implies that the low absorbability of plant-based protein sources is not an inherent property of a plant-based protein *per se*, but simply a result of the whole-food matrix of the protein source.

Protein absorbability has long been recognised as a crucial component of the nutritional quality of a protein source [44]. Currently, The Food and Agriculture Organization of the United Nations (FAO) and World Health Organization (WHO) recommend the Digestible Indispensable Amino Acid Score (DIAAS) to quantify dietary protein quality [45]. The DIAAS of a protein is based on its capacity to meet the requirements of each indispensable amino acid, which is reflected by the amino acid profile and absorbability of each individual indispensable amino acid. However, a limitation of the DIAAS score is that it only accounts for overall protein absorbability (cumulative absorption) and not for amino acid absorption kinetics (the rate at which amino acids are being absorbed). Several studies suggest that a more rapid rate of amino acid absorption forms an independent factor that modulates the muscle protein synthetic response to feeding [15, 46-48], although such association is not always observed [49, 50]. There are few data available on the amino acid absorption kinetics following the ingestion of plant-based protein sources or plant-derived protein isolates or concentrates. With regards to the post-prandial rise in circulating amino acid concentrations as a proxy for protein digestion and amino acid absorption, data seem to suggest that plant-derived protein isolates or concentrates are rapidly digestible [11, 14, 36, 51, 52] and do not seem to differ substantially from most animal-derived proteins or protein sources. It is more than likely that the anti-nutritional factors in plant-based protein sources (whole foods) not only compromise overall protein absorbability, but also attenuate the post-prandial rise in amino acid absorption rates. Because of the apparent differences in protein absorbability and protein digestion and amino acid absorption kinetics, we need to be careful when referring to plant-based proteins as either plant-based protein sources or rather as plant-derived protein isolates or concentrates.

3 AMINO ACID COMPOSITION OF PROTEIN

Following dietary protein digestion and amino acid absorption, a large proportion of the dietary protein derived amino acids is released in the circulation. The post-prandial increase in plasma amino acid concentration activates the protein synthetic machinery in skeletal muscle tissue while also providing the necessary precursors to allow muscle protein synthesis rates to increase [5, 53, 54]. The essential amino acids are considered to be mainly responsible for the post-prandial stimulation of muscle protein synthesis [54]. In agreement, a dose-dependent relationship has been reported between the amount of essential amino acids ingested and the post-prandial muscle protein synthetic response [55]. Consequently, proteins with high(er) essential amino acid contents are generally considered high(er) quality proteins and are also more likely to (strongly) stimulate post-prandial muscle protein synthesis. Previously we have shown that the essential amino acid contents of plant-based proteins are generally lower when compared with animal-derived proteins [37, 56]. In the current review, we included an extended overview of the amino acid composition of a wide variety of protein (sources) we have analysed (**Figure 2.1A**). However, there are also plant-based proteins (such as: soy, brown rice, canola, pea, corn and potato protein) that have relatively high essential amino acid contents, meeting the requirements recommended by the World Health Organization (WHO) / Food and Agriculture Organization (FAO) / United Nations University (UNU) [57]. In fact, the essential amino acid contents of canola (29%), pea (30%), corn (32%) and potato (37%) derived protein are comparable or even greater than casein (34%) or egg (32%) protein [37]. Therefore, certain plant-based proteins could, in theory, provide sufficient essential amino acids to allow a robust post-prandial increase in skeletal muscle protein synthesis rate.

Among all of the essential amino acids, leucine represents the amino acid with the strongest anabolic properties. Leucine is sensed by Sestrin2, which promotes translocation of mammalian target of rapamycin complex 1 (mTORC1) to the lysosome membrane where it gets activated, resulting in the activation of the downstream anabolic signalling pathways that control muscle tissue protein synthesis [58-60]. The current leucine requirement within a given protein source is set at 5.9% by the WHO/FAO/UNU [57]. Whereas plant-based proteins like hemp (5.1% leucine) and lupin (5.2%) fall short, other proteins like oat (5.9%), spirulina (6.0%) and wheat (6.1%) protein provide close to the recommended leucine content. Moreover, plant-based proteins like soy (6.9%), canola (6.9%), pea (7.2%), brown rice (7.4%), potato (8.3%) and corn (13.5%) protein have leucine contents that exceed the recommended requirements. The leucine content of potato protein (8.3%) is even higher when compared with casein (8.0%) or egg (7.0%) protein. Furthermore, the leucine content of corn protein (13.5%) is even higher than whey protein (11.0%), the latter of which is typically regarded as the protein with the highest leucine content and the strongest anabolic potential among the animal-derived proteins (**Figure 2.1B**).

Besides having a relatively low essential amino acid content (i.e. low leucine content), many plant-based proteins are deficient in one or more specific amino acid. Plant-based proteins are often particularly low in lysine and/or methionine content (ranging from 1.4 - 6% and 0.2

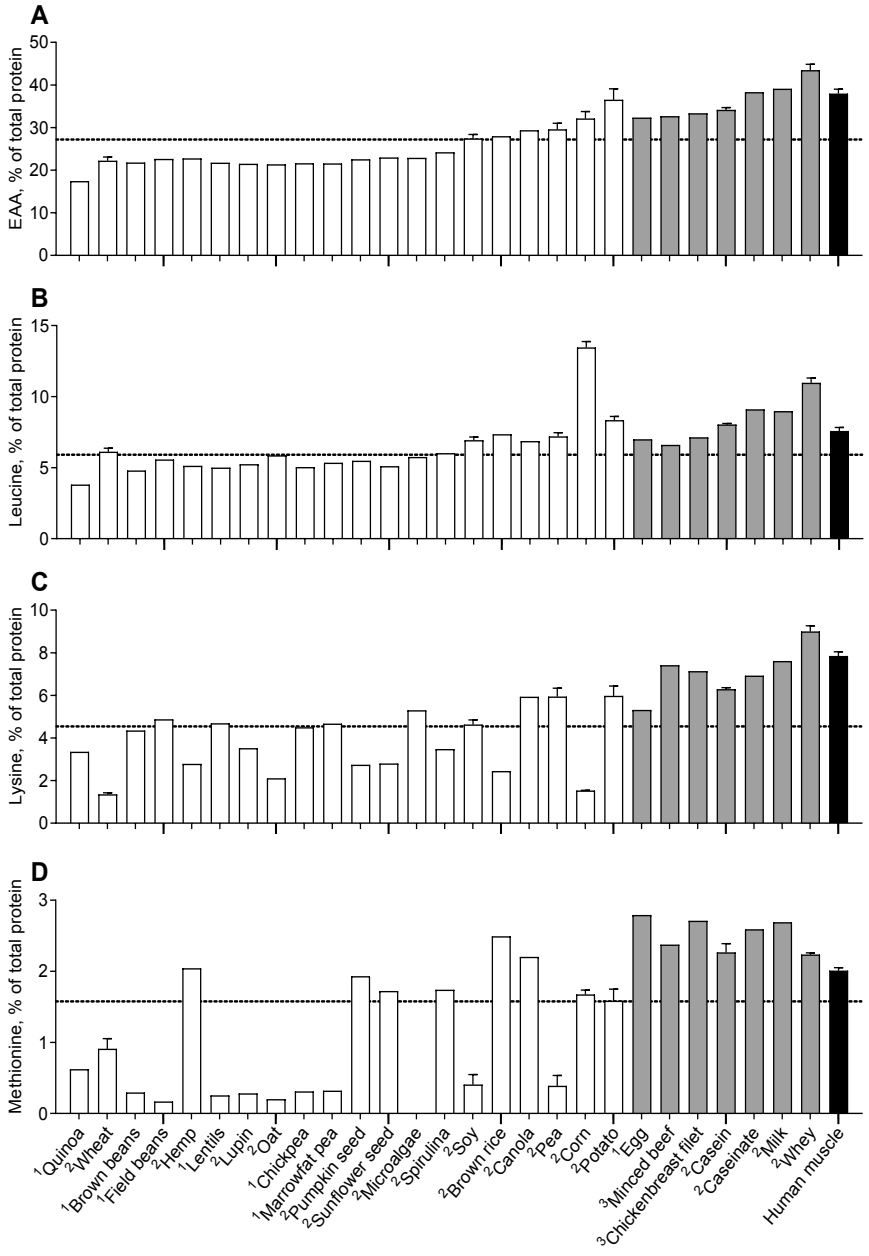

FIGURE 2.1 Essential amino acid (EAA, Panel A), leucine (Panel B), lysine (Panel C), and methionine (Panel D) contents (expressed as % of total protein) of various dietary protein sources and human skeletal muscle protein. White bars represent plant-based protein sources, grey bars represent animal-derived protein sources, and the black bar represents human skeletal muscle protein. Dashed line represents the amino acid requirements for adults (WHO/FAO/UNU Expert Consultation 2007 [57]). Note: EAA is the sum of histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, and valine, as tryptophan was not measured. Values obtained from multiple products are expressed as mean (± SEM). This figure represents an extension from data previously presented by Gorissen et al. 2018 [37], assessed using the same method. 1 Flour, 2 Protein concentrate/isolate, 3 Freeze dried raw product.

- 2.5%, respectively) when compared with animal-based proteins (ranging from 5.3 - 9.0% and 2.2 - 2.8%, respectively; **Figure 2.1C** and **2.1D**). The lysine content of wheat (1.4%), corn (1.5%), oat (2.1%), brown rice (2.4%), pumpkin seeds (2.7%), sunflower seeds (2.8%), hemp (2.8%), quinoa (3.3%), spirulina (3.5%), and lupin (3.5%) protein are well below the WHO/FAO/UNU requirements (4.5%) and substantially lower when compared with soy (4.6%), canola (5.9%), pea (5.9%), and potato (6.0%) protein (**Figure 2.1C**). A considerable number of plant-based proteins also fall short for methionine requirements (1.6%), with oat (0.2%), field bean (0.2%), brown bean (0.3%), lentil (0.3%), chickpea (0.3%), marrowfat pea (0.3%), lupin (0.3%), pea (0.4%), soy (0.4%), quinoa (0.6%), and wheat (0.9%) protein providing much less methionine. In contrast, other plant-based proteins (e.g. potato (1.6%), corn (1.7%), spirulina (1.7%), sunflower seed (1.7%), pumpkin seed (1.9%), hemp (2.0%), canola (2.2%), and brown rice (2.5%) protein) tend to meet the methionine content requirements (**Figure 2.1D**). Clearly, there is quite some variability in amino acid composition between the many different plant-based proteins and plant-based protein sources.

Only a handful of studies have directly compared post-prandial muscle protein synthesis rates following ingestion of plant- versus animal derived proteins [11, 12, 14, 34-36]. Ingestion of soy protein has been shown to be less effective in stimulating post-prandial muscle protein synthesis rates when compared with the ingestion of an equivalent amount of whey protein in both young and older adults at rest and during recovery from exercise [11, 12, 34], but more effective than casein protein [11]. Furthermore, Yang *et al.* [12] showed that ingesting a greater amount (40 vs 20 g) of soy protein did not compensate for the lesser muscle protein synthetic response when compared with the ingestion of 20 g whey protein isolate. We observed no significant post-prandial increase in muscle protein synthesis rates following the ingestion of 35 g wheat protein hydrolysate in a group of healthy, older men [14]. When we increased the amount of wheat protein hydrolysate to 60 g, thereby providing the same amount of leucine as provided in 35 g whey protein, we observed a robust increase in muscle protein synthesis rates. Clearly, these data seem to support the hypothesis that differences in amino acid composition can be, at least partly, compensated for by ingesting greater amounts of the specific protein source.

More recently, we observed no differences in post-prandial muscle protein synthesis rates following the ingestion of 30 g wheat protein hydrolysate or the same amount of milk protein concentrate [36]. In contrast to the earlier work in our group, this study was performed in young, recreationally active adults. The greater sensitivity of skeletal muscle tissue to the anabolic properties of amino acids due to the higher habitual activity level in younger, more active, adults [65, 66] may have been responsible for the absence of any measurable differences in the post-prandial muscle protein synthetic response to the ingestion of 30 g wheat versus milk derived protein. Clearly, we need to understand that differences in the anabolic responses to the ingestion of plant versus animal-based protein sources will also depend on the amount of protein provided and the specific population in which the comparison is made.

In short, the amino acid composition of plant-based protein sources can be highly variable. Therefore, more studies are warranted to assess the anabolic properties of various plant- and animal-derived proteins and protein sources beyond the few comparisons that are currently available (soy and wheat protein). Furthermore, it should be noted that the outcome of these comparisons will likely differ depending on the amount of protein ingested and the population and setting in which the comparisons are being made.

4 IMPROVING THE ANABOLIC PROPERTIES OF PLANT-BASED PROTEINS

As discussed previously, the proposed lesser anabolic properties of plant-based versus animal-based proteins may be attributed to differences in protein absorbability, protein digestion and amino acid absorption kinetics, and/or amino acid composition of the proteins. There are various nutritional strategies that may be applied to improve the anabolic properties of plant-based proteins depending on the factor(s) responsible for the proposed lower anabolic capacity (**Figure 2.2**).

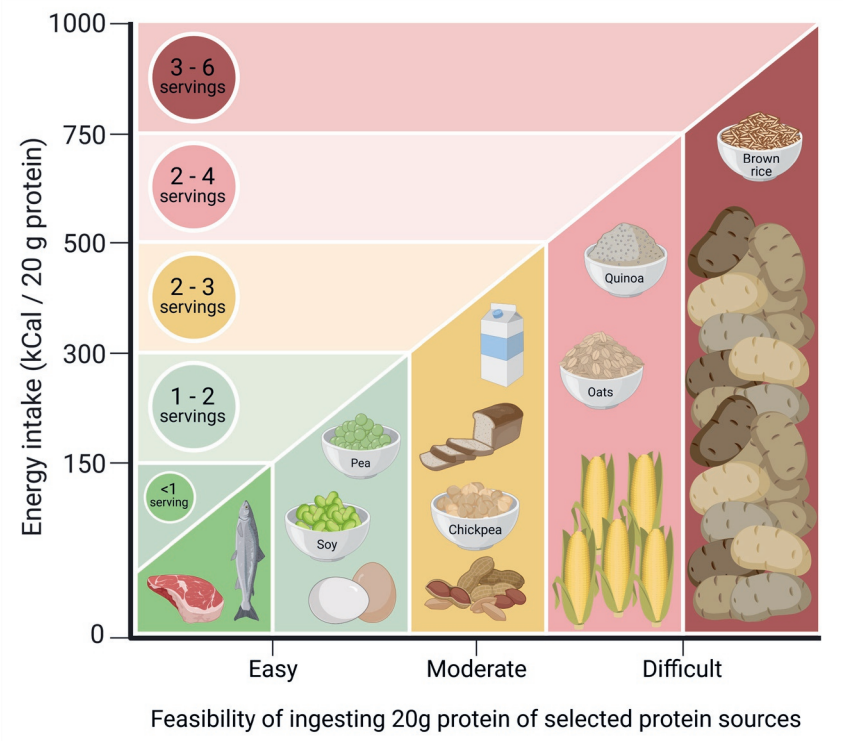

FIGURE 2.2 Categorical representation of the feasibility to consume 20 g protein provided by ingesting the whole food source (x-axis), with the amount of food that needs to be consumed expressed as servings with the concomitant energy intake equivalent (y-axis). Serving sizes: meat/salmon: ~100 g, egg: ~120 g (2 eggs), soy: ~100 g, pea: ~150 g, chickpea: ~150 g, peanut: ~50 g, bread (wheat): ~70 g (2 slices), milk: ~200 mL, corn: ~150 g, oats ~40 g (raw), Quinoa: ~75 g (raw), brown rice: ~75 g (raw), potato: 175 g.

The absorbability of a plant-based protein source is often compromised by the presence of anti-nutritional factors in plant-based protein sources, such as fibre and polyphenolic tannins [41]. Processing of whole-foods can strongly increase the absorbability of intrinsic protein. Dehulling of beans prior to consumption has been shown to represent an effective means to increase the capacity to absorb the intrinsic protein [42]. Extraction of protein and purification from anti-nutritional factors to produce a plant-derived protein isolate or concentrate further improves the efficiency by which plant-based proteins can be absorbed [43]. Furthermore, heat treatment and hydrolysation of the protein further increase digestibility and/or improve protein digestion and amino acid absorption kinetics [3, 67]. These processes are typically applied in most plant- as well as animal-based protein sources that we purchase either as (processed) food products or as protein isolates or concentrates. Clearly, when dealing with foods the various processes from harvest, processing, storage, cooking, chewing and ingestion all contribute to the absorbability of the final protein source and the rate of its protein digestion and amino acid absorption. These processes also differ between the various foods that together form our composite meals. Future work will need to address the anabolic properties of actual foods and, more importantly, the muscle protein synthetic response to the ingestion of complete meals.

The lesser anabolic properties of some plant-based proteins may be attributed to the low(er) essential amino acid content and/or specific amino acid deficiencies of that protein. The easiest way to compensate for the lower protein quality of a plant-based versus animal-based protein source is to simply consume a greater amount of the lesser quality protein (**Figure 2.2**). In support, we observed that ingestion of 60 g as opposed to 35 g of a wheat protein hydrolysate effectively increased post-prandial muscle protein synthesis rates in a group, healthy older men [14]. Although this strategy may not apply to all plant-based proteins [63], increasing the protein dosage to compensate for either the lower essential amino content or a specific amino acid deficiency should theoretically improve the post-prandial protein synthetic response. Although such a strategy would be easy to apply when considering the use of a plant-derived protein isolate or concentrate, it may not always be practical or feasible when considering plant-based (whole) foods. The lower protein density of most plant-based protein sources would greatly increase both the total caloric content and volume of the plant-based food that would need to be consumed. Simply consuming 20 g protein in the form of a plant-based protein source is already challenging, both from a perspective of food volume as well as caloric content (**Figure 2.3** and **2.4**). Current research has focused on evaluating the anabolic properties of plant-based protein isolates or hydrolysates. Ingesting ample amounts of a single plant-based protein in the form of its whole food will not always be feasible, especially in a more clinical setting in which food intake is generally compromised, or in a sport setting where athletes need to adhere to strict caloric intakes.

An alternative strategy to increase the anabolic potential of a plant-based protein is to combine different protein types and/or sources to provide a protein blend with a more balanced amino acid profile. Whereas some plant-based proteins are particularly deficient in lysine, others are deficient in methionine [37]. For example, corn, hemp, brown rice, soy

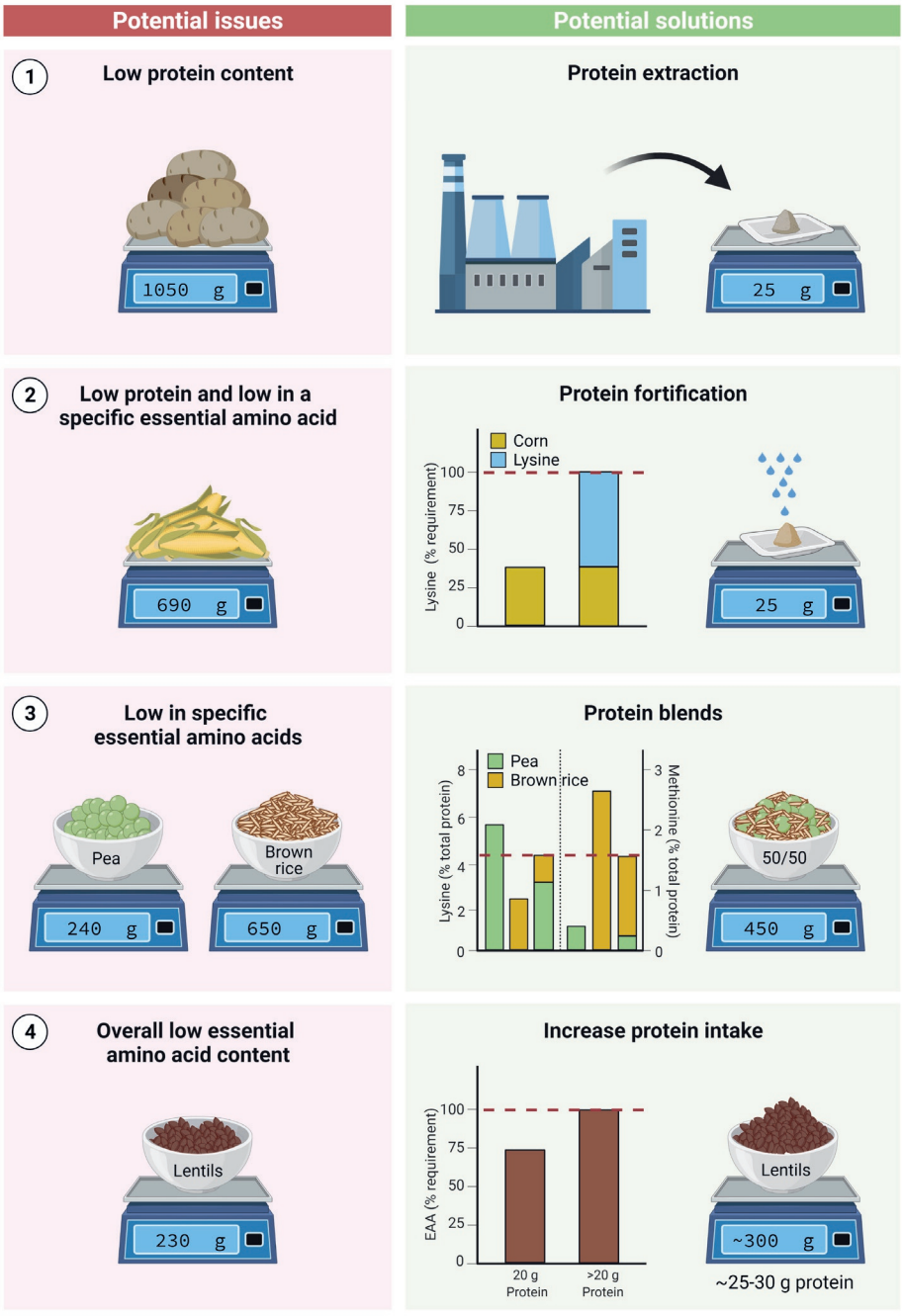

FIGURE 2.3 Overview of potential issues and solutions to optimize the anabolic response following plant-based protein consumption. 1) For plant-based foods with a high protein quality, but low protein content (e.g. potato), extraction of high-quality protein isolates forms an effective method to allow ingestion of a desired amount of protein. 2) For plant-based food sources with deficiencies in specific amino acids (e.g. corn: low in lysine), a protein isolate or concentrate can be fortified with the deficient free amino acid(s) to improve the amino acid content profile. 3) Plant-based food sources with deficiencies

in specific essential amino acids can be combined to improve the overall amino acid profile of the protein blend. For example, peas are low in methionine but high in lysine, in contrast, brown rice is high in methionine but low in lysine. A blend combining pea and brown rice would meet overall amino acid requirements. 4) When plant-based food sources (or protein isolates) are deficient in one or more amino acids (e.g. lentils, wheat) this may be compensated for by simply ingesting a greater amount of the plant based protein source. Illustrations: the scale balance represents the amount of food to be consumed to provide 20 g protein, unless otherwise indicated. Weight for brown rice and lentils represent cooked amounts. Dashed horizontal line in graphs represents the amino acid requirements for adults (WHO/FAO/UNU Expert Consultation 2007 [57]). EAA: Essential amino acid

and pea protein are low in lysine and/or methionine content. For each protein source, this deficiency could be compensated for by consuming 2 to 4 times more of the same protein. However, combining corn, hemp, or brown rice protein (low lysine and high methionine content) with an equal amount of soy or pea protein (low methionine and high lysine content) provides a blend with a more balanced amino acid profile (**Figure 2.3**). Such blends would require only 1.1-1.9 times more protein to be consumed to compensate for specific amino acid deficiencies [37]. Besides exclusive plant-based protein blends, combinations of plant- plus animal-derived proteins may also play an important role in the trend to lower animal-derived food consumption without compromising protein quality. Oat, lupin, quinoa, and wheat protein are low in both lysine and methionine contents, which could theoretically be compensated for by ingesting 3-8 times more of the respective protein. However, blending these proteins with an equal amount of an animal-derived protein would require only 1.05–1.4 times more of the respective protein blend to be consumed to provide sufficient amounts of all essential amino acids [37]. Such protein blends would represent the composition of an omnivorous diet, in which ~40-50% of the consumed protein is generally derived from plant-based sources [68]. In support, robust increases in post-prandial muscle protein synthesis rates have been reported following ingestion of whey, casein and soy protein blends [69-71]. More recently, we observed no differences in the post-prandial muscle protein synthetic response following ingestion of 30 g milk or a 30 g protein blend combining wheat and milk protein [36]. Many more protein blends combining two or more protein sources at various ratios can be composed to achieve particular aims regarding amino acid composition, price, taste, and sustainability without compromising the capacity to stimulate muscle protein synthesis.

If a specific amino acid deficiency forms the limiting factor for a plant-based protein to increase post-prandial muscle protein synthesis rates, an alternative option would be to fortify the protein with one or more specific (free) amino acids. As leucine is considered to be fundamental to the post-prandial muscle protein synthetic response, fortification with free leucine could represent a feasible strategy to augment post-prandial muscle protein synthesis rates. In support, leucine fortification of a bolus of intact protein, amino acid mixture, or mixed meal has been reported to further increase post-prandial muscle protein synthesis rates [16, 18, 72, 73]. To our knowledge, there are not many data available on the impact of leucine fortification of plant-based proteins on subsequent post-prandial muscle protein synthesis rates. A study in rodents demonstrated lower muscle protein synthesis rates after feeding wheat versus whey protein [74]. Fortification of the wheat protein with free leucine, to match the leucine content in an equivalent amount of whey protein, increased muscle

protein synthesis rates to a level that was no longer different from the response observed after whey protein feeding. In contrast, we did not observe higher post-prandial muscle protein synthesis rates following ingestion of 20 g soy protein fortified with 2.5 g free leucine compared with 20 g soy protein only during recovery from exercise in young adults [35]. In fact, we observed no measurable differences in post-prandial muscle protein synthesis rates following ingestion of 20 g whey, 20 soy, or 20 g soy fortified with 2.5 g free leucine to match the amount of leucine present in 20 g whey [12]. We can only assume that under these conditions the leucine content was not a limiting factor to the post-prandial rise in muscle protein synthesis rates. This may be explained by the exercise induced increase in skeletal muscle tissue sensitivity to the stimulating properties of an increase in circulating leucine concentration. With many plant-based proteins being deficient in lysine and/or methionine, it has been hypothesized that fortification of these plant-based proteins with their respective deficient amino acid(s) may amplify their anabolic potential (**Figure 2.3**). Though fortification with selected free amino acids is commonly applied in plant-based products designed to replace meat or dairy products, there are no studies that have assessed the efficacy of such a strategy as a means to improve the anabolic properties of plant-based protein ingestion.

5 POST-PRANDIAL PROTEIN HANDLING FOLLOWING MEAL INGESTION

Work on the anabolic properties of plant-based proteins has been largely confined to the comparison of post-prandial muscle protein synthesis rates following ingestion of a handful of plant- versus animal-derived protein isolates or concentrates. However, dietary protein is generally consumed in the form of a whole-food or food product and as part of a more complete, composite meal. This automatically provides a blend of different plant-based proteins sources, improving the post-prandial muscle protein synthetic response. Furthermore, when consuming protein as part of a product and/or meal other nutrients such as carbohydrates, fats, micronutrients, and other (anti-) nutritional compounds may modify post-prandial protein digestion and amino acid absorption kinetics and subsequent muscle protein synthesis rates [75]. In support, we [76-78] have shown that post-prandial protein digestion and amino acid absorption may be delayed when carbohydrate or fat are co-ingested with protein. However, this does not seem to have much impact on post-prandial muscle protein synthesis rates [76, 79]. In addition, it has been suggested that co-ingestion of carbohydrate with protein could increase post-prandial muscle protein synthesis rates by stimulating post-prandial insulin release. However, the impact of endogenous insulin release on post-prandial muscle protein synthesis rate has proven permissive rather than stimulatory and the modest increase in insulin release observed following protein ingestion only is already sufficient to allow post-prandial muscle protein synthesis to reach maximal values [80]. In support, co-ingestion of carbohydrate with protein has been proven not to augment post-prandial muscle protein synthesis rates neither at rest [76, 77, 81] or during recovery from exercise [78, 82, 83].

Although such studies provide insight in the impact of co-ingesting other macronutrients on protein digestion and amino acid absorption kinetics and the subsequent post-prandial stimulation of muscle protein synthesis, they do not necessarily reflect the anabolic response to the ingestion of the whole-foods from which they are derived. Whereas several studies have assessed post-prandial muscle protein synthesis rates following the ingestion of whole-foods such as milk [30], meat [8, 30-32], and eggs [84], there are less data available on the anabolic responses to the ingestion of plant-based whole-foods. This knowledge gap prevents us from understanding the true anabolic properties of consuming plant-based foods as the food matrix of plant-based foods may compromise protein digestion and amino acid absorption kinetics and, as such, attenuate the postprandial rise in muscle protein synthesis rates. Previous work has shown substantial differences in post-prandial plasma amino acid responses following ingestion of an egg- versus cereal-based breakfast, providing an isonitrogenous amount of protein [85]. The observed differences in the post-prandial rise in plasma amino acid concentrations following the egg- versus cereal-based breakfast did not result in differences in muscle protein synthesis rates. This clearly shows that the muscle protein synthetic response to meal ingestion is complex and can't be predicted by simply assessing protein amino acid composition or post-prandial plasma amino acid profiles.

The matrix of whole-foods, food products and/or composite meals is, at least partly, defined by the combination of a variety of macronutrients, micronutrients, and (anti-)nutritional compounds. However, the food matrix is also modified by commercial food processing as well as in-house food preparation, which often includes heating and/or cooking [3, 86, 87]. Prior to consumption food is subsequently cut or mashed and chewed upon, which will also impact the rate of protein digestion and amino acid absorption [3, 88, 89]. Numerous factors play a role in determining the post-prandial muscle protein synthetic response to food ingestion. Besides the impact of individual food matrices on protein digestion and amino acid absorption kinetics, it is important to consider that a composite meal often includes a variety of animal and plant-based foods, or at least various plant-based foods. There is currently limited information within the literature on the (potential) interaction between different protein sources within a single meal on protein digestion and amino acid absorption kinetics and the post-prandial muscle protein synthetic response to feeding.

Although we have gained much insight in the various factors that modulate dietary protein absorbability, protein digestion and amino acid absorption, and post-prandial muscle protein synthesis, we lack insights on post-prandial protein handling following ingestion of whole-foods and mixed meals. Future studies are warranted to assess the anabolic properties of composite meal ingestion and the impact this can have on muscle conditioning in both health and disease.

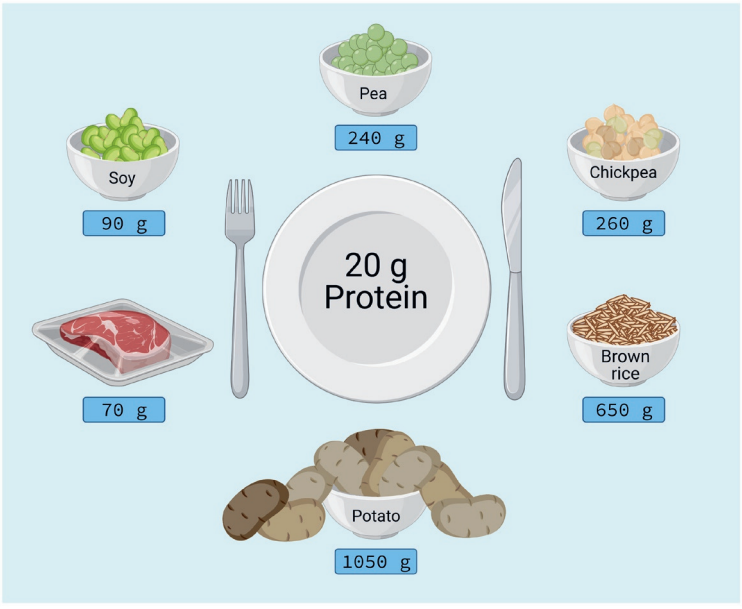

FIGURE 2.4 Amount of the selected whole-foods protein sources to be consumed to allow ingestion of 20 g protein. Illustrated are meat, soy, pea, chick-pea, brown rice, and potato in order of protein content (from high to low).

6 PLANT-BASED PROTEINS IN SPORTS NUTRITION

The transition towards a more plant-based diet has gained much interest among athletes. Not surprisingly, this also raises questions regarding the impact of the (lower) quality of plant-based proteins on muscle conditioning during recovery from exercise. There are only a handful studies that have compared post-exercise muscle protein synthetic responses following the ingestion of plant- versus animal-derived proteins [11, 12, 14, 34-36]. In these studies, the main plant-derived protein that has been applied is soy protein. Some [11, 12, 34], but certainly not all [35] studies have reported less of an increase in post-exercise muscle protein synthesis rates following ingestion of soy protein when compared with an equivalent amount of milk or whey protein. Furthermore, soy protein has been shown to result in greater muscle protein synthesis rates during 3 hours of post-exercise recovery when compared with casein protein [11]. As exercise makes the muscle more sensitive to the anabolic properties of amino acid or protein administration, it could be speculated that the post-prandial rise in circulating plasma leucine concentration is of lesser importance when protein is consumed following exercise. Therefore, the lower leucine content of most plant-based proteins may no longer restrict post-prandial muscle protein synthesis rates during recovery from exercise. Consequently, the capacity of a protein to stimulate post-exercise muscle protein synthesis is more likely to be determined by the amount of amino acids provided as precursors for protein synthesis. Therefore, an ample provision of all amino acids without deficiencies in specific amino acids may be of primary importance when determining the optimal plant-

based protein (blend) to support post-exercise muscle conditioning. Clearly, research is warranted to compare muscle protein synthesis rates during recovery from exercise while ingesting different plant- versus animal-based proteins or protein sources. Those studies will provide insight in the preferred characteristics of a dietary protein (blend) that would optimize the skeletal muscle adaptive response to exercise.

Longer-term intervention studies assessing the impact of protein supplementation on the adaptive response to resistance type exercise training tend to show greater gains in muscle mass and strength when applying protein supplementation [90, 91]. Increases in daily muscle protein synthesis rates and/or gains in muscle mass have been reported following resistance type exercise training while supplementing plant-derived protein sources, such as soy [92-95], pea [96], rice [97] and potato [98] protein. However, whether these gains in muscle mass and strength during resistance type exercise training differ from the gains observed when an equivalent amount of animal-based protein was supplemented remains equivocal. A recent meta-analysis concluded that the animal- or plant-based origin of the supplemented protein source does not impact the gains in lean mass or muscle strength following prolonged resistance type exercise training [99]. However, it seems evident that this conclusion would depend also on the population, the type of training, the training status of the volunteers, and most of all the amount of protein supplemented and the overall habitual protein intake. Recent work by Hevia-Larrain *et al.* [100] reported no differences in muscle mass and strength accrual following prolonged resistance exercise training while consuming either an exclusively plant-based versus an omnivorous diet. This may not be too much of a surprise as the untrained subjects were consuming a high-protein intake diet (~ 1.6 g/kg body mass/day) throughout the exercise intervention period, with substantial amounts of protein (soy or whey protein isolates) being supplemented twice daily.

Based upon the described differences in protein absorbability, protein digestion and amino acid kinetics, and post-prandial muscle protein synthesis rates following ingestion of plant- versus animal-based protein sources, we could hypothesise that when transitioning towards a more plant-based diet more dietary protein would be required to allow the same stimulation of muscle protein synthesis rates. This would also imply that more plant-based proteins should be consumed and/or supplemented to achieve the same level of muscle mass accretion during prolonged resistance type exercise training. However, most athletes already consume ample amounts of protein due to their higher energy intake. A nation-wide survey of well-trained athletes reports a protein intake of ~1.5 g protein per kg body mass per day [101]. Although this represents a daily protein intake well above the Recommended Daily Allowance (RDA) proposed by the World Health Organisation (0.8 g/kg/day), it has been argued that a protein intake of 1.6 g/kg would maximize gains in muscle mass and strength during prolonged resistance type exercise training [91]. Consequently, it could be speculated that a diet providing low(er) quality protein could compromise the skeletal muscle adaptive response to exercise training. However, the latter represents more an academic concept as small differences in protein quality will not have much impact on the adaptive response to exercise training when such large amounts of protein are habitually consumed. Furthermore,

omnivorous athletes already derive more than 40% of their habitual daily protein intake from plant based sources [101].

More important is the potential negative impact of a transition towards a more plant-based diet in conditions where athletes lower their energy intake and, as such, reduce protein consumption. Athletes trying to reduce body weight by caloric restriction or athletes recovering from an injury would actually require a similar or even higher (absolute) protein intake while consuming less food. In such conditions the quality of the consumed protein is of the utmost importance, and transitioning to a diet with less anabolic properties could compromise muscle maintenance or attenuate muscle regain. Therefore, we need to evaluate the positive as well as the potentially negative aspects of transitioning towards a more plant-based diet. Furthermore, we need to evaluate whether this is accompanied by a transition towards greater dietary protein intake requirements. Work is needed to evaluate the impact of structurally consuming a more exclusive plant-based whole-foods diet on muscle mass and function in various populations, in both health and disease.

7 ALTERNATIVE PROTEIN SOURCES

Huge investments are presently being made in the search for a more sustainable production of high-quality protein sources that are not derived from animals. This process has now expanded from plant-based protein sources to various other protein sources, including the growing of yeast, fungi, micro-algae, the breeding of insects, and even the cultivation of lab grown meat as potential protein sources for human consumption. Although a discussion on these alternative, sustainable protein sources is beyond the scope of this review, we will address two of these protein sources as they have recently been assessed for their capacity to stimulate post-prandial muscle protein synthesis rates *in vivo* in humans.

Recent work has addressed the anabolic properties of a food source derived from cultivating a fungus (*Fusarium venenatum*), resulting in what has been coined mycoprotein [102-104]. This protein source has been reported to have a high protein content (~45%) with the protein showing an amino acid composition that does not differ much from dairy protein [105]. Prior work suggested good digestibility based upon the observation that post-prandial plasma essential amino acid (and leucine) concentrations were comparable following ingestion of mycoprotein when compared with the ingestion of an equivalent amount of milk protein. More recently, they followed up by showing that ingestion of a single bolus of mycoprotein (70 g, providing 31.5 g protein) increased both resting and post-exercise muscle protein synthesis rates in young males, with a post-prandial muscle protein synthetic response that was greater than the response observed after ingesting a leucine-matched bolus of milk protein (31 g, providing 26.2 g protein) [102]. These data show that fungi can provide a viable, high-quality protein source that is effective in stimulating muscle protein synthesis.

Another alternative dietary protein source that has gained much interest is edible insects. Although technically insects also classify as animals, they can be produced on a more viable and sustainable commercial scale and, as such, they form another promising candidate to contribute to ensuring global food security [106, 107]. Insects have a high protein content and their protein has an amino acid composition that closely resembles conventional high-quality animal-derived proteins [106]. Recently, we produced intrinsically labelled lesser mealworms by feeding these larvae with stable isotope labelled amino acids [23], allowing us to directly quantitate protein digestion and amino acid absorption kinetics and the subsequent muscle protein synthetic response at rest and during recovery from exercise following ingestion of a single bolus of mealworms. The mealworm derived protein was rapidly digested and absorbed and strongly increased post-prandial muscle protein synthesis rates. In fact, the observed post-prandial muscle protein synthetic response did not differ from the response observed after the ingestion of an equivalent amount of milk protein [108].

These are just two examples of other alternative, high-quality protein sources that can be produced on a viable and more sustainable commercial scale and that seem to have anabolic properties that do not differ from the conventional animal-based protein sources. Clearly, more work will be performed to establish the digestion and absorption kinetics of many of these novel protein sources and evaluate their post-prandial anabolic properties. There seem to be many opportunities for the production of alternative protein sources to successfully meet the future global dietary protein demands.

8 CONCLUSIONS

There is a global trend of a transition towards the consumption of a more plant-based diet. Ingestion of plant-derived proteins are generally considered to result in lower post-prandial muscle protein synthesis responses when compared with the ingestion of an equivalent amount of animal-derived protein. The lesser anabolic properties of plant-based versus animal-derived proteins have been attributed to differences in their protein digestion and amino acid absorption kinetics and amino acid composition. Most plant-based proteins have a low(er) essential amino acid content and are often deficient in one or more specific amino acids, such as lysine and methionine. However, there are large differences in amino acid composition between various plant-derived proteins or plant-based protein sources. So far, only few studies have directly compared the muscle protein synthetic response following the ingestion of a plant- versus animal-derived protein. The proposed lower anabolic properties of plant- versus animal-derived proteins may be compensated for by 1) consuming a greater amount of the plant-derived protein or plant-based protein source to compensate for the lesser quality; 2) using specific blends of plant-derived proteins to create a more balanced amino acid profile; 3) fortifying the plant-based protein (source) with the specific free amino acid(s) that is (are) deficient. Clinical studies are warranted to assess the anabolic properties of the various plant-based proteins and their protein sources and to identify the factors that may or may not compromise the capacity to stimulate post-prandial muscle protein synthesis rates

in vivo in humans. Healthy, active athletes typically consume a diet that provides well above ~1.5 g protein per day. The consumption of more plant-based protein(s) should, therefore, not necessarily lead to a less than optimal protein intake. Accordingly, there are ample data to show that protein supplementation with plant-derived proteins can (also) support greater gains in muscle mass and strength when combined with prolonged resistance type exercise training. Under conditions of a low energy intake, as observed during dietary interventions to support body fat loss or in clinically compromised patients, it could be speculated that transition towards a more plant-based diet could compromise the post-prandial stimulation of muscle protein synthesis rates. Consequently, future work will need to establish whether the transition towards a more exclusive plant-based diet is accompanied by a transition towards greater dietary protein intake requirements.

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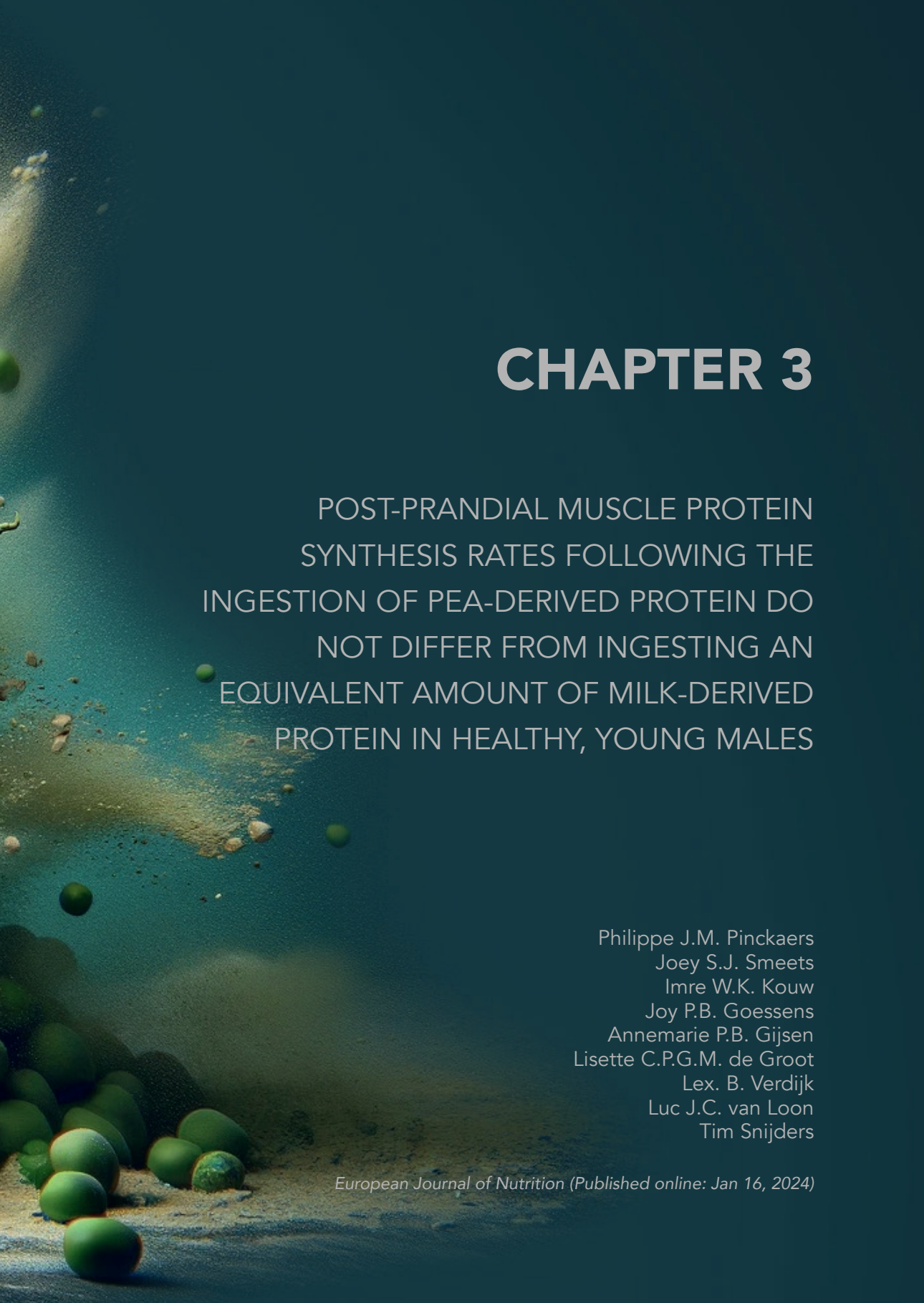

CHAPTER 3

POST-PRANDIAL MUSCLE PROTEIN SYNTHESIS RATES FOLLOWING THE INGESTION OF PEA-DERIVED PROTEIN DO NOT DIFFER FROM INGESTING AN EQUIVALENT AMOUNT OF MILK-DERIVED PROTEIN IN HEALTHY, YOUNG MALES

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ABSTRACT

Background

Plant-derived proteins have received considerable attention as an alternative to animal-derived proteins. However, plant-derived proteins are considered to have less anabolic properties when compared with animal-derived proteins. The lower muscle protein synthesis rates following ingestion of plant- compared with animal-derived protein have been attributed to the lower essential amino acid content of plant-derived proteins and/or their specific amino acid deficiencies.

Objective

This study aimed to compare post-prandial muscle protein synthesis rates following the ingestion of 30 g pea-derived protein with 30 g milk-derived protein in healthy, young males.

Methods

In a randomized, double-blind, parallel-group design, 24 young males (24±3 y) received a primed continuous L-[ring-$^{13}C_6$]-phenylalanine infusion after which they ingested 30 g pea (PEA) or 30 g milk-derived protein (MILK). Blood and muscle biopsies were collected frequently for 5 h to assess post-prandial plasma amino acid profiles and subsequent post-prandial muscle protein synthesis rates.

Results

MILK increased plasma essential amino acid concentrations more than PEA over the 5 h post-prandial period (incremental area under curve 151±31 vs 102±15 mmol·300 min·L^{-1}, respectively; P<0.001). Ingestion of both MILK and PEA showed a robust muscle protein synthetic response with no significant differences between treatments (0.053±0.013 and 0.053±0.017 %·h^{-1}, respectively; P=0.96).

Conclusion

Post-prandial muscle protein synthesis rates following the ingestion of 30 g pea-derived protein do not differ from the response following ingestion of an equivalent amount of milk-derived protein. International Clinical Trials Registry Platform (NTR6548; 27-06-2017).

INTRODUCTION

Protein ingestion increases muscle protein synthesis rates [1, 2]. The increase in muscle protein synthesis rate is driven by the post-prandial increase in plasma essential amino acid (EAA) concentrations [3], with the rise in circulating leucine concentration being of particular relevance [4-8]. The anabolic properties of different proteins or protein sources seem to be largely determined by their EAA content, amino acid profile, and their protein digestion and amino acid absorption kinetics [9-11]. Consequently, post-prandial muscle protein synthesis rates can differ substantially following ingestion of the same amount of protein derived from different protein sources [12-14].

Within the wide variety of dietary protein sources, the main categories are animal (e.g. milk, meat) and non-animal proteins (e.g. wheat, soy). Within the non-animal proteins, plant proteins comprise a large part of our daily protein intake [15] and are likely to become more important with respect to future global protein needs and more sustainable protein production [16, 17]. However, plant-derived proteins are considered to have lesser anabolic properties when compared to animal-derived proteins, due to their lower digestibility and incomplete amino acid profile [17, 18]. So far, only a few studies have directly compared the muscle protein synthetic response following the ingestion of a plant-derived protein versus high(er) quality animal-derived proteins, demonstrating equivocal results, with muscle protein synthesis rates being either lower [14, 19-21], higher [14], or not different [22-24]. Furthermore, these studies have mainly focused on investigating soy- [14, 20-22] and wheat- [19, 25] derived proteins (and more recently also potato derived protein [24]). Most plant-derived proteins are generally low in essential amino acid content and often deficient in one or more specific amino acids, particularly leucine, lysine, and/or methionine [26]. The amino acid composition and deficiencies can be quite variable between different plant-based proteins. To what degree this may have an impact on their properties to stimulate post-prandial muscle protein synthesis rates remains to be determined.

Pea-derived protein has received considerable interest as an alternative for animal-derived proteins, as together with soy protein it forms one of the main plant based protein sources used in meat substitutes [27-30]. Pea-derived protein is considered of interest given its high nutritional value, availability, non-allergenic properties, and low production costs [31]. Pea-derived protein contains a sufficient amount of total essential amino acids (30%) and has a leucine (7.2 %) and lysine content (5.9 %) that exceeds the WHO/FAO/UNU amino acid requirements [32]. The latter is the proposed amino acid requirement that indicates the amount of amino acids that needs to be ingested to maintain skeletal muscle mass in healthy adults [32]. However, total essential amino acid content of pea-derived protein is less when compared with most animal-based proteins. Furthermore, pea-derived protein is particularly low in methionine. Whether this lower total essential amino acid content and low methionine content compromises the capacity to stimulate post-prandial muscle protein synthesis remains to be assessed.

In the present study, we aimed to compare the impact of ingesting 30 g pea- *vs* 30 g milk-derived protein on post-prandial muscle protein synthesis rates *in vivo* in healthy, young males. We hypothesize that the ingestion of 30 g pea-derived protein would result in lower post-prandial muscle protein synthesis rates when compared with the ingestion of an equivalent amount of milk-derived protein.

MATERIALS AND METHODS

Participants

A total of 24, healthy, recreationally active males aged 18-35 y were recruited to participate in this parallel group, double-blind, randomized controlled trial to compare the impact of ingesting 30 g pea and 30 g milk derived protein on post-prandial muscle protein synthesis rates *in vivo* in humans. As we provided the same absolute amount of protein (30 g) we decided to select only a single sex in the present study, to limit the range of the amount of protein provided when expressed per kg muscle mass. Participants were recreationally active and generally performed between 2-4 exercise sessions per week in various sports (e.g. soccer, basketball, weight lifting, running, cycling, etc.), but were not involved in any structured progressive exercise training regimen. This study was part of a larger trial registered at the International Clinical Trials Registry Platform (NTR6548) and was conducted between June 2017 and April 2019 at Maastricht University in Maastricht, The Netherlands (See **Supplemental Figure 3.1** for the CONSORT (Consolidated Standards of Reporting Trials) flow diagram, indicating the specific comparison that the current study was based on). The data of the milk-derived protein group were used in various comparisons and, as such, have been published previously, as well as the procedures applied in this trial [23, 33]. All participants were informed about the purpose of the study, the experimental procedures, and possible risks before providing informed written consent to participate. The procedures followed were in accordance with the ethical standards of the medical ethics committee of Maastricht University Medical Centre+ (METC 173001), and in accordance with the Helsinki Declaration of 1975 as revised in October 2013. The study was independently monitored and audited by the Clinical Trial Centre Maastricht.

Preliminary testing

Participants aged 18-35 y, with BMI >18.5 and <27.5 $kg \cdot m^{-2}$ underwent an initial screening session to assess eligibility. Height, weight, blood pressure, and body composition (by dual-energy X-ray absorptiometry; Discovery A, Hologic; (National Health and Nutrition Examination Survey - Body composition analysis (NHANES BCA) enabled) were determined. Participants were deemed healthy based on their responses to a medical questionnaire. The screening sessions and experimental trials were separated by at least 3 days.

Study design

Participants were randomly assigned to ingest a 400 mL beverage containing either 30 g milk-derived protein concentrate (MILK), or 30 g pea-derived protein concentrate (PEA).

TABLE 3.1 Participants' characteristics

	MILK	PEA
Age (y)	26 ± 4	23 ± 2
Height (m)	1.76 ± 0.06	1.77 ± 0.07
Mass (kg)	71.5 ± 9.0	71.7 ± 9.1
BMI ($kg \cdot m^{-2}$)	23.0 ± 2.1	22.7 ± 1.9
Systolic blood pressure (mmHg)	119 ± 6	122 ± 12
Diastolic blood Pressure (mmHg)	71 ± 9	69 ± 8
Resting heart rate (bpm)	64 ± 10	63 ± 8
Lean body mass (kg)	53.2 ± 7.9	53.6 ± 6.8
Body fat (%)	23.1 ± 3.2	22.2 ± 4.0

Values represent mean ± standard deviation. *n* = 12 per nutritional intervention group. MILK: 30 g milk-derived protein, PEA: 30 g pea-derived protein. Independent samples T-test all $P>0.05$

After beverage ingestion, the bottle was rinsed with 150 mL of water, which was also ingested by the participants. Milk-derived protein concentrate (Refit MPC80) was obtained from FrieslandCampina (Wageningen, the Netherlands), and pea-derived protein concentrate (Nutralys S85F) was supplied by Kellogg (Battle Creek, MI, USA). Participants were allocated to a treatment according to a block randomization list performed using a computerized randomizer (http://www.randomization.com/). An independent researcher was responsible for random assignment (n=12 per group) and preparation of the study treatment beverages, which were sequentially numbered according to subject number. The beverages were provided in identical, non-transparent protein shakers.

Diet and physical activity

Participants refrained from sports and strenuous physical activities (e.g. lifting heavy weights), and alcohol consumption for 3 days prior to the experimental trial. In addition, all participants were instructed to complete a food and activity record for 3 days prior to the experimental trial (See **Supplemental Table 3.1** for an overview of participants' habitual food intake in the 3 days prior to the experimental trial). The evening before the trial, all participants consumed a standardized meal containing 2.8 MJ, with 20% energy provided as carbohydrate, 65% as fat, and 15% as protein, before 10:00 PM after which they remained fasted.

Experimental protocol

The procedures applied in this trial, have previously been described elsewhere [23]. At ~7:30 AM, participants arrived at the laboratory in an overnight post-absorptive state. A cannula was inserted into an antecubital vein for stable isotope amino acid infusion. A second cannula was inserted retrogradely into a dorsal hand vein on the contralateral arm for arterialized blood sampling. To obtain arterialized blood samples, the hand was placed in a hot box (60°C) for 10 min prior to blood sample collection.

After taking a baseline blood sample (*t*= -180 min), the plasma phenylalanine pool was primed with a single dose of L-[ring-$^{13}C_6$]-phenylalanine (2.25 μmol·kg^{-1}). Thereafter, a continuous intravenous infusion of L-[ring-$^{13}C_6$]-phenylalanine (0.05 μmol·kg^{-1}·min^{-1}) was initiated (*t*= -180 min) using a calibrated IVAC 598 pump (San Diego, CA, USA). Subsequently, arterialized blood samples were collected at *t*= -90, -60 and -30 min. At *t*= 0 min an arterialized blood sample was obtained as well as a muscle biopsy from the *M. vastus lateralis*. Immediately following the muscle biopsy, participants ingested a 400 mL beverage corresponding to their randomized treatment allocation i.e.: MILK (*n*=12), or PEA (*n*=12). To minimize dilution of the steady-state plasma L-[ring-$^{13}C_6$]-phenylalanine precursor pool, the phenylalanine content of the protein drink was enriched with 3.85 % L-[ring-$^{13}C_6$]-phenylalanine. Arterialized blood samples were then collected at *t*= 15, 30, 45, 60, 90, 120, 150, 180, 210, 240, and 300 min after protein ingestion in the post-prandial period. Blood samples were collected into EDTA-containing tubes and centrifuged at 1200*g* for 10 min at 4°C. Aliquots of plasma were frozen in liquid nitrogen and stored at -80°C. A second and third muscle biopsy from the *M. vastus lateralis* were collected at *t*= 120 and *t*= 300 min to determine post-prandial skeletal muscle protein synthesis rates over the 0-120, 120-300, and 0-300 min post-prandial periods. Muscle biopsy collection was alternated between legs and obtained with the use of a 5 mm Bergström needle [34], custom-adapted for manual suction. Samples were obtained from separate incisions from the middle region of the *M. vastus lateralis*, ~15 cm above the patella and ~3 cm below entry through the fascia. Local anesthetic (1 % xylocaine with adrenaline 1:100,000) was applied to numb the skin and fascia. Muscle samples were freed from any visible non-muscle material, immediately frozen in liquid nitrogen, and stored at -80°C until further processing. When the experimental protocol was complete, cannulae were removed and participants were provided with food and monitored for ~30 min before leaving the laboratory. For a schematic representation of the infusion protocol, see Figure 3.1.

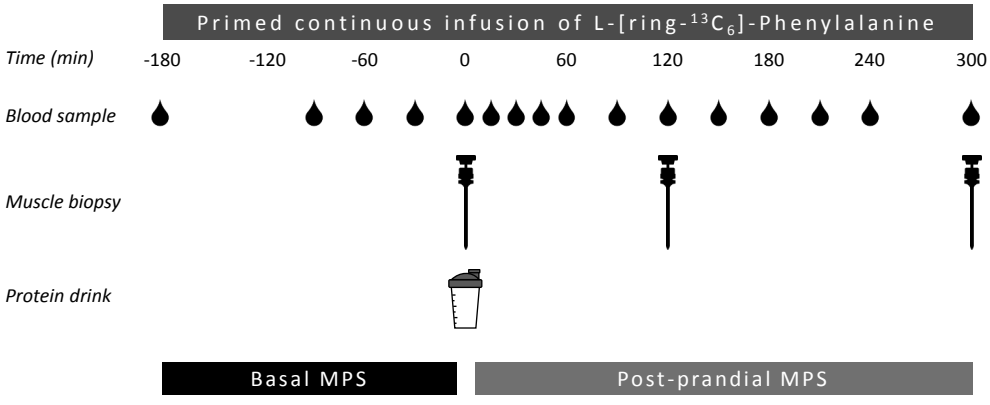

FIGURE 3.1 Schematic representation of the experimental design.

Protein powder analysis

Batch specific nitrogen contents for milk and pea-derived protein concentrates were provided by the manufacturers. The protein content of the milk-derived protein was determined as

nitrogen content x 6.38, and the protein content of pea-derived protein was determined as nitrogen x 6.25 [35, 36]. Amino acid contents of the protein powders were determined by acid hydrolysis in triplicate, and subsequent analysis of the free amino acids using ultra-performance liquid chromatochraphy-mass spectrometry (UPLC-MS; ACQUITY UPLC H-Class with QDa; Waters, Saint-Quentin, France), as previously described [23]. The amino acid composition of the protein powders are presented in Table 3.2.

TABLE 3.2 Amino acid composition of protein or protein blend consumed

	MILK	PEA
Alanine	0.9	1.1
Arginine	0.8	1.7
Aspartic acid	1.8	2.5
Cystine	0.1	0.1
Glutamic acid	5.1	3.9
Glycine	0.5	1.1
Histidine	0.6	0.5
Isoleucine	0.9	0.6
Leucine	2.4	1.8
Lysine	2.0	1.7
Methionine	0.7	0.2
Phenylalanine	1.2	1.2
Proline	2.9	1.1
Serine	1.2	1.4
Threonine	0.9	0.8
Tyrosine	0.6	0.4
Valine	1.1	0.8
TAA	23.8	20.9
EAA	9.8	7.7
BCAA	4.4	3.2
Nitrogen content (%)	13.4	13.6
Protein content (%)	85.5[1]	84.7[2]

Values for amino acid contents are in grams per 30 g protein. [1]Protein as nitrogen content * 6.38; [2]Protein as nitrogen content * 6.25; MILK: 30 g milk-derived protein, PEA: 30 g pea-derived protein. BCAA: branched chain amino acids, EAA: essential amino acids, TAA: total amino acids.

Plasma analysis

Plasma glucose and insulin concentrations were analyzed using commercially available kits (ref. no. A11A01667, Glucose HK CP, ABX Diagnostics, Montpellier, France; and ref. no. HI-14K, Millipore, St. Louis, MO, respectively). Plasma amino acid concentrations were determined by UPLC-MS, as previously described [23].

Plasma L-[ring-$^{13}C_6$]-phenylalanine enrichments were determined by gas chromatography-mass spectrometry (GC-MS; Agilent 7890A GC/5975C MSD; Agilent Technologies), as previously described [23]. In short, the free amino acids from deproteinized plasma samples were purified using cation exchange resin columns (AG 50W-X8, mesh size: 100-200, ionic form: hydrogen (Bio-Rad Laboratories, Hercules, CA, USA)), and subsequently converted to their tert-butyl dimethylsilyl (TBDMS) derivative before analysis by GC-MS.

Basal (post-absorptive) muscle protein synthesis rates were assessed to confirm that protein ingestion increases muscle protein synthesis rates. The single biopsy approach was applied to assess post-absorptive muscle protein synthesis rates without the need to collect an additional muscle biopsy [37]. In short, plasma protein obtained prior to tracer infusion (*t*= -180 min) was used to determine background L-[ring-$^{13}C_6$]-phenylalanine enrichments. For this purpose, the plasma sample was precipitated by adding perchloric acid. Subsequently, similarly as for the myofibrillar protein fraction, the denaturized plasma protein pellet was hydrolyzed, passed over a cation exchange resin column (AG 50W-X8, mesh size: 100-200, ionic form: hydrogen (Bio-Rad Laboratories, Hercules, CA, USA)), and the resulting amino acid samples were derivatized to their N(O,S)-ethoxycarbonyl-ethylesters before being measured by gas chromatography-combustion-isotope ratio mass spectrometry (GC-IRMS; Mat 253, Thermo Scientific, Bremen, Germany) using a DB5MS (30m) column (Agilent technologies, Santa Clara, Ca, USA), as previously described [23].

Muscle analysis

Muscle analysis for the determination of muscle protein bound L-[ring $^{13}C_6$]-phenylalanine enrichments has previously been explained in detail [23]. In short, a piece of wet muscle (~50-70 mg) was homogenized and a myofibrillar protein-enriched fraction was obtained by removal of the collagen enriched fraction. Subsequently, the amino acids from the resulting dried myofibrillar protein-enriched fractions were liberated by adding 2 mL of 6 M HCl and heating to 110°C for 16 h, passed over a cation exchange resin column (AG 50W-X8, mesh size: 100-200, ionic form: hydrogen (Bio-Rad Laboratories, Hercules, CA, USA)), and derivatized to their N(O,S)-ethoxycarbonyl-ethylesters. The ratio of $^{13}C/^{12}C$ of myofibrillar protein-bound phenylalanine was determined using GC-IRMS.

Calculations

The plasma free and muscle protein-bound L-[ring-$^{13}C_6$]-phenylalanine enrichments were used to calculate fractional myofibrillar protein synthesis rates (%·h^{-1}). This calculation was performed by the standard precursor-product equation [38]:

$$FSR = \left(\frac{(E_{b2} - E_{b1})}{(E_{precursor} \cdot t)}\right) \cdot 100$$

Where E_b is the increment in myofibrillar protein-bound L-[ring-$^{13}C_6$]-phenylalanine enrichment (mole % excess, MPE) during the tracer incorporation period, and t is the tracer incorporation time in h. Weighted mean plasma L-[ring-$^{13}C_6$]-phenylalanine enrichments were calculated by taking the measured enrichment between consecutive time points and correcting for the time

between these sampling time points ($E_{precursor}$). For calculation of post-prandial FSR, skeletal muscle biopsy samples at *t*= 0, 120 and 300 min were used. For the calculation of basal FSR, E_{b2} represented the protein-bound L-[ring-$^{13}C_6$]-phenylalanine enrichments in muscle at *t*= 0 min, and E_{b1} represented the protein bound L-[ring-$^{13}C_6$]-phenylalanine enrichments in plasma protein at *t*= -180 min.

Net incremental area under curve (iAUC) was determined for plasma amino acid concentrations during the 5 h post-prandial period following protein ingestion. The iAUC was calculated using the trapezoid rule, with plasma concentrations before beverage ingestion (*t*= 0 min) serving as baseline. Time to reach peak plasma amino acid concentrations were determined for each individual and subsequently averaged per group.

Outcome measures

Myofibrillar FSR over the entire (i.e. 0 – 300 min) post-prandial period, comparing MILK *vs* PEA was defined as the primary outcome measure. Secondary outcome measures were myofibrillar FSR in the early (i.e. 0 – 120 min) and late (i.e. 120 – 300 min) post-prandial period, plasma glucose, insulin, and amino acid concentrations and plasma amino acid iAUC. Plasma glucose, insulin, and amino acid peak concentrations and time to peak were tertiary outcomes.

Statistical analysis

A sample size calculation was performed with differences in post-prandial myofibrillar FSRs between the 2 treatments as primary outcome measure. Based on previous work in this area, a sample size of 12 participants per treatment, including a 10 % dropout rate was calculated using a power of 80 %, a significance level of 0.05, a difference in FSR of 0.008 %·h^{-1} (or ~20% when expressed as relative difference, e.g. 0.040 vs 0.048 %·h^{-1}) [39], and a within-group standard deviation of 0.0065 %·h^{-1}(or~16 %) [40, 41].

The primary outcome, post-prandial (0-300 min) muscle protein synthesis rates between the two treatments, was analyzed by independent samples *t*-test. Likewise, basal post-absorptive, (-180-0 min) and post-prandial myofibrillar protein synthesis rates during the early (0-120 min) and late (120-300 min) post-prandial period were analyzed by independent samples *t*-test. As secondary analyses, a two-way repeated measures ANOVA was performed to evaluate changes over time and the increase in post-prandial muscle protein synthesis rates above basal post-absorptive rates. Plasma glucose, insulin, and amino acid concentrations and amino acid enrichments over time were compared between groups using a two-way (*time x treatment*) repeated measures ANOVA, with time as within-subjects factor, and treatment as between-subjects factor. In case a significant *time x treatment* interaction was observed, post-hoc analyses were performed to determine significant differences between treatments for each time point. Participants' characteristics, plasma glucose, insulin, and amino acid concentrations, expressed as peak values, time to peak and iAUC, were analyzed by independent samples *t*-test to locate differences between groups. Statistical analyses were performed with a software package (IBM SPSS statistics for Windows, version 26.0, IBM

Corp., Armonk, NY, USA). Means were considered to be significantly different for *P* values <0.05. Data are expressed as means±SD. Except for plasma insulin concentrations (*n*=11 for MILK), no missing values were present for any of the outcome parameters.

RESULTS

Participants' characteristics

Twenty-four healthy, recreationally active males (24±3 y; 1.77±0.06 m; 71.6±8.9 kg) volunteered to participate in this parallel group, double-blind, randomized controlled trial (Table 3.1).

Plasma glucose and insulin concentrations

Plasma glucose concentrations were maintained following protein ingestion, with no differences between treatments (*time x treatment*: *P*=0.27; Figure 3.2A). Plasma insulin concentrations increased following protein ingestion, with no differences between the MILK and PEA treatment group over time (*time x treatment*: *P*=0.32; Figure 3.2B). Similarly, peak plasma insulin concentrations (28±8 *vs* 25±7 mU·L^{-1} respectively; independent-samples *t*-test: *P*=0.34), and post-prandial plasma insulin availability (iAUC) did not differ following MILK *vs* PEA ingestion (1058±331 *vs* 797±498 mU·300 min·L^{-1}, respectively; independent-samples *t*-test: *P*=0.16).

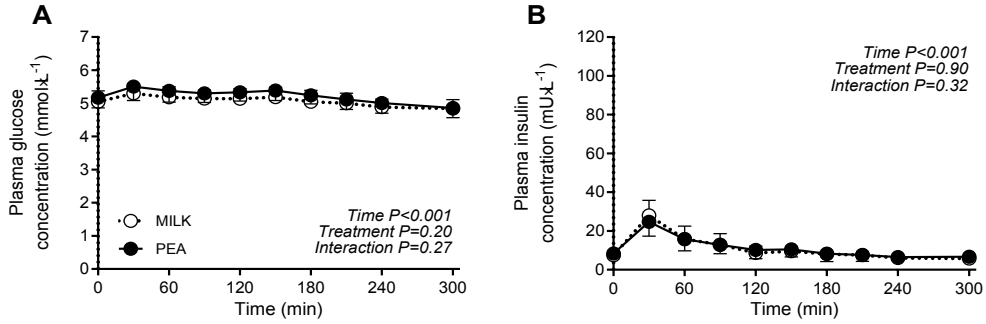

FIGURE 3.2 Post-prandial plasma glucose (Panel A) and insulin (Panel B) concentrations during the 5 hour period following the ingestion of MILK *vs* PEA in healthy, young males (*n*=12 per group). Time 0 min represents time of beverage intake. MILK: 30 g milk-derived protein, PEA: 30 g pea-derived protein. Values represent means ± standard deviation; Repeated measures ANOVA with time as within-subjects variable and interventional drink (treatment) as between-subjects variable.

Plasma AA concentrations

Plasma EAA concentrations increased following protein ingestion, with a greater rise in circulating EAA concentrations following MILK *vs* PEA ingestion (*time x treatment: P*=0.03; Figure 3.3A). Plasma EAA concentrations were increased above basal post-absorptive

concentrations for the entire 300 min post-prandial period after MILK and PEA ingestion. In accordance with the significant *time x treatment* interaction, peak plasma EAA concentrations following MILK *vs* PEA ingestion were reached at 36±10 min and 56±32 min (independent samples *t*-test: *P*=0.05), reaching levels of 1871±124 and 1601±162 μmol·L^{-1} (independent samples *t*-test: *P*<0.001), respectively. The overall increase in plasma EAA availability over the entire 300 min post-prandial period, expressed as iAUC, was ~48 % greater for MILK *vs* PEA (151±31 *vs* 102±15 mmol·300 min·L^{-1}; independent samples *t*-test: *P*<0.001; **Figure 3.3B**).

The post-prandial increase in plasma leucine concentrations following protein ingestion (**Figure 3.3C**) differed between MILK *vs* PEA (*time x treatment: P*<0.01). Plasma leucine concentrations increased for the entire 300 min post-prandial period following ingestion of both MILK and PEA. In accordance with the significant *time x treatment* interaction, peak plasma leucine concentrations were ~25 % higher for MILK *vs* PEA (353±45 *vs* 282±30 μmol·L^{-1}, respectively; *P*<0.001) and were reached 46±43 and 58±31 min after protein ingestion, respectively (independent samples *t*-test: *P*=0.47). The overall increase in plasma leucine availability over the entire 300 min post-prandial period, expressed as iAUC, was ~44 % greater for MILK *vs* PEA (36±7 *vs* 25±4 mmol·300 min·L^{-1}; independent samples *t*-test: *P*<0.001; **Figure 3.3D**).

The post-prandial increase in plasma lysine concentrations following protein ingestion was not different following MILK *vs* PEA ingestion (*time x treatment P*=0.33; **Figure 3.3E**). Plasma lysine concentrations increased for 240 and 210 min after MILK and PEA ingestion, respectively. Peak plasma lysine concentrations were not different following MILK *vs* PEA ingestion (370±29 *vs* 339±50 μmol·L^{-1}, respectively; independent samples *t*-test: *P*=0.08), but were reached ~16 min earlier (34±7 vs 50±21 min after protein ingestion respectively, independent samples *t*-test: *P*=0.02). Peak plasma lysine concentrations increased ~137 % above baseline values for MILK, and ~106 % above baseline for PEA. Consequently, the overall increase in plasma lysine availability over the entire 300 min post-prandial period, expressed as iAUC, was ~25 % greater for MILK *vs* PEA (25±8 *vs* 20±4 mmol·300 min·L^{-1}; independent samples *t*-test: *P*=0.03; **Figure 3.3F**).

The post-prandial increase in plasma methionine concentrations following protein ingestion was significantly greater following MILK *vs* PEA ingestion (*time x treatment: P*<0.001; **Figure 3.3G**). Plasma methionine concentrations increased for 240 and 90 min after MILK and PEA ingestion, respectively. After which methionine concentrations became lower when compared to post-absorptive values in the PEA group. In accordance with the significant *time x treatment* interaction, peak plasma methionine concentrations were ~114 % greater for MILK *vs* PEA (60±5 and 28±4 μmol·L^{-1}, independent samples t-test: *P*<0.001), and were reached ~30 min after protein ingestion (34±9 *vs* 35±22 min; independent samples t-test: *P*=0.86). As a result, peak plasma methionine concentrations increased ~190 % above baseline values for MILK, but only increased ~33 % above baseline values for PEA. The overall increase in plasma methionine availability over the entire 300 min post-prandial period, expressed as iAUC, was

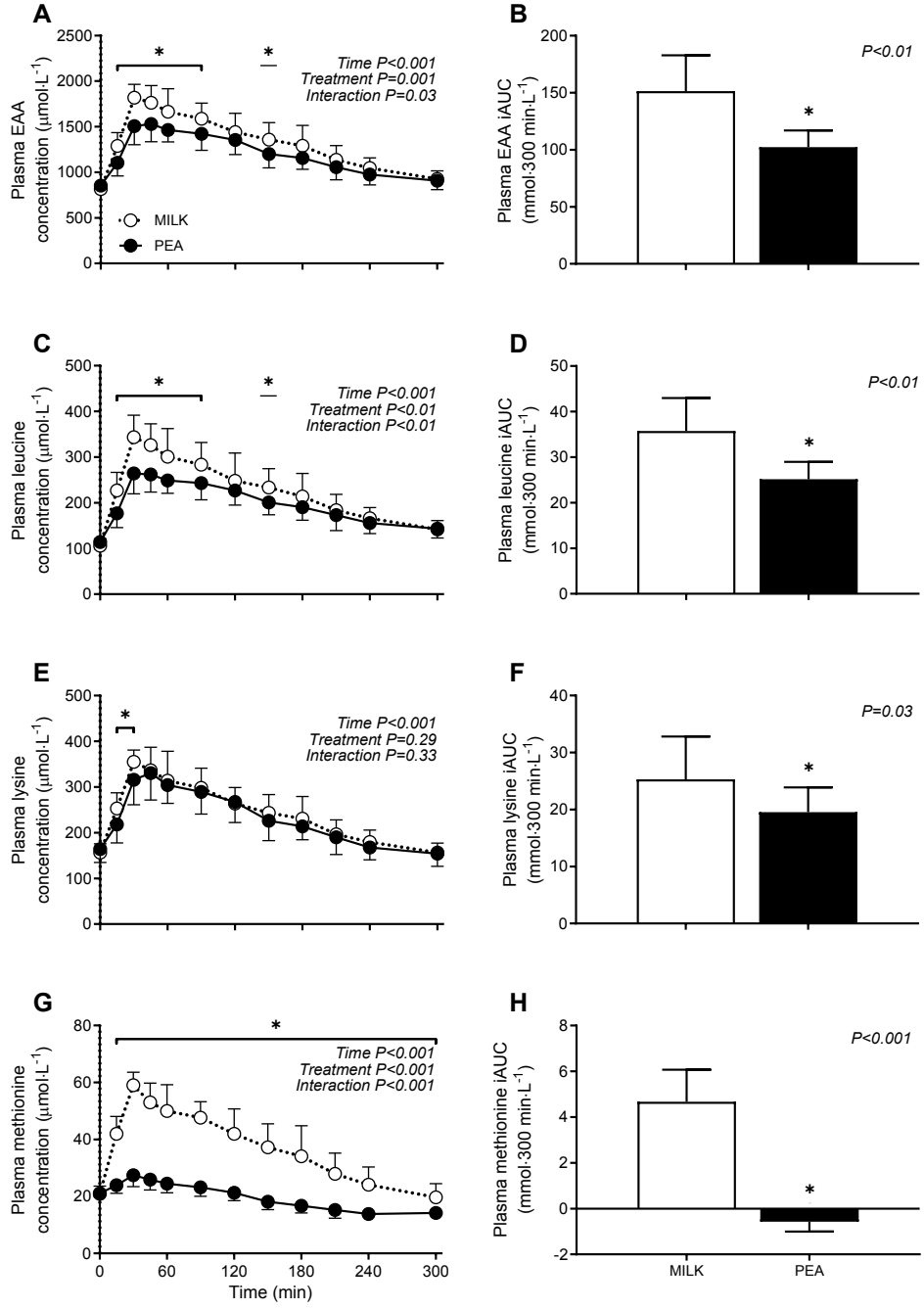

FIGURE 3.3 Post-prandial plasma essential amino acid (EAA, Panel A), leucine (Panel C), lysine (Panel E), and methionine (Panel G) concentrations during the 5 hour post-prandial period following the ingestion of MILK *vs* PEA in healthy, young males (*n*=12 per group). Time 0 min represents time of beverage intake. Panels B, D, F and H represent the 0-5 h incremental area under curve (iAUC) following protein ingestion. MILK: 30 g milk-derived protein, PEA: 30 g pea-derived protein. Values represent means ± standard deviation; * significantly different for MILK *vs* PEA (*P*<0.05). Repeated measures ANOVA with time as within-subject variable and interventional drink (treatment) as between-subject variable.

several fold greater for MILK *vs* PEA (4.7±1.4 *vs* -0.6±0.4 mmol·300 min·L^{-1}; independent samples t-test: *P*<0.001; **Figure 3.3H**).

In general, post-prandial increases in plasma amino acid concentrations revealed significant differences over time following MILK *vs* PEA ingestion for most amino acids (**Supplemental Figure 3.2**; *time x treatment: P*<0.05). The post-prandial increases in plasma alanine, BCAA, cystine, proline, threonine, tryptophan, tyrosine, and valine availability over the entire 300 min post-prandial period (iAUC) were greater for MILK *vs* PEA (independent samples t-test: *P*<0.05), with an exception for plasma arginine, asparagine, glycine, and ornithine, which were lower for MILK *vs* PEA (independent samples t-test: *P*<0.05, **Supplemental Figure 3.2**).

Plasma free and muscle protein-bound L-[ring-$^{13}C_6$]-phenylalanine enrichments

Plasma L-phenylalanine concentrations and L-[ring-$^{13}C_6$]-phenylalanine enrichments over time are presented in **Figure 3.4A** and **3.4B**, respectively. Plasma L-[ring-$^{13}C_6$]-phenylalanine enrichments over time did not differ following MILK *vs* PEA ingestion during the post-prandial period (*time x treatment: P*=0.18). Mean plasma L-[ring-$^{13}C_6$]-phenylalanine enrichments averaged 7.11±0.65 and 6.63±0.58 MPE during the basal post-absorptive period (independent samples *t*-test: *P*=0.07), and 6.64±0.53 and 6.33±0.27 MPE throughout the 5 h post-prandial period (independent samples *t*-test: *P*=0.08) following MILK and PEA ingestion, respectively. Myofibrillar protein-bound L-[ring-$^{13}C_6$]-phenylalanine enrichments were higher following ingestion of MILK and PEA from 0.0032±0.0032 and 0.0028±0.0029 MPE at *t*= 0 min, to 0.0115±0.0041 and 0.0104±0.0035 MPE at *t*= 120 min, reaching 0.0214±0.0049 and 0.0205±0.0047 MPE at *t*= 300 min after protein ingestion, respectively.

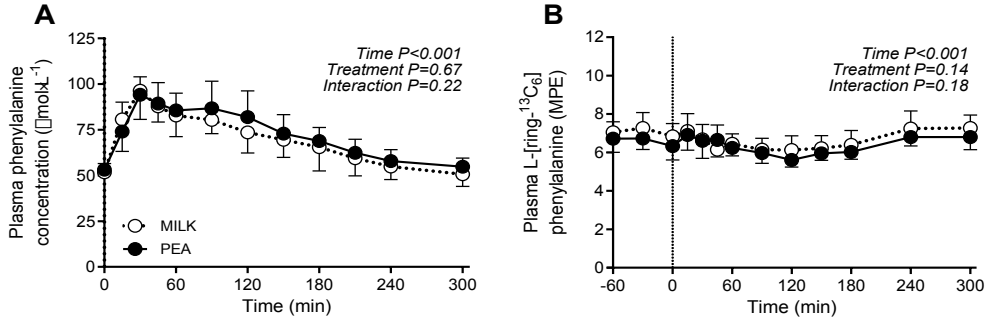

FIGURE 3.4 Post-prandial plasma phenylalanine concentrations (Panel A) and plasma L-[ring-$^{13}C_6$]-phenylalanine enrichments (Panel B) during the 5 h post-prandial period following the ingestion of MILK *vs* PEA in healthy, young males (*n*=12 per group). Time 0 min represents time of beverage intake. MILK: 30 g milk-derived protein, PEA: 30 g pea-derived protein. Values represent means ± standard deviation. Repeated measures ANOVA with time as within-subject variable and interventional drink (treatment) as between-subject variable.

Muscle protein synthesis rates

Post-absorptive fractional myofibrillar protein synthesis rates averaged 0.014±0.014 and 0.015±0.017 %·h^{-1} in the MILK and PEA experiment, with no differences between groups (independent samples *t*-test: *P*=0.94). Post-prandial muscle protein synthesis rates (0-300 min), did not differ between MILK *vs* PEA, (independent samples *t*-test: *P*=0.96). Additionally, muscle protein synthesis rates did not differ for the early (0-120 min; independent samples *t*-test: *P*=0.71), and late (120-300 min; independent samples *t*-test: *P*=0.55) post-prandial period. Post-prandial muscle protein synthesis rates averaged 0.053±0.013 and 0.053±0.017 %·h^{-1} assessed over the entire 5 h post-prandial period, for the MILK and PEA treatment group, respectively (**Figure 3.5**). Secondary analyses using two-way repeated measure ANOVA showed that protein ingestion increased myofibrillar protein synthesis rates to 0.059±0.024 and 0.054±0.031 %·h^{-1} during the early post-prandial period (0-120 min) and to 0.049±0.017 and 0.053±0.015 %·h^{-1} during the late post-prandial period (120-300 min) in MILK and PEA, respectively (main effect of time *P*<0.001), with no *time x treatment* interaction.

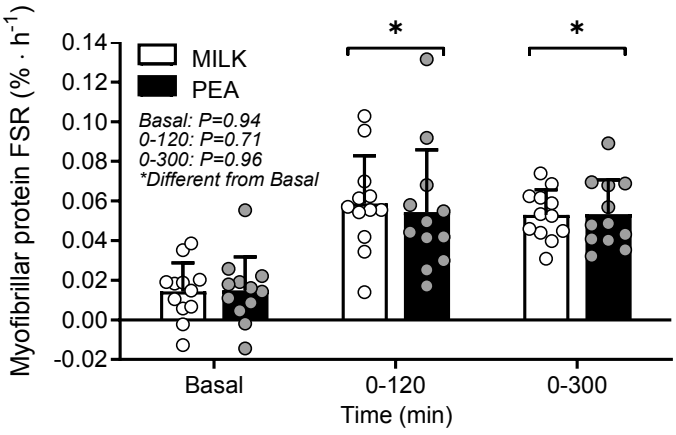

FIGURE 3.5 Myofibrillar protein fractional synthetic rates (FSR) at different time points following ingestion of MILK vs PEA in healthy, young males (n=12 per group). MILK: 30 g milk-derived protein, PEA: 30 g pea-derived protein. Bars represent means ± standard deviation, dots represent individual values. *significantly effect of time P<0.001.

DISCUSSION

The present study shows that ingestion of a pea-derived protein is followed by a substantial increase in muscle protein synthesis rates in healthy, young males. Despite lower post-prandial plasma essential amino acid concentrations, post-prandial muscle protein synthesis rates following the ingestion of 30 g pea-derived protein did not differ from the rates observed after ingesting an equivalent amount of milk-derived protein.

Plant-derived proteins are known to have deficiencies in specific EAA according to the WHO/FAO/UNU requirements [32], and can be particularly low in leucine, lysine, and/or methionine contents [26]. Pea-derived protein contains a sufficient amount of leucine and a lysine content that is higher than most plant-derived protein sources [26]. In contrast, pea-derived protein has a particularly low methionine content [26]. In the present study, EAA (9.8 *vs* 7.7 g), leucine (2.4 *vs* 1.8 g), and methionine (0.7 *vs* 0.2 g) contents were all substantially higher in the milk compared with the pea-derived protein (**Table 3.2**). Furthermore, although pea-derived protein is considered to be very rich in lysine, its content was still lower when compared to milk-derived protein (**Table 3.2**). These differences in amino acid composition translated into lower post-prandial peak plasma EAA, leucine, and methionine concentrations (**Figure 3.3**) and a lesser post-prandial plasma amino acid availability (**Figure 3.3**) following ingestion of a single bolus of 30 g pea- when compared with milk-derived protein. The observed differences in post-prandial plasma amino acid profiles appear to be in line with previous publications showing an attenuated rise in circulating plasma amino acids following ingestion of various plant-derived proteins (such as soy, wheat, and potato protein) when compared with the ingestion of an equivalent amount of animal-derived protein [22-24]. The attenuated amino acid response may be attributed to differences in protein structure and function of plant-derived proteins that may compromise digestion and amino acid absorption and/or amino acid retention in splanchnic tissues [2, 42-44]. In this study we assessed whether such differences in the post-prandial amino acid responses also lead to differences in post-prandial muscle protein synthesis rates.

The post-prandial rise in plasma amino acid concentrations following the ingestion of pea-derived protein resulted in a strong post-prandial stimulation of muscle protein synthesis (**Figure 3.5**). Interestingly, we show that despite the lower post-prandial plasma amino acid availability following pea *vs* milk-derived protein ingestion, the post-prandial muscle protein synthetic response to pea-derived protein did not differ from milk-derived protein ingestion. Clearly, the provided pea-derived protein has sufficient potential to strongly stimulate muscle protein synthesis *in vivo* in humans. This is in line with our previous work [19], demonstrating that the ingestion of sufficient amounts (e.g. 30 g) of wheat- or potato-derived protein does not result in a lesser muscle protein synthetic response when compared to the ingestion of an equivalent amount of dairy protein in young individuals, despite a low(er) lysine and/or methionine availability. Consequently, we need to conclude that overall plasma amino acid availability, as a resultant of both endogenous and exogenous amino acid release, is sufficient to allow maximal stimulation of post-prandial muscle protein synthesis rates following the ingestion of pea-derived protein. Collectively, these findings imply that pea-derived protein represents a viable, high-quality protein source to support human nutrition, and further research might consider its utility in a wider range of contexts.

To date, most studies comparing anabolic properties of animal- versus non-animal proteins have assessed muscle protein synthesis rates following the ingestion of protein isolates or protein concentrates [14, 19-22, 24, 25]. However, our daily protein intake is generally not consumed in the form of protein isolates or concentrates, but rather in the form of whole-foods.

The matrix in which proteins are embedded in whole-foods can differ substantially between animal- and non-animal protein sources [45-47]. Most plant based whole-foods contain anti-nutritional factors (*e.g.,* dietary fiber, trypsin inhibitors or phytates) that compromise protein digestibility, attenuate the post-prandial rise in circulating amino acid concentrations and, as such, lower the capacity to increase muscle protein synthesis rates [48, 49]. Therefore, our data are restricted to (pea and milk) protein concentrates and are not necessarily reflective of the metabolic response to the ingestion of all (pea and milk) derived products. Furthermore, it should be noted that a specific amino acid deficiency of a protein or protein source may be compensated for by other proteins or protein sources, as most proteins or protein sources are typically consumed as part of a more complex meal or protein blend [47]. Therefore, we would encourage the exploration of anabolic responses to the ingestion of protein sources in the form of whole-foods and more complex, composite meals. The latter may provide even more insight in the impact of our food processing and consumption on post-prandial protein handling and subsequent muscle maintenance.

In conclusion, ingestion of 30 g pea-derived protein stimulates muscle protein synthesis rates in young, healthy males. Post-prandial muscle protein synthesis rates following the ingestion of 30 g pea-derived protein do not differ from rates observed after ingesting 30 g milk-derived protein. Ingestion of a meal-like (30 g) dose of plant-derived protein can be as effective as ingesting an equivalent amount of animal-derived protein to increase muscle protein synthesis rates *in vivo* in healthy, young males.

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Conflicts of Interest

PJMP, IWKK, JPBG, LCPGMdG, LBV, TS and LJCvL have the following interests: This study was funded by TiFN, Wageningen, The Netherlands. The sponsors Tereos Syral (Marckolsheim, France), Cargill (Minneapolis, MN, USA), and Kellogg (Battle Creek, MI, USA) partly financed the project. The researchers are responsible for the study design, data collection and analysis, decision to publish, and preparation of the manuscript. The industrial partners have contributed to the project through regular discussion, and were involved in the study design. More specifically: the choice of interventional products which were produced by these sponsors. The funders had no role in data collection and analysis, decision to publish, or preparation of the manuscript. In addition: LJCvL and LBV have received research grants, consulting fees, speaking honoraria, or a combination of these from Friesland Campina, Tereos Syral, and Pepsico. The other authors report no conflicts of interest.

Authorship

The author contributions were as follows: LJCvL, TS, LBV, LCPGMdG, and PJMP designed research; PJMP, JSJS, IWKK, JPBG, and APBG conducted research; PJMP, TS, and LJCvL analyzed data; PJMP and LJCvL wrote paper; PJMP, TS, and LJCvL had primary responsibility for final content. All authors read and approved the final manuscript.

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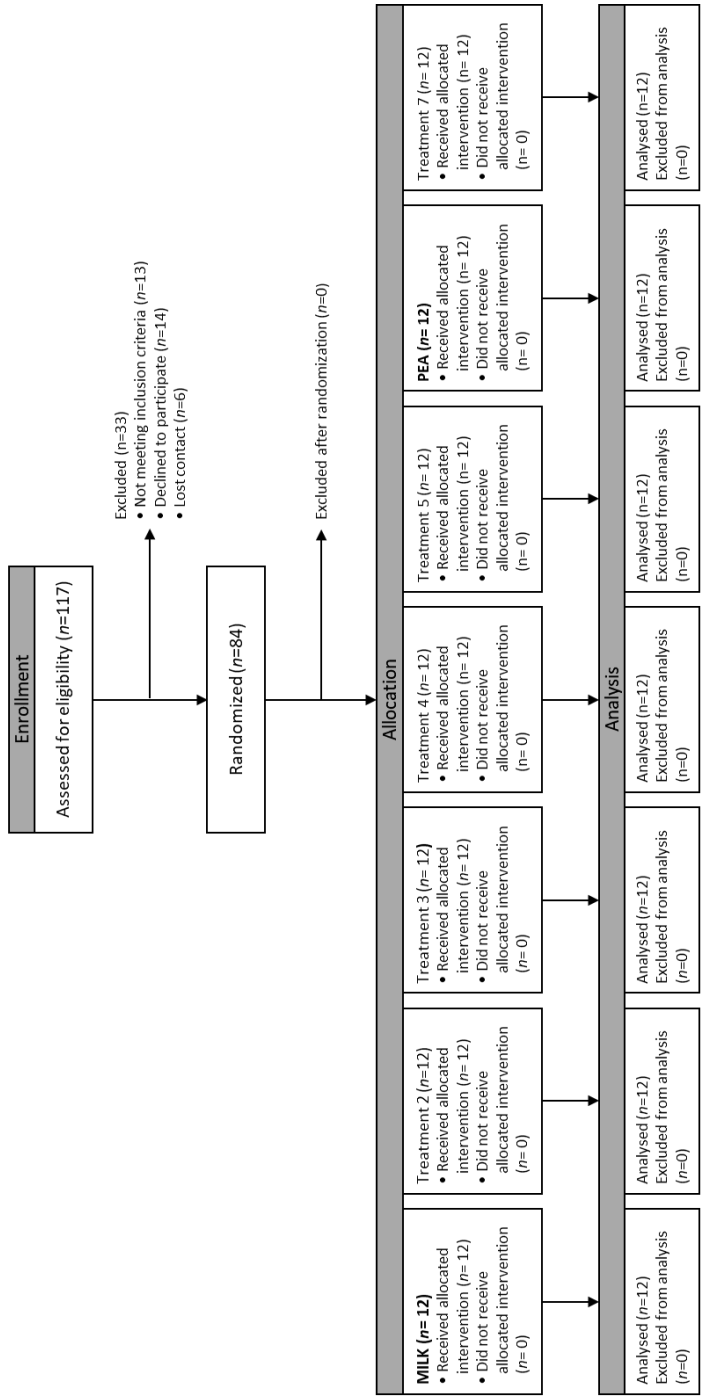
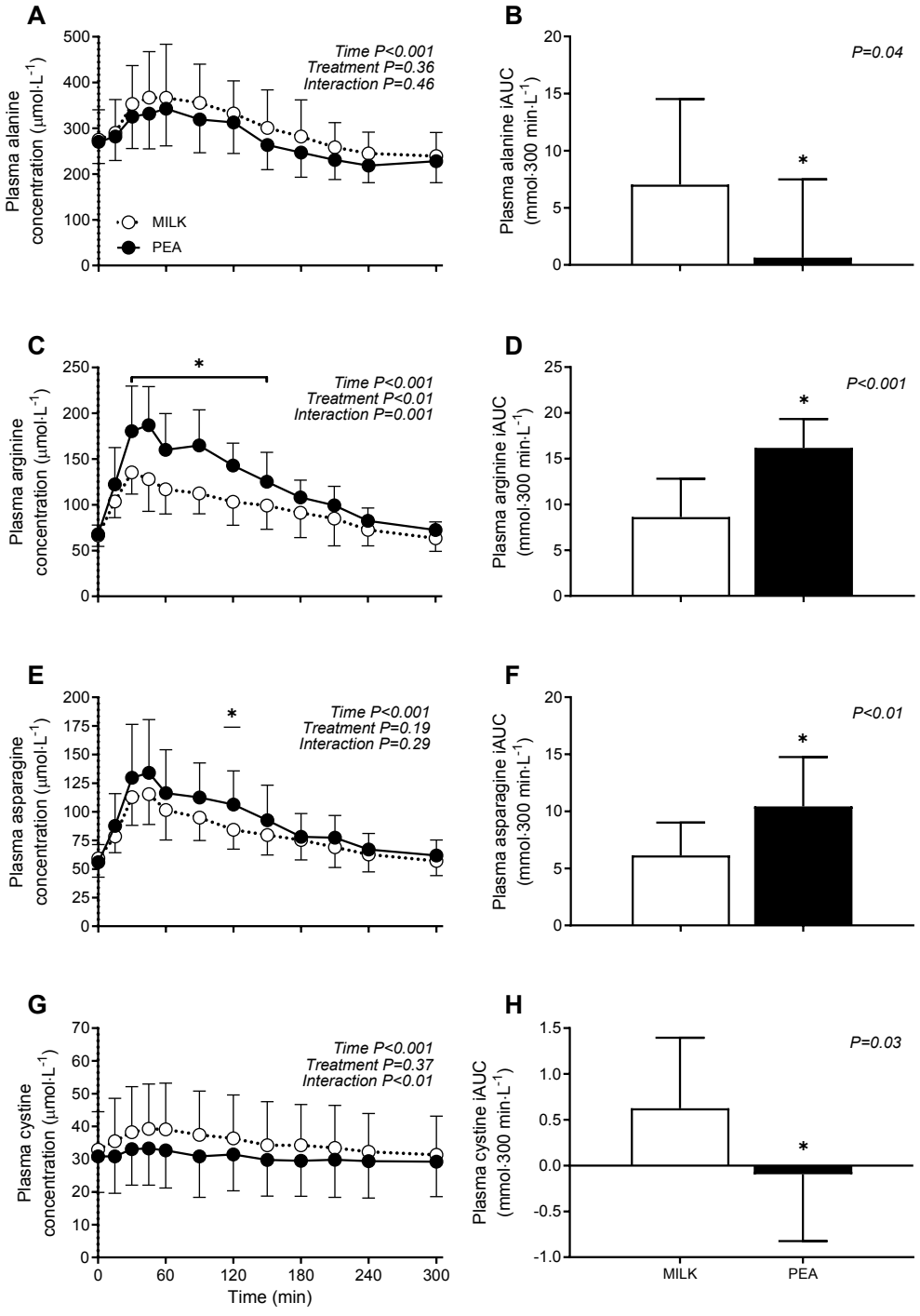
SUPPLEMENTAL MATERIAL



SUPPLEMENTAL FIGURE 3.1 CONSORT flow diagram. CONSORT, Consolidated Standards of Reporting Trials. The current study was part of a larger trial with a total of 7 parallel groups (n = 12 per group) as indicated in the flow diagram. MILK (30 g milk protein), PEA (30 g pea protein).



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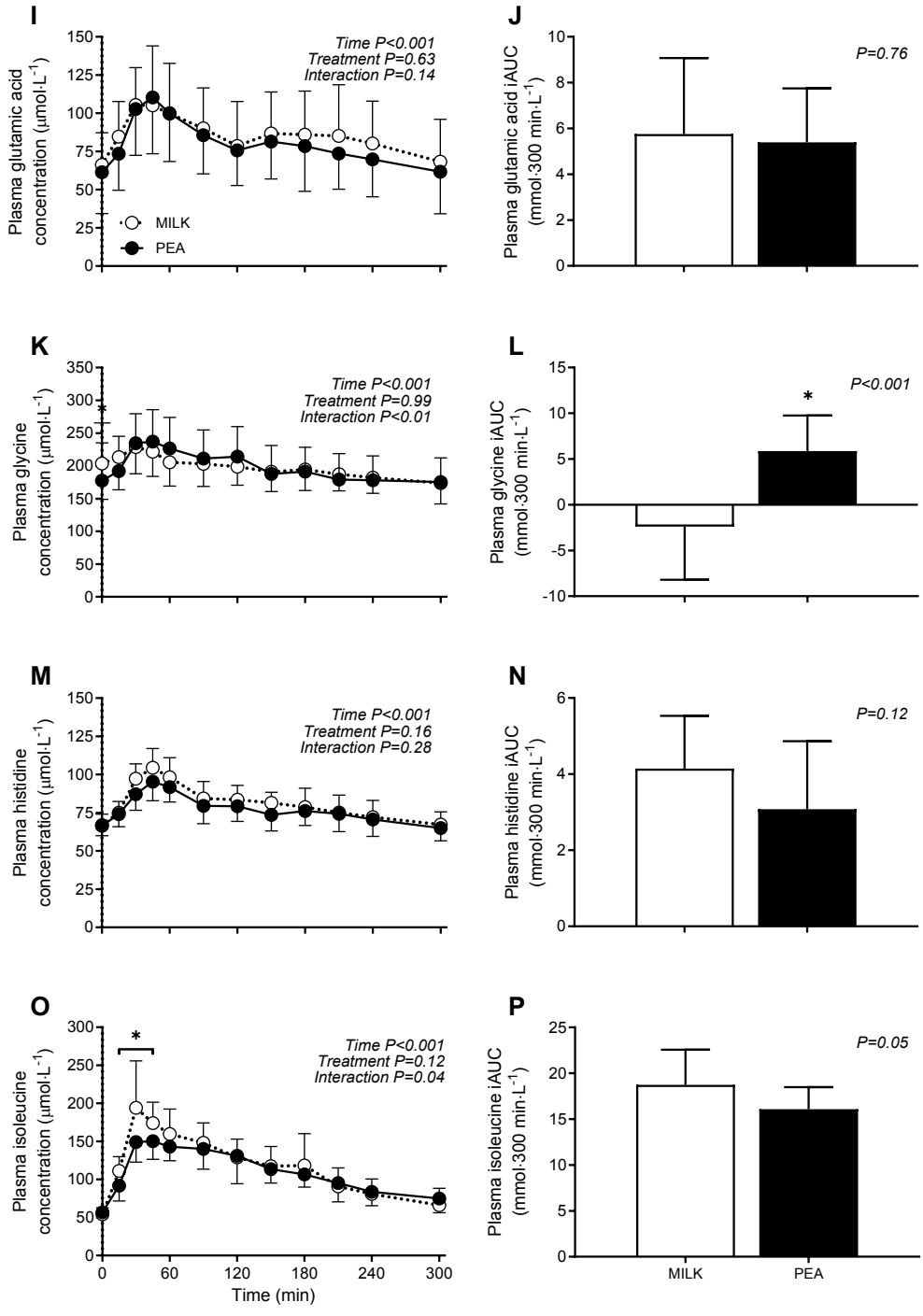

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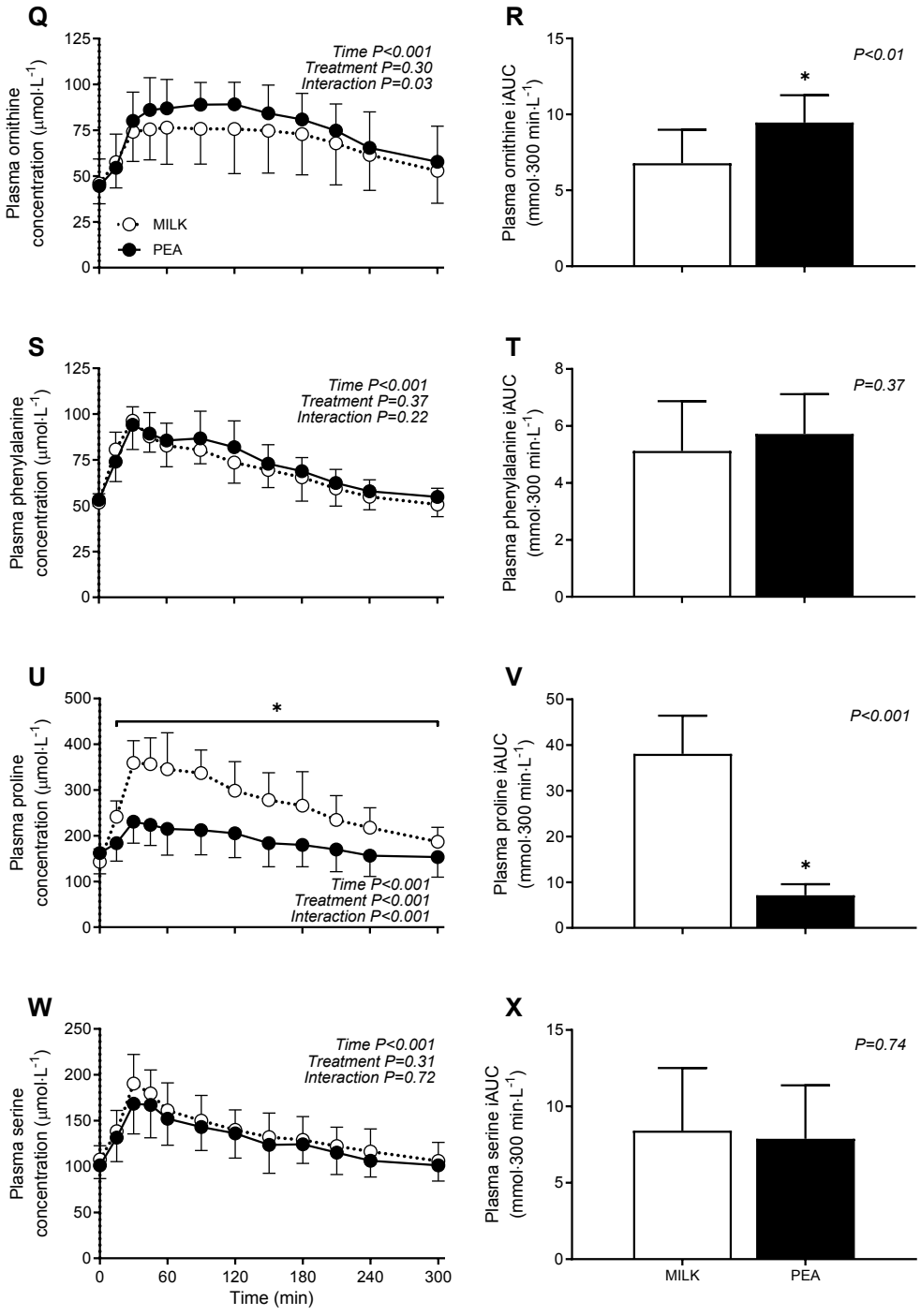

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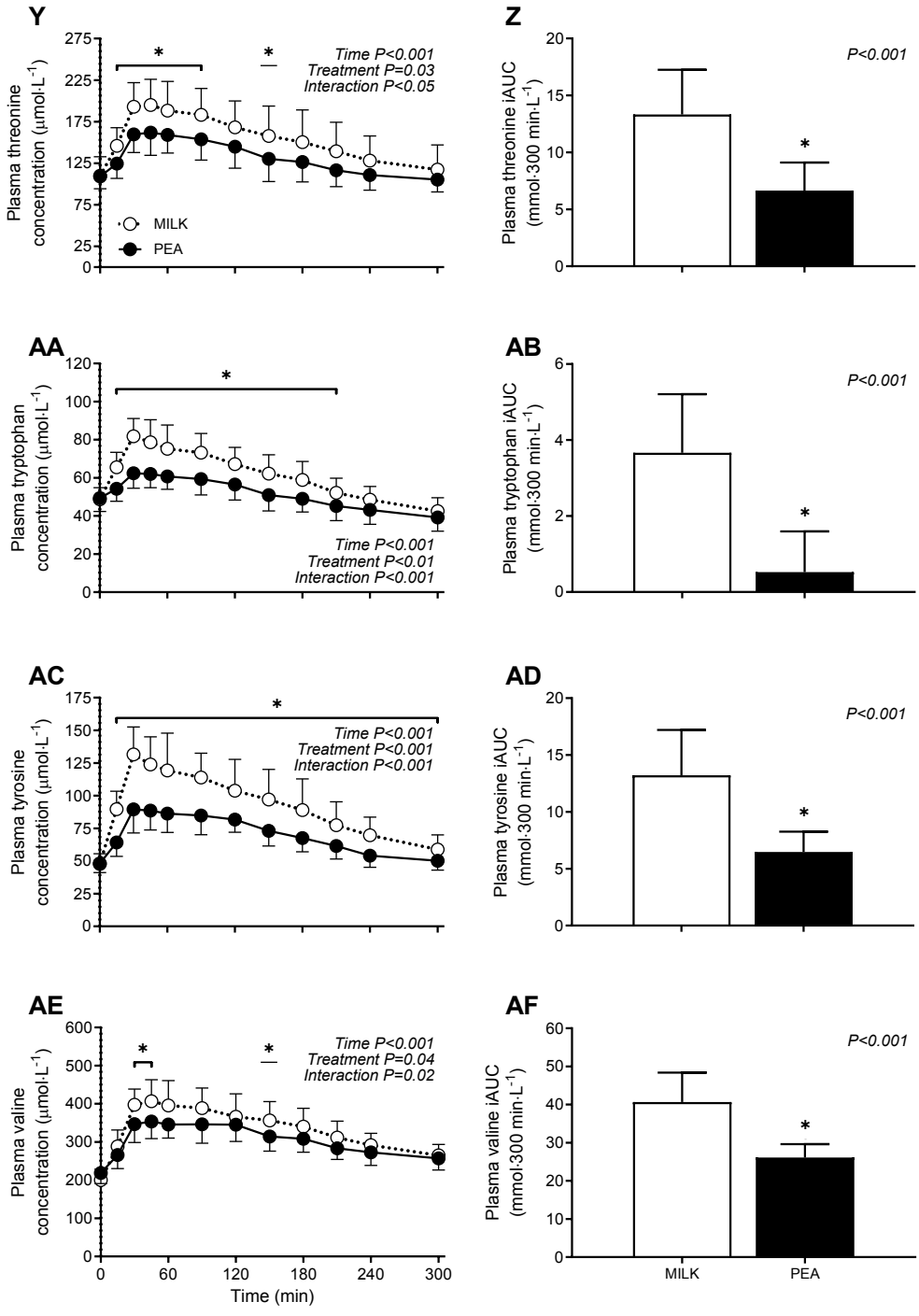

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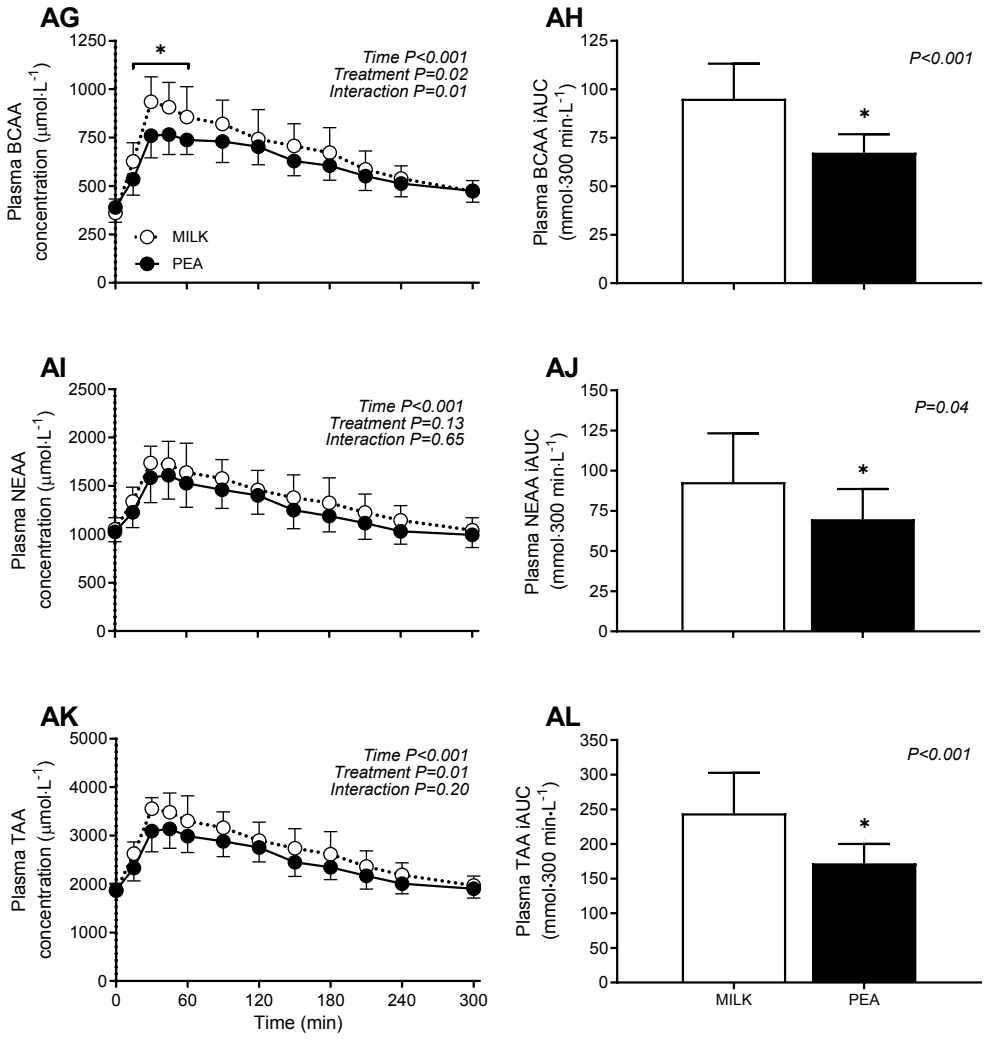

SUPPLEMENTAL FIGURE 3.2 Post-prandial plasma amino concentrations during the 300 min post-prandial period following the ingestion of MILK vs PEA. Time 0 min represents time of beverage intake. Panels B, D, F, H, J, L, N, P, R, T, V, X, Z, AB, AD, AF, AH, AJ, AL represent the 0-5 h incremental area under curve (iAUC) following protein ingestion. MILK (30 g milk protein), PEA (30 g pea protein). Values represent means ± standard deviation; *significantly different between interventions (P<0.05). Repeated measures ANOVA with time as within-subject variable and interventional drink (treatment) as between-subject variable, and independent samples t-test were used to determine differences between groups.

SUPPLEMENTAL TABLE 3.1 Average 3 day dietary intake of study participants

	MILK	PEA
Energy ($MJ \cdot d^{-1}$)	9.3 ± 2.2	9.2 ± 2.2
Carbohydrate ($g \cdot d^{-1}$)	267 ± 63	246 ± 47
Fat ($g \cdot d^{-1}$)	78 ± 27	87 ± 33
Protein ($g \cdot d^{-1}$)	97 ± 29	100 ± 40
Energy ($kJ \cdot kg^{-1} \cdot d^{-1}$)	131 ± 26	130 ± 37
Carbohydrate ($g \cdot kg^{-1} \cdot d^{-1}$)	3.8 ± 0.9	3.5 ± 0.8
Fat ($g \cdot kg^{-1} \cdot d^{-1}$)	1.1 ± 0.3	1.2 ± 0.5
Protein ($g \cdot kg^{-1} \cdot d^{-1}$)	1.3 ± 0.4	1.4 ± 0.6
Carbohydrate (% total energy)	50 ± 7	47 ± 8
Fat (% total energy)	33 ± 8	35 ± 7
Protein (% total energy)	18 ± 3	18 ± 5

Values represent mean ± standard deviation. *n*=12 per nutritional intervention group. MILK: 30 g milk protein, PEA: 30 g pea protein. Independent samples *t*-test for MILK *vs* PEA. Independent samples *t-test* between groups: all *P*>0.05. 3 Day food records were analyzed using "Mijn Eetmeter" (https://mijn.voedingscentrum.nl/nl/eetmeter/), online software available from the Netherlands Nutrition Centre.

CHAPTER 4

NO DIFFERENCES IN MUSCLE PROTEIN SYNTHESIS RATES FOLLOWING INGESTION OF WHEAT PROTEIN, MILK PROTEIN, AND THEIR PROTEIN BLEND IN HEALTHY, YOUNG MALES

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ABSTRACT

Background

Plant-derived proteins have been suggested to have less anabolic properties when compared with animal-derived proteins. Whether blends of plant- and animal-derived proteins can compensate for their lesser anabolic potential has not been assessed.

Objective

This study compares post-prandial muscle protein synthesis rates following the ingestion of milk protein with wheat protein or a blend of wheat plus milk protein in healthy, young males.

Methods

In a randomized, double blind, parallel-group design, 36 males (23±3 y) received a primed continuous L-[ring-$^{13}C_6$]-phenylalanine infusion after which they ingested 30 g milk protein (MILK), 30 g wheat protein (WHEAT), or a 30 g blend combining 15 g wheat plus 15 g milk protein (WHEAT+MILK). Blood and muscle biopsies were collected frequently for 5 hours to assess post-prandial plasma amino acid profiles and subsequent myofibrillar protein synthesis rates.

Results

Ingestion of protein increased myofibrillar protein synthesis rates in all treatments ($P<0.001$). Post-prandial myofibrillar protein synthesis rates did not differ between MILK *vs* WHEAT (0.053±0.013 *vs* 0.056±0.012 %·h-1, respectively; *t*-test $P=0.56$) or between MILK vs WHEAT+MILK (0.053±0.013 *vs* 0.059±0.025 %·h-1, respectively; *t*-test $P=0.46$).

Conclusion

Ingestion of 30 g milk protein, 30 g wheat protein, or a blend of 15 g wheat plus 15 g milk protein increases muscle protein synthesis rates in young males. Furthermore, muscle protein synthesis rates following the ingestion of 30 g milk protein do not differ from rates observed after ingesting 30 g wheat protein or a blend with 15 g milk plus 15 g wheat protein in healthy, young males.

INTRODUCTION

Protein ingestion increases muscle protein synthesis rates [1, 2]. The increase in muscle protein synthesis rate is believed to be driven by the post-prandial increase in plasma essential amino acid (EAA) concentrations [3], with the rise in plasma leucine concentration being of particular relevance [4-8]. The anabolic properties of different types of protein seem to be largely determined by their EAA content, amino acid profile, as well as their protein digestion and amino acid absorption kinetics [9-11]. As a result, post-prandial muscle protein synthesis rates may differ substantially following ingestion of the same amount of protein derived from different protein sources [12-14].

The various dietary protein sources can be classified as animal- or plant-derived proteins. Plant based proteins are suggested to provide a lesser anabolic stimulus due to their lower digestibility and incomplete amino acid (AA) profile with typically low levels of leucine, lysine, and/or methionine [15, 16]. However, plant-derived proteins comprise a large part of our daily protein intake [17] and will become more important with respect to future global protein needs and more sustainable protein production, as plant-based protein sources require less water, land, and energy resources when compared to the production of animal-based proteins [15, 18]. So far, few studies have assessed the muscle protein synthetic response to the ingestion of plant-derived proteins in vivo in humans [14, 19-21]. Ingestion of soy protein has been shown to result in lower [19, 20] or similar [14, 21] post-prandial muscle protein synthesis rates when compared to the ingestion of dairy protein. More plant-derived proteins should be investigated for their properties to stimulate muscle protein synthesis rates.

Wheat protein is the most abundant plant-based protein source [17]. Wheat protein contains an insufficient amount of EAA according to the WHO/FAO/UNU amino acid requirements [22] and a lower amount of leucine when compared to animal proteins [23]. Theoretically, this should compromise its capacity to stimulate post-prandial muscle protein synthesis rates. Recently, Gorissen et al. [24] reported a lower muscle protein synthetic response following ingestion of 35 g wheat protein when compared to 35 g casein in older males. However, the lesser muscle protein synthetic response could be compensated for by ingesting nearly double the amount of wheat protein. Of course, simply increasing the amount of protein intake is not always practical. Therefore, other strategies such as the fortification of plant-derived proteins with their limiting amino acids have been suggested as a means to improve the overall quality of plant-derived proteins [15]. Alternatively, blends of different protein sources may provide a more practical and feasible strategy to improve overall protein quality [25], thereby increasing the anabolic response to protein feeding [26]. Since more than half of the worldwide protein consumption originates from plants [17], blends of both plant- and animal-derived proteins may represent an effective and practical strategy to improve the overall quality of the ingested protein, while reducing the amount of animal-derived protein in our diet.

We hypothesize that the ingestion of 30 g milk protein results in higher post-prandial muscle protein synthesis rates when compared with the ingestion of the same amount of wheat protein. However, when wheat and milk protein are combined in a 1/1 ratio, we expect these differences to not be present. To test these hypotheses, we included 36 healthy, young males to participate in a study in which we compared the impact of ingesting 30 g milk protein with the ingestion of 30 g wheat protein or a protein blend combining 15 g wheat plus 15 g milk protein on post-prandial muscle protein synthesis rates in vivo in humans.

MATERIALS AND METHODS

Participants

Thirty-six healthy males (23±3 y; 1.79±0.06 m; 71.5±8.3 kg) volunteered to participate in this parallel group, double blind, randomized controlled trial (participants' characteristics are presented in **Table 4.1**). Participants were recreationally active and generally performed between 2-4 exercise sessions per week in various sports (e.g. soccer, basketball, weight lifting, running, cycling, etc.), but were not involved in any structured progressive exercise training regimen. This study was part of a larger trial registered at the Netherlands Trial Register (NTR6548, https://www.trialregister.nl/trial/6364), and was conducted between June 2017 and April 2019 at Maastricht University in Maastricht, The Netherlands (See **Supplemental Figure 4.1** for the CONSORT (Consolidated Standards of Reporting Trials) flow diagram). All participants were informed about the purpose of the study, the experimental procedures, and possible risks before providing informed written consent to participate. The procedures followed were in accordance with the ethical standards of the medical ethics committee of Maastricht University Medical Centre+ (METC 173001), and in accordance with the Helsinki Declaration of 1975 as revised in October 2013. The study was independently monitored and audited by the Clinical Trial Centre Maastricht.

Preliminary testing

Participants aged 18-35 y, with BMI >18.5 and <27.5 $kg \cdot m^{-2}$ underwent an initial screening session to assess eligibility. Height, weight, blood pressure and body composition (by dual-energy X-ray absorptiometry; Discovery A, Hologic; (National Health and Nutrition Examination Survey - Body composition analysis (NHANES BCA) enabled) were determined. Participants were deemed healthy based on their responses to a medical questionnaire. The screening sessions and experimental trials were separated by at least 3 days.

Study design

Participants were randomly assigned to ingest a 400 mL beverage containing either 30 g milk protein concentrate (MILK), 30 g wheat protein hydrolysate (WHEAT), or 15 g wheat protein hydrolysate plus 15 g milk protein concentrate (WHEAT+MILK). After beverage ingestion, the bottle was rinsed with 150 mL of water, which was also ingested by the participants. Milk protein concentrate (Refit MPC80) was obtained from FrieslandCampina (Wageningen, the Netherlands) and wheat protein hydrolysate (Meripro 500) was supplied by Tereos Syral

TABLE 4.1 Participants' characteristics

	MILK	WHEAT+MILK	WHEAT
Age (y)	26 ± 4	22 ± 3	23 ± 3
Height (m)	1.76 ± 0.06	1.80 ± 0.06	1.80 ± 0.07
Mass (kg)	71.5 ± 9.0	72.8 ± 6.9	70.5 ± 9.7
BMI ($kg \cdot m^{-2}$)	23.0 ± 2.1	22.5 ± 1.5	21.7 ± 2.0
Systolic blood pressure (mmHg)	119 ± 6	123 ± 13	121 ± 10
Diastolic blood Pressure (mmHg)	71 ± 9	70 ± 11	67 ± 9
Resting heart rate (bpm)	64 ± 10	62 ± 8	63 ± 10
Lean body mass (kg)	53.2 ± 7.9	56.2 ± 5.8	54.1 ± 6.0
Body fat (%)	23.1 ± 3.2	21.4 ± 5.5	20.0 ± 2.8

Values represent mean ± standard deviation. *n* = 12 per nutritional intervention group. MILK: 30 g milk protein, WHEAT+MILK: 15 g wheat protein plus 15 g milk protein, WHEAT: 30 g wheat protein. Independent samples *t*-test for MILK *vs* WHEAT and MILK *vs* WHEAT+MILK all $P>0.05$.

(Marckolsheim, France). Participants were allocated to a treatment according to a block randomization list (blocks of 7) performed using a computerized randomizer (http://www.randomization.com/). An independent researcher was responsible for random assignment (*n*=12 per group) and preparation of the study treatment beverages, which were sequentially numbered according to subject number. The beverages were prepared in non-transparent protein-shakers.

Diet and physical activity

Participants refrained from sports and strenuous physical activities (e.g. lifting heavy weights), and alcohol consumption for 3 days prior to the experimental trial. In addition, all participants were instructed to complete a food and activity record for 3 days prior to the experimental trial (See **Supplemental Table 4.1** for an overview of participants' habitual food intake in the 3 days prior to the experimental trial). The evening before the trial, all participants consumed a standardized meal containing 2.8 MJ of energy, with 65% energy provided as carbohydrate, 20% as fat, and 15% as protein, before 10:00 PM after which they remained fasted.

Experimental protocol

At ~7:30 AM, participants arrived at the laboratory in an overnight post-absorptive state. A cannula was inserted into an antecubital vein for stable isotope amino acid infusion. A second cannula was inserted retrogradely into a dorsal hand vein on the contralateral arm for arterialized blood sampling. To obtain arterialized blood samples, the hand was placed in a hot box (60°C) for 10 min prior to blood sample collection.

After taking a baseline blood sample (t= -180 min), the plasma phenylalanine pool was primed with a single dose of L-[ring-$^{13}C_6$]-phenylalanine (2.25 $\mu mol \cdot kg^{-1}$). Thereafter, a continuous intravenous infusion of L-[ring-$^{13}C_6$]-phenylalanine (0.05 $\mu mol \cdot kg^{-1} \cdot min^{-1}$) was initiated (t= -180 min) using a calibrated IVAC 598 pump (San Diego, CA, USA). Subsequently, arterialized

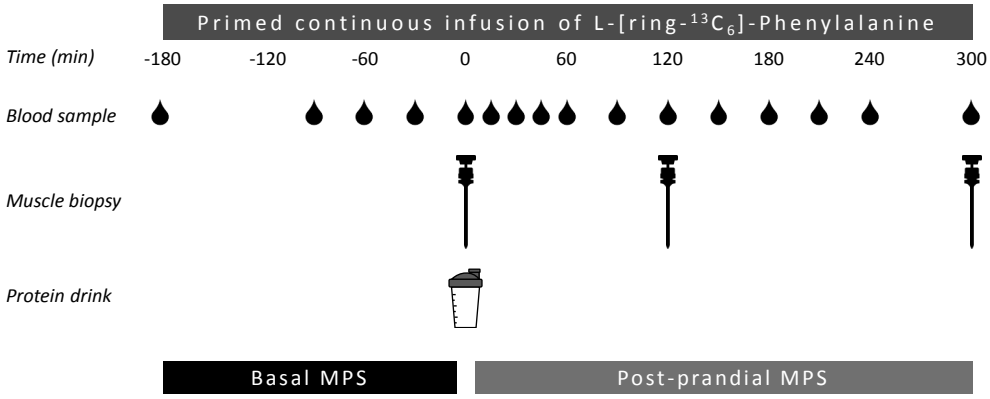

FIGURE 4.1 Schematic representation of the experimental design.

blood samples were collected at t=-90, -60 and -30 min. At t= 0 min an arterialized blood sample was obtained as well as a muscle biopsy from the M. vastus lateralis. Immediately following the muscle biopsy, participants ingested a 400 mL beverage corresponding to their randomized treatment allocation i.e.: MILK (*n*=12), WHEAT (*n*=12), or WHEAT+MILK (*n*=12). To minimize dilution of the steady-state plasma L-[ring-$^{13}C_6$]-phenylalanine precursor pool, the phenylalanine content of each protein drink was enriched with 3.85% free, crystalline L-[ring-$^{13}C_6$]-phenylalanine [21, 27]. Arterialized blood samples were then collected at t= 15, 30, 45, 60, 90, 120, 150, 180, 210, 240, and 300 min after protein ingestion in the post-prandial period. Blood samples were collected into EDTA-containing tubes and centrifuged at 1200g for 10 min at 4°C. Aliquots of plasma were frozen in liquid nitrogen and stored at -80°C. A second and third muscle biopsy from the M. vastus lateralis were collected at t= 120 and t= 300 min to determine post-prandial skeletal muscle protein synthesis rates over the 0-120, 120-300, and 0-300 min post-prandial period. Muscle biopsy collection was alternated between legs and obtained with the use of a 5 mm Bergström needle [28], custom-adapted for manual suction. Samples were obtained from separate incisions from the middle region of the M. vastus lateralis, ~15 cm above the patella and ~3 cm below entry through the fascia. Local anesthetic (1% Xylocaine with adrenaline 1:100,000) was applied to numb the skin and fascia. Muscle samples were freed from any visible non-muscle material, immediately frozen in liquid nitrogen, and stored at -80°C until further processing. When the experimental protocol was complete, cannulae were removed and participants were provided with food and monitored for ~30 min before leaving the laboratory. For a schematic representation of the infusion protocol, see **Figure 4.1**.

Protein powder analysis

Batch specific nitrogen contents of both milk protein concentrate and wheat protein hydrolysate were provided by the manufacturer. The protein content of the milk protein was determined as nitrogen content x 6.38 and the protein content of wheat protein powder was determined as nitrogen content x 5.7 [29, 30]. Amino acid contents of the protein powders were determined by acid hydrolysis in triplicate. Specifically, the amino acids were liberated

from the protein powders (~4 mg) by adding 2 mL of 6M HCl and heating to 110°C for 12 h. The hydrolyzed free amino acids were subsequently dried under a nitrogen stream while heated to 120°C. Before analysis using ultra-performance liquid chromatography-mass spectrometry (UPLC-MS; ACQUITY UPLC H-Class with QDa; Waters, Saint-Quentin, France), the hydrolysate was dissolved in 5 mL of 0.1 M HCl and 20 µL of AccQ/Tag derivatizing reagent solution (Waters, Saint-Quentin, France) was added as described below for the plasma amino acid concentration analysis. The amino acid composition of the protein powders and the protein blend are presented in **Table 4.2**.

Plasma analysis

Plasma glucose and insulin concentrations were analyzed using commercially available kits (GLUC3, Roche, Ref: 05168791190, and immunologic, Roche, Ref: 12017547122, respectively). Plasma amino acid concentrations were determined by UPLC-MS. Specifically, 50 µL blood plasma was deproteinized using 100 µL of 10% SSA with 50 µM of MSK-A2 internal standard (Cambridge Isotope Laboratories, Massachusetts, USA). Subsequently, 50 µL of ultra-pure demineralized water was added and samples were centrifuged. After centrifugation, 10 µL of supernatant was added to 70 µL of Borate reaction buffer (Waters, Saint-Quentin, France). In addition, 20 µL of AccQ/Tag derivatizing reagent solution (Waters, Saint/Quentin, France) was added after which the solution was heated to 55 °C for 10 min. Of this 100 µL derivative, 1 µL was injected and measured using UPLC-MS.

Plasma L-[ring-$^{13}C_6$]-phenylalanine enrichments were determined by gas chromatography-mass spectrometry (GC-MS; Agilent 7890A GC/5975C MSD; Agilent Technologies). Specifically, the plasma was deproteinized on ice with dry 5-sulfosalicyclic acid. Free amino acids were purified using cation exchange resin columns (AG 50W-X8, mesh size: 100-200, ionic form: hydrogen (Bio-Rad Laboratories, Hercules, CA, USA)). The free amino acids were converted to their tert-butyl dimethylsilyl (TBDMS) derivative before analysis by GC-MS using selected ion monitoring of masses 336 and 342 for unlabeled and [ring-$^{13}C_6$]-labelled phenylalanine, respectively. Standard regression curves were applied from a series of known standard enrichment values against the measured values to assess the linearity of the mass spectrometer and to account for any isotope fractionation which may have occurred during the analysis.

Basal muscle protein synthesis rates were assessed to confirm that protein ingestion increases muscle protein synthesis rates. The single biopsy approach was applied to assess post-absorptive muscle protein synthesis rates without the need to collect an additional muscle biopsy [31]. In short, plasma protein obtained prior to tracer infusion (t= -180 min) was used to determine background L-[ring-$^{13}C_6$]-phenylalanine enrichments. For this purpose, the plasma sample was precipitated by adding perchloric acid. Subsequently, similarly as for the myofibrillar protein fraction, the denaturized plasma protein pellet was hydrolyzed, passed over a cation exchange resin column (AG 50W-X8, mesh size: 100-200, ionic form: hydrogen (Bio-Rad Laboratories, Hercules, CA, USA)), and the resulting amino acid samples were derivatized to their N(O,S)-ethoxycarbonyl-ethylesters before being measured by gas

TABLE 4.2 Protein drink amino acid composition

	MILK	WHEAT+MILK[1]	WHEAT
Alanine	0.9	0.8	0.7
Arginine	0.8	0.8	0.8
Aspartic acid	1.8	1.3	0.8
Cystine	0.1	0.2	0.3
Glutamic acid	5.1	7.8	10.5
Glycine	0.5	0.8	1.1
Histidine	0.6	0.5	0.5
Isoleucine	0.9	0.7	0.6
Leucine	2.4	2.1	1.8
Lysine	2.0	1.2	0.4
Methionine	0.7	0.6	0.4
Phenylalanine	1.2	1.3	1.4
Proline	2.9	3.5	4.1
Serine	1.2	1.3	1.4
Threonine	0.9	0.8	0.7
Tyrosine	0.6	0.5	0.4
Valine	1.1	0.9	0.7
TAA	23.8	25.2	26.7
EAA	9.8	8.2	6.5
BCAA	4.4	3.7	3.1
Nitrogen content (%)	13.4	13.6	13.8
Protein content (%)	85.5[2]	82.2	78.9[3]

Values for amino acid contents are in grams per 30 g protein. MILK: 30 g milk protein, WHEAT+MILK: 15 g wheat protein plus 15 g milk protein, WHEAT: 30 g wheat protein. [1]Values are obtained by averaging the measured values for wheat and milk protein. [2]Protein as nitrogen content x 6.38; [3]Protein as nitrogen content x 5.7.

chromatography-combustion-isotope ratio mass spectrometry (GC-IRMS; Mat 253, Thermo Scientific, Bremen, Germany) using a DB5MS (30m) column (Agilent technologies, Santa Clara, Ca, USA), as explained below.

Muscle analysis

A piece of wet muscle (~50-70 mg) was homogenized on ice using a Teflon pestle in ice-cold homogenization buffer (7 µL/mg; 67 mM sucrose, 50 mM Tris/HCl, 50 mM KCl, 10 mM EDTA) containing Complete Mini protease inhibitor cocktail and PhosSTOP (Roche Applied Science). After ~3 min of hand homogenization, the homogenate was centrifuged at 2,200*g* for 5 min at 4°C to precipitate the myofibrillar proteins. The protein pellet was washed once with MilliQ water and centrifuged at 250g for 10 min at 4°C. The myofibrillar proteins were solubilized by adding 1 mL of 0.3 M NaOH and heating to 50°C for 30 min with vortex mixing

every 10 min. Samples were centrifuged at 11,000g for 5 min at 4°C and the supernatant containing the myofibrillar protein-enriched fraction was collected. The collagen pellets were washed once with 0.3 M NaOH and centrifuged at 11,000g for 5 min at 4°C. The resulting supernatant was added to the already collected myofibrillar protein-enriched fraction and the collagen pellets were discarded. Myofibrillar proteins were precipitated by the addition of 1 mL of 1 M perchloric acid and centrifuged at 800g for 10 min at 4°C. The myofibrillar protein-enriched fraction was washed twice with 70% ethanol and centrifuged at 450g. The amino acids were liberated from the myofibrillar protein-enriched fraction by adding 2 mL of 6 M HCl and heating to 110°C for 16 h. The hydrolyzed myofibrillar protein fractions were dried under a nitrogen stream while heated to 120°C. The dried myofibrillar protein fraction was dissolved in a 50% acetic acid solution. The amino acids from the myofibrillar protein fraction were passed over a cation exchange resin column (AG 50W-X8, mesh size: 100-200, ionic form: hydrogen (Bio-Rad Laboratories, Hercules, CA, USA)). Subsequently, the purified amino acid solution was dried under a nitrogen stream at room temperature, followed by derivatization to their N(O,S)-ethoxycarbonyl-ethylesters. The ratio of $^{13}C/^{12}C$ of myofibrillar protein-bound phenylalanine was determined using GC-IRMS by monitoring ion masses 44, 45 and 46. Standard regression curves were applied from a series of known standard enrichment values against the measured values to assess the linearity of the mass spectrometer and to account for any isotope fractionation which may have occurred during the analysis.

Muscle intra-cellular enrichments were determined from a separate piece of muscle. Specifically, a piece of wet muscle (~50-70 mg) was freeze dried for 48 h. Collagen, excessive blood and other non-muscle materials were subsequently removed from the muscle fibers under a light microscope. The isolated muscle fiber mass was weighed and 35 volumes (7x wet weight of isolated muscle fibers x wet-to-dry ratio 5:1) of ice-cold 2% perchloric acid was added. Thereafter, the tissue was homogenized by sonification and centrifuged to separate the supernatant from the protein. The supernatants containing the muscle intra-cellular free amino acids were purified, and derivatized before analysis by GC-MS, similarly as for the plasma L-[ring $^{13}C_6$]-phenylalanine enrichments.

Calculations

Fractional myofibrillar protein synthesis rates (%·h^{-1}) were calculated by the standard precursor-product equation [32]:

$$FSR = \left(\frac{(E_{b2} - E_{b1})}{(E_{precursor} \cdot t)}\right) \cdot 100$$

Where E_b is the increment in myofibrillar protein-bound L-[ring-$^{13}C_6$]-phenylalanine enrichment (mole % excess, MPE) during the tracer incorporation period, and t is the tracer incorporation time in h. Weighted mean plasma L-[ring-$^{13}C_6$]-phenylalanine enrichments were calculated by taking the measured enrichment between consecutive time points and correcting for the time between these sampling time points ($E_{precursor}$). For calculation of post-prandial FSR, skeletal muscle biopsy samples at t= 0, 120 and 300 min were used. For the calculation of basal

FSR, E_{b2} represented the protein-bound L-[ring-$^{13}C_6$]-phenylalanine enrichments in muscle at t= 0 min, and E_{b1} represented the protein bound L-[ring-$^{13}C_6$]-phenylalanine enrichments in plasma protein at t= -180 min.

Net incremental area under curve (iAUC) was determined for plasma amino acid concentrations during the 5 h post-prandial period following protein ingestion. The iAUC was calculated using the trapezoid rule, with plasma concentrations before beverage ingestion (t= 0 min) serving as baseline.

Outcome measures

Myofibrillar FSR over the entire (i.e. 0 – 300 min) post-prandial period, comparing MILK *vs* WHEAT and MILK *vs* WHEAT+MILK was defined as the primary outcome measure. Secondary outcome measures were myofibrillar FSR in the early (i.e. 0 – 120 min) and late (i.e. 120 – 300 min) post-prandial period, plasma glucose, insulin, and amino acid concentrations and plasma amino acid iAUC, comparing MILK *vs* WHEAT and MILK *vs* WHEAT+MILK. Plasma glucose, insulin, and amino acid peak concentrations and time to peak were tertiary outcomes, comparing MILK *vs* WHEAT and MILK *vs* WHEAT+MILK.

Statistical analysis

A power calculation was performed with differences in postprandial myofibrillar FSRs between 2 treatments as primary outcome measure. A sample size of 12 participants per treatment, including a 10% dropout rate was calculated using a power of 80%, a significance level of 0.05, a standard deviation of 0.0065 %·h^{-1}, and a difference in FSR of 0.008 %·h^{-1} between treatments (or ~20% when expressed as a relative difference). Participant characteristics were analyzed by independent samples *t*-test for MILK *vs* WHEAT and MILK *vs* WHEAT+MILK. Plasma glucose, insulin, and amino acid concentrations and amino acid enrichments were analyzed by a two-way (time x treatment) repeated measures ANOVA for MILK *vs* WHEAT and MILK *vs* WHEAT+MILK. Bonferroni post hoc analysis were performed if a significant F ratio was found to isolate specific differences. Plasma glucose, insulin, and amino acid concentrations, expressed as peak values, time to peak and iAUC, were analyzed by independent samples *t*-test for MILK *vs* WHEAT and MILK *vs* WHEAT+MILK. Basal post-absorptive, and post-prandial myofibrillar protein synthesis rates during the early (0-120 min) and entire (0-300 min) post-prandial period were analyzed by independent samples *t*-test for MILK *vs* WHEAT and MILK *vs* WHEAT+MILK. Statistical analyses were performed with a software package (IBM SPSS statistics for Windows, version 26.0, IBM Corp., Armonk, NY, USA). Means were considered to be significantly different for $P < 0.05$. Data are expressed as means±SD.

RESULTS

Plasma glucose and insulin concentrations

Plasma glucose concentrations did not change following protein ingestion (**Figure 4.2A**), and did not differ between MILK *vs* WHEAT (*time x treatment: P*=0.09) or MILK *vs* WHEAT+MILK (*time x treatment: P*=0.71). Plasma insulin concentrations increased following protein ingestion, with no differences in peak plasma insulin concentrations and iAUC between MILK and WHEAT (*P*=0.79 and *P*=0.12, respectively) or between MILK and WHEAT+MILK (*P*=0.08 and *P*=0.77, respectively; **Figure 4.2B**).

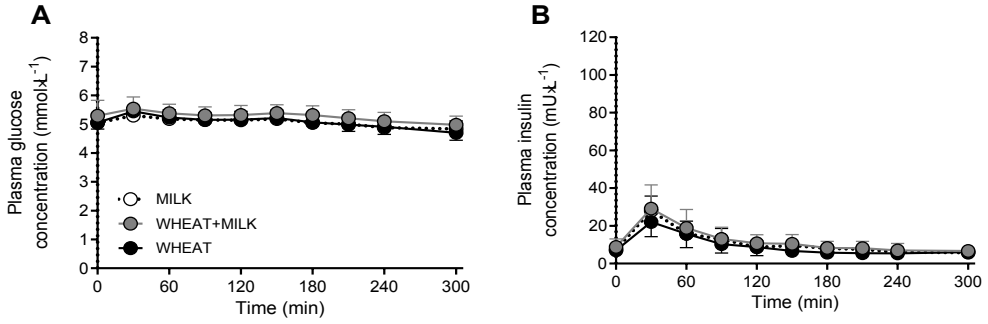

FIGURE 4.2 Post-prandial plasma glucose (Panel A) and insulin (Panel B) concentrations during the 300 min period following the ingestion of MILK *vs* WHEAT and MILK *vs* WHEAT+MILK in healthy, young males (*n*=12 per group). Time 0 min represents time of beverage intake. MILK: 30 g milk protein, WHEAT: 30 g wheat protein, WHEAT+MILK: 15 g wheat protein + 15 g milk protein. Values represent means ± standard deviation; Repeated measures ANOVA with time as within-subjects variable and interventional drink (treatment) as between-subjects variable. *Time x treatment:* Panel A: MILK *vs* WHEAT *P*=0.09, MILK *vs* WHEAT+MILK *P*=0.71; Panel B: MILK *vs* WHEAT *P*=0.12, MILK *vs* WHEAT+MILK *P*=0.97.

Plasma AA concentrations

Plasma EAA concentrations increased following protein ingestion over time for all treatments (**Figure 4.3A**). This increase was greater for MILK *vs* WHEAT (time x treatment: *P*<0.001), but did not differ between MILK and WHEAT+MILK (time x treatment: *P*=0.06). MILK ingestion resulted in higher peak EAA concentrations *vs* WHEAT (1871±124 *vs* 1449±144 μmol·L^{-1}; *P*<0.001) and *vs* WHEAT+MILK (1871±124 *vs* 1611±160 μmol·L^{-1}; *P*<0.001). These peak EAA concentrations were reached faster following MILK *vs* WHEAT (36±10 *vs* 63±18 min; *P*<0.001), but were not different in MILK *vs* WHEAT+MILK (36±10 *vs* 43±19 min; *P*=0.26). The overall increase in plasma EAA concentrations over the entire 300 min post-prandial period, expressed as iAUC, was 110 % greater for MILK *vs* WHEAT (151±31 *vs* 72±9 mmol·300 min·L^{-1}; *P*<0.001) and 58 % greater for MILK *vs* WHEAT+MILK (151±31 *vs* 96±31 mmol·300 min·L^{-1}; *P*<0.001; **Figure 4.3B**).

Plasma leucine concentrations increased over time for all treatments following protein ingestion (**Figure 4.3C**). This increase was greater for MILK *vs* WHEAT (time x treatment: *P*<0.001), but did not differ between MILK and WHEAT+MILK (time x treatment: *P*=0.09).

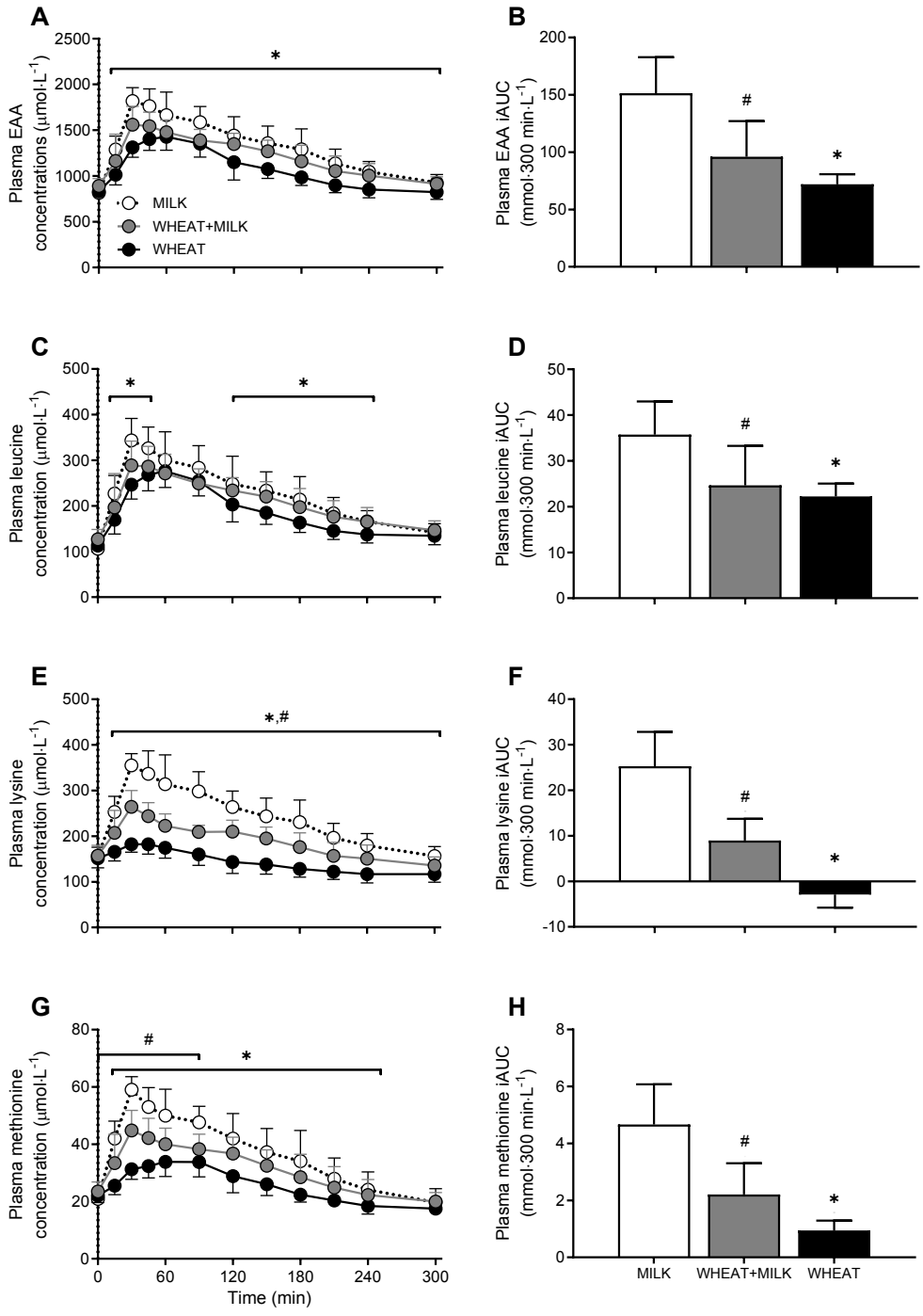

FIGURE 4.3 Post-prandial plasma essential amino acid (EAA, Panel A), leucine (Panel C), lysine (Panel E), and methionine (Panel G) concentrations during the 300 min period following the ingestion of MILK *vs* WHEAT and MILK *vs* WHEAT+MILK in healthy, young males (*n*=12 per group). Time 0 min represents time of beverage intake. Panels B, D, F and H represent the 0-5 h net incremental area under curve

(iAUC) following protein ingestion. MILK: 30 g milk protein, WHEAT: 30 g wheat protein, WHEAT+MILK: 15 g wheat protein + 15 g milk protein. Values represent means ± standard deviation; * significantly different for MILK *vs* WHEAT (P<0.05), # significantly different for MILK *vs* WHEAT+MILK (P<0.05). Repeated measures ANOVA with time as within-subject variable and interventional drink (treatment) as between-subject variable. *Time x treatment:* Panel A: MILK *vs* WHEAT P<0.001, MILK *vs* WHEAT+MILK P=0.06, Panel C: MILK *vs* WHEAT P=0.001, MILK *vs* WHEAT+MILK P=0.09, Panel E: MILK *vs* WHEAT P<0.001, MILK *vs* WHEAT+MILK P<0.001, Panel G: MILK *vs* WHEAT P<0.001, MILK *vs* WHEAT+MILK P<0.01.

MILK ingestion resulted in higher peak leucine concentrations *vs* WHEAT (353±45 *vs* 280±37 μmol·L^{-1}; P<0.001) and *vs* WHEAT+MILK (353±45 *vs* 301±44 μmol·L^{-1}; P=0.01). Time to reach these peak concentrations did not differ between interventions (MILK *vs* WHEAT: 46±43 *vs* 58±19 min; P=0.42 and MILK *vs* WHEAT+MILK: 46±43 *vs* 64±51 min; P=0.31). The overall increase in plasma leucine concentrations over the entire 300 min post-prandial period, expressed as iAUC, was 61 % greater for MILK *vs* WHEAT (36±7 *vs* 22±3 mmol·300 min·L^{-1}; P<0.001), and 45 % greater for MILK *vs* WHEAT+MILK (36±7 *vs* 25±9 mmol·300 min·L^{-1}; P<0.01; **Figure 4.3D**).

Plasma lysine concentrations increased over time for MILK and WHEAT+MILK, but not for WHEAT (**Figure 4.3E**). This increase was greater for MILK *vs* WHEAT (time x treatment: P<0.001), as well as for MILK *vs* WHEAT+MILK (time x treatment: P<0.001). MILK ingestion resulted in higher peak lysine concentrations *vs* WHEAT (370±29 *vs* 186±20 μmol·L^{-1}; P<0.001) and *vs* WHEAT+MILK (370±29 *vs* 268±32 μmol·L^{-1}; P<0.001). Time to reach these peak concentrations did not differ between interventions (MILK *vs* WHEAT: 34±7 *vs* 41±11 min; P=0.06 and MILK *vs* WHEAT+MILK: 34±7 *vs* 41±26 min; P=0.31). The overall increase in plasma lysine concentrations over the entire 300 min post-prandial period, expressed as iAUC, was much greater for MILK *vs* WHEAT (25±8 *vs* -3±3 mmol·300 min·L^{-1}; P<0.001), and 183 % greater for MILK *vs* WHEAT+MILK (25±8 *vs* 9±5 mmol·300 min·L^{-1}; P<0.001; **Figure 4.3F**).

Plasma methionine concentrations increased over time for all treatments following protein ingestion (**Figure 4.3G**). This increase was greater for MILK *vs* WHEAT (time x treatment: P<0.001), as well as for MILK *vs* WHEAT+MILK (time x treatment: P=0.002). MILK ingestion resulted in higher peak methionine concentrations *vs* WHEAT (60±5 *vs* 35±5 μmol·L^{-1}; P<0.001) and *vs* WHEAT+MILK (60±5 *vs* 46±7 μmol·L^{-1}; P<0.001). These peak methionine concentrations were reached faster following MILK ingestion *vs* WHEAT (34±9 *vs* 73±24 min; P<0.001), but were not different *vs* WHEAT+MILK (34±9 *vs* 41±24 min; P=0.63). The overall increase in plasma methionine concentrations over the entire 300 min post-prandial period, expressed as iAUC, was 393 % greater for MILK *vs* WHEAT (5±1 *vs* 1±0.3 mmol·300 min·L^{-1}; P<0.001), and 112 % greater for MILK *vs* WHEAT+MILK (5±1 *vs* 2±1 mmol·300 min·L^{-1}; P<0.001; **Figure 4.3H**).

In general, increases in plasma amino acid concentrations revealed significant differences over time between MILK and WHEAT for all measured amino acids except alanine, arginine, glutamic acid and ornithine (**Supplemental Figure 4.2**), while the increased plasma amino

acid concentrations did not differ between MILK and WHEAT+MILK. The increases in plasma amino acid concentrations over the entire 300 min post-prandial period (iAUC) were greater for asparagine, isoleucine, threonine, tryptophan, tyrosine and valine, and smaller for cysteine, glycine and proline for MILK *vs* WHEAT (P<0.05). For MILK *vs* WHEAT+MILK, plasma iAUC were greater for isoleucine, threonine, tryptophan, tyrosine, and valine (P<0.05, Supplemental Figure 4.2).

Plasma and muscle L-[ring-$^{13}C_6$]-phenylalanine enrichments

Plasma L-phenylalanine concentrations and L-[ring-$^{13}C_6$]-phenylalanine enrichments over time are presented in Figure 4.4A and 4.4B, respectively. Plasma L-[ring-$^{13}C_6$]-phenylalanine enrichments over time were different between MILK *vs* WHEAT at t= 60, 90, 120, and 300 min following protein ingestion (time x treatment: P<0.001), but not between MILK *vs* WHEAT+MILK (Figure 4.4B; time x treatment: P=0.51). Mean plasma L-[ring-$^{13}C_6$]-phenylalanine enrichments averaged 7.11±0.65, 6.80±0.61 and 6.65±0.51 MPE during the basal post-absorptive period, and 6.64±0.53, 6.34±0.44, and 6.25±0.36 MPE during the full 300 min post-prandial period for MILK, WHEAT+MILK, and WHEAT respectively.

Myofibrillar protein-bound L-[ring-$^{13}C_6$]-phenylalanine enrichments increased following ingestion of MILK, WHEAT+MILK and WHEAT from 0.0032±0.0032, 0.0033±0.0024, and 0.0038±0.0018 MPE at t= 0 min, to 0.0116±0.0041, 0.0123±0.0063, and 0.0107±0.0044 MPE at t= 120 min reaching 0.0214±0.0049, 0.0227±0.0094, and 0.0219±0.0047 MPE, respectively, at 300 min after protein ingestion, with no differences observed between MILK *vs* WHEAT (all P>0.56) and MILK *vs* WHEAT+MILK (all P>0.68) at any time point.

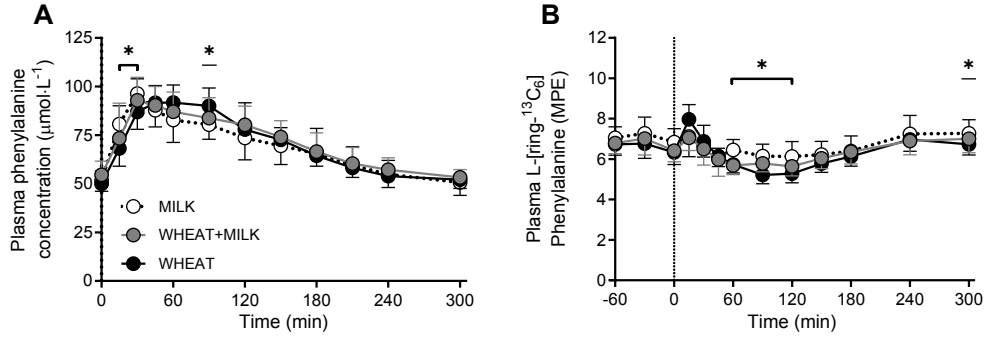

FIGURE 4.4 Post-prandial plasma phenylalanine concentrations (Panel A) and plasma 1-[$^{13}C_6$]-phenylalanine enrichments (Panel B) during the 300 min period following the ingestion of MILK *vs* WHEAT and MILK *vs* WHEAT+MILK in healthy, young males (n=12 per group). Time 0 min represents time of beverage intake. MILK: 30 g milk protein, WHEAT: 30 g wheat protein, WHEAT+MILK: 15 g wheat protein + 15 g milk protein. Values represent means ± standard deviation; * significantly different for MILK *vs* WHEAT (P<0.05). Repeated measures ANOVA with time as within-subject variable and interventional drink (treatment) as between-subject variable. *Time x treatment:* Panel A: MILK *vs* WHEAT P<0.001, MILK *vs* WHEAT+MILK P=0.29, Panel B: MILK *vs* WHEAT P<0.001, MILK *vs* WHEAT+MILK P=0.51.

Muscle protein synthesis rates

Post-absorptive fractional myofibrillar protein synthesis rates averaged 0.014±0.014, 0.016±0.011 and 0.018±0.009 %·h^{-1} in MILK, WHEAT+MILK, and WHEAT, with no differences between MILK *vs* WHEAT (*P*=0.41) and MILK *vs* WHEAT+MILK (*P*=0.81). Protein ingestion increased myofibrillar protein synthesis rates to 0.059±0.024, 0.067±0.032 and 0.053±0.025 %·h^{-1} during the early post-prandial period (0-120 min) and to 0.049±0.017, 0.054±0.036, 0.058±0.013 %·h^{-1} during the late post-prandial period (120-300 min). Post-prandial muscle protein FSR averaged 0.053±0.013, 0.059±0.025 and 0.056±0.012 %·h^{-1} assessed over the entire 300 min post-prandial period after protein ingestion (**Figure 4.5**). Post-prandial myofibrillar protein synthesis rates did not differ between MILK *vs* WHEAT, for the early (0-120 min; *P*=0.58), late (120-300 min; *P*=0.15), and entire (0-300 min; *P*=0.56) post-prandial period. Similarly, post-prandial myofibrillar protein synthesis rates did not differ between MILK *vs* WHEAT+MILK, for the early (0-120 min; *P*=0.47), late (120-300 min; *P*=0.69), and entire (0-300 min; *P*=0.46) post-prandial period (**Figure 4.5**). Myofibrillar protein synthesis rates determined with the intra-cellular L-[ring-$^{13}C_6$]-phenylalanine enrichments used as precursor pool resulted in similar findings with no differences in FSR values between Milk *vs* WHEAT and MILK *vs* WHEAT+MILK at any time point (**Supplemental Figure 4.3**).

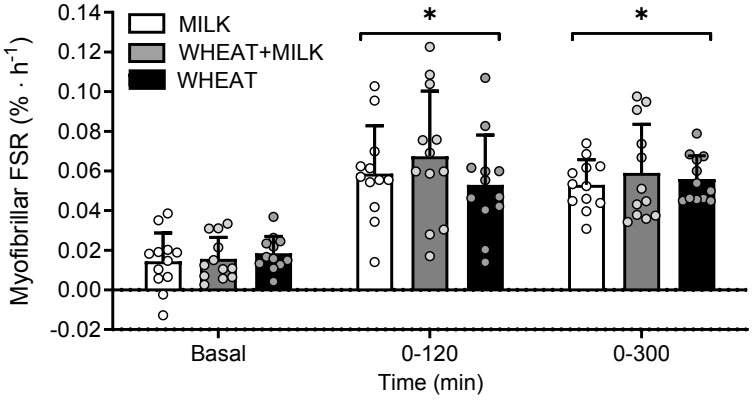

FIGURE 4.5 Myofibrillar fractional synthetic rate (FSR) at different time points following ingestion of MILK *vs* WHEAT and MILK *vs* WHEAT+MILK in healthy, young males (*n*=12 per group). MILK: 30 g milk protein, WHEAT: 30 g wheat protein, WHEAT+MILK: 15 g wheat protein + 15 g milk protein. Values represent means ± standard deviation. *significantly different from basal; *P*<0.05. Independent samples *t*-test: MILK *vs* WHEAT *P*=0.41, *P*=0.58, and *P*=0.56 for basal, 0-120, and 0-300 min, respectively. MILK *vs* WHEAT+MILK *P*=0.81, *P*=0.47, and *P*=0.46 for basal, 0-120, and 0-300 min, respectively.

DISCUSSION

The present study shows that ingestion of 30 g protein as either milk, wheat, or a blend of wheat and milk protein is followed by a robust increase in circulating amino acid concentrations in healthy, young males. Despite the observation of greater post-prandial plasma essential

amino acid availability following milk when compared to wheat or wheat plus milk protein ingestion, post-prandial myofibrillar protein synthesis rates did not differ between treatments.

Plant-derived proteins are generally considered to have a lesser capacity to stimulate post-prandial muscle protein synthesis due to among others their incomplete amino acid profile, with typical low levels of EAA, and in particular low leucine, lysine, and/or methionine contents [15, 16]. Indeed, in the present study, EAA (9.8 *vs* 6.5 g), leucine (2.4 *vs* 1.8 g), lysine (2.0 *vs* 0.4 g) and methionine (0.7 *vs* 0.4 g) contents were all substantially higher in the milk protein when compared to the wheat protein that was provided (**Table 4.2**). These differences also translated into greater post-prandial plasma EAA (+110%), leucine (+61%), lysine (+868%) and methionine (+393%) availability following milk compared with wheat protein ingestion (**Figure 4.3**). Although the amino acid profile of the various proteins were reflected by the post-prandial plasma amino acid concentrations, these differences did not affect the post-prandial increase in myofibrillar protein synthesis rates following the ingestion of 30 g milk or wheat protein (**Figure 4.5**). These findings may seem to be in contrast with our previous work, where we failed to observe a significant increase in muscle protein synthesis rates following ingestion of 35 g wheat protein hydrolysate, as opposed to the ingestion of an equivalent amount of casein [24]. However, the apparent discrepancy is likely explained by the inclusion of healthy, active young males in the present study as opposed to the selection of older males in Gorissen *et al*. [24]. In that study [24], anabolic resistance in the older volunteers [33] likely prevented a measurable increase in muscle protein synthesis following ingestion of a similar bolus of wheat protein hydrolysate. Accordingly, it has been suggested that the amount of leucine necessary to induce a robust stimulation of muscle protein synthesis is lower in young when compared to older individuals [8, 34, 35]. Whether this is merely attributed to an anabolic resistance of aging or simply secondary to a more sedentary lifestyle remains a topic of debate [36]. In the present study, the 30 g wheat protein provided 1.8 g leucine, which has been reported to be sufficient to stimulate muscle protein synthesis in healthy, young individuals [14]. In line, we observed a strong stimulation of muscle protein synthesis following wheat protein ingestion, despite the lower EAA content and incomplete amino acid profile in these healthy, young males.

There are only few studies that have assessed the capacity of plant-derived proteins to directly increase post-prandial muscle protein synthesis rates [14, 19-21]. Some have reported measurable increases in muscle protein synthesis rates following ingestion of high quality plant-derived proteins such as soy [14, 19]. Despite the lower essential amino acid content and incomplete amino acid profile, our data show that even the ingestion of an ample amount of a low(er) quality plant-derived protein source such as wheat protein can also effectively increase muscle protein synthesis rates in healthy, young males.

We anticipated a lesser muscle protein synthetic response following the ingestion of 30 g wheat protein when compared to the ingestion of milk protein. Therefore, we also included a third treatment in which we aimed to augment the anabolic properties of the wheat protein by composing a protein blend with equal amounts of both wheat and milk protein. We

hypothesized that a protein blend would restore the anabolic properties, thereby allowing a robust post-prandial muscle protein synthetic response while consuming less animal-derived protein. The amino acid composition of the wheat plus milk protein blend remained different from the milk protein, with the EAA (9.8 *vs* 8.2 g), leucine (2.4 *vs* 2.1 g), lysine (2.0 *vs* 1.2 g) and methionine (0.7 *vs* 0.6 g) contents being higher in the milk protein when compared to the protein blend. (**Table 4.2**). The differences in the protein amino acid profile translated to a greater post-prandial EAA (+58%), leucine (+45%), lysine (+182%) and methionine (+111%) availability following ingestion of milk when compared to the milk plus wheat protein blend (**Figure 4.3**). The smaller differences in plasma amino acid availability clearly showed that the ingested protein blend improved post-prandial EAA availability when compared with the ingestion of wheat protein only. In line with observations discussed above, the differences in amino acid profile and subsequent post-prandial plasma amino acid availability did not modify post-prandial muscle protein synthesis rates (**Figure 4.5**).

The present study extends on prior work showing no impairments in muscle protein synthesis following ingestion of protein blends combining soy and dairy protein during recovery from exercise in healthy, young adults [26, 37]. The present study is the first to compare muscle protein synthesis rates following ingestion of a blend combining a high-quality animal protein source (milk) plus a low-quality plant-derived protein source (wheat) with the same amount of milk protein at rest in healthy, young adults. The findings support the concept that ingestion of an ample, meal-like amount (30 g) of plant-derived protein or plant- plus animal-derived protein blend robustly stimulates muscle protein synthesis rates in healthy, young males to an extent that it does not differ from the response observed after ingesting the same amount of a high-quality animal-derived protein. The lower levels of leucine, lysine, and methionine in wheat protein or the wheat plus milk protein blend do not restrict the capacity to induce a significant and sustained muscle protein synthetic response. This is in contrast to current beliefs in which the low(er) levels of certain amino acids in plant-derived proteins are thought to compromise the post-prandial muscle protein synthetic response to protein ingestion [15].

There has been a growing interest in the use of plant-based diets and plant-derived proteins, both from a consumer and scientific perspective [38]. These data may alleviate the restraints many nutritionists have with regards to the media driven hype to consume more plant based as opposed to animal-based proteins. From the perspective of post-prandial muscle protein synthesis, the general public is unlikely to compromise post-prandial muscle protein synthesis rates when plant-derived protein(s) are consumed in a single meal containing ~30 g protein. Although, it should be noted that this study only investigated wheat protein as a plant-derived protein source, as more research is needed to evaluate the anabolic properties of many other plant-derived protein sources. It could be suggested that a more sustained use of plant-based proteins could lead to a (relative) deficit of specific amino acids. However, this argument would only hold true if a very limited variety of plant-derived protein sources was consumed over a prolonged time period. The present study was performed in healthy, young and active males who are highly sensitive to the anabolic properties of amino acids [39]. Although our data are likely to translate to most healthy, active individuals, we need to stress

that these findings are unlikely to translate to older adults, sedentary, and/or more clinically compromised populations. These populations suffer from anabolic resistance and typically consume less protein per meal [33, 40-42]. Consequently, in these populations a greater post-prandial rise in circulating plasma essential amino acids, and leucine in particular, may be needed to induce a proper post-prandial muscle protein synthetic response, which is essential for the maintenance of muscle mass. Therefore, research is warranted to establish the anabolic response following the consumption of more plant-based versus animal-based protein meals and diets in older and/or more clinically compromised populations.

In conclusion, ingestion of 30 g milk protein, 30 g wheat protein, or a blend of 15 g wheat plus 15 g milk protein increases muscle protein synthesis rates in young, healthy males. Post-prandial muscle protein synthesis rates following the ingestion of 30 g milk protein do not differ from rates observed after ingesting 30 g wheat protein or a blend providing 15 g milk plus 15 g wheat protein in healthy, young males. Ingestion of a meal-like (30 g) dose of plant-derived protein can be as effective as ingesting the same amount of animal-derived protein to increase muscle protein synthesis rates *in vivo* in healthy, young males.

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Conflicts of Interest

PJMP, IWKK, LCPGMdG, LBV, TS and LJCvL have the following interests: This study was funded by TiFN, Wageningen, The Netherlands. The sponsors Tereos Syral (Marckolsheim, France), Cargill (Minneapolis, MN, USA), and Kellogg (Battle Creek, MI, USA) partly financed the project. The researchers are responsible for the study design, data collection and analysis, decision to publish, and preparation of the manuscript. The industrial partners have contributed to the project through regular discussion, and were involved in the study design. More specifically: the choice of interventional products which were produced by these sponsors. The funders had no role in data collection and analysis, decision to publish, or preparation of the manuscript. In addition: LJCvL and LBV have received research grants, consulting fees, speaking honoraria, or a combination of these from Friesland Campina, Tereos Syral, and Pepsico. The other authors report no conflicts of interest.

Authorship

The author contributions were as follows: LJCvL, TS, LBV, LCPGMdG, and PJMP designed research; PJMP, IWKK, FKH and JMXvK conducted research; PJMP, TS and LJCvL analyzed data; PJMP and LJCvL wrote paper; PJMP, TS and LJCvL had primary responsibility for final content. All authors read and approved the final manuscript.

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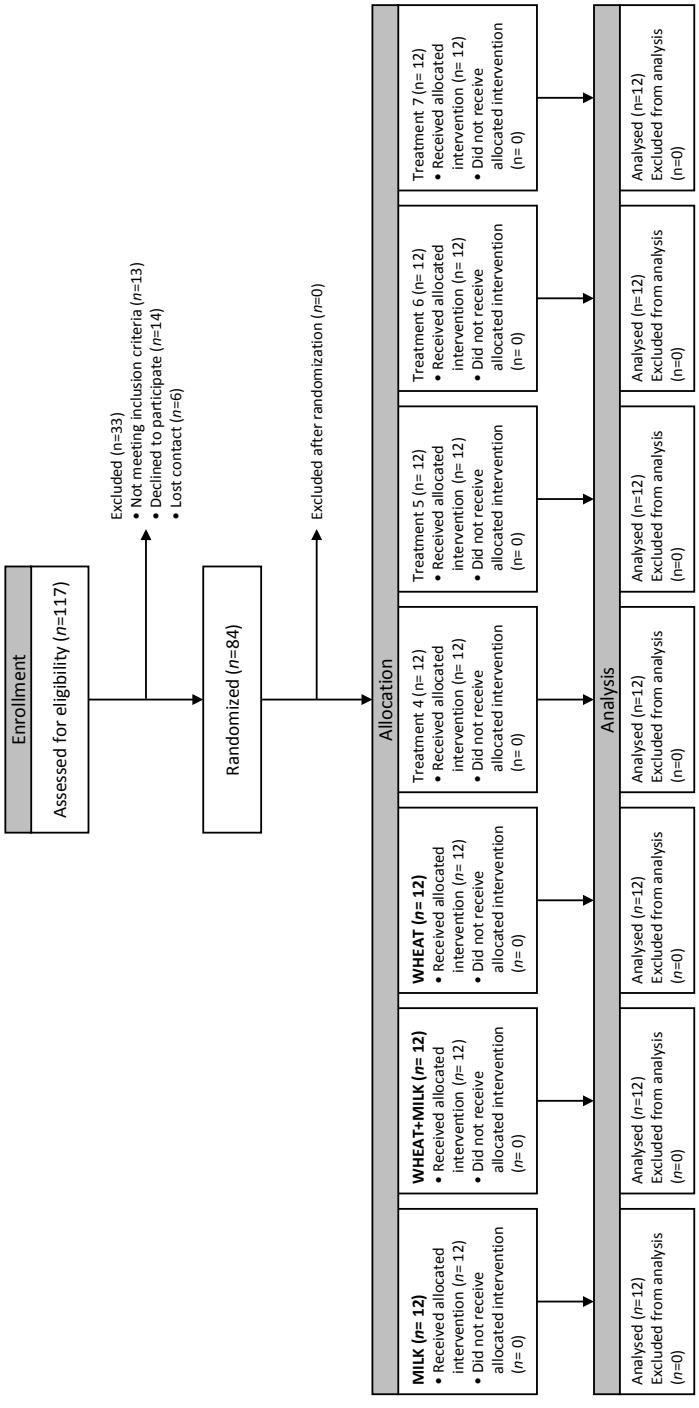
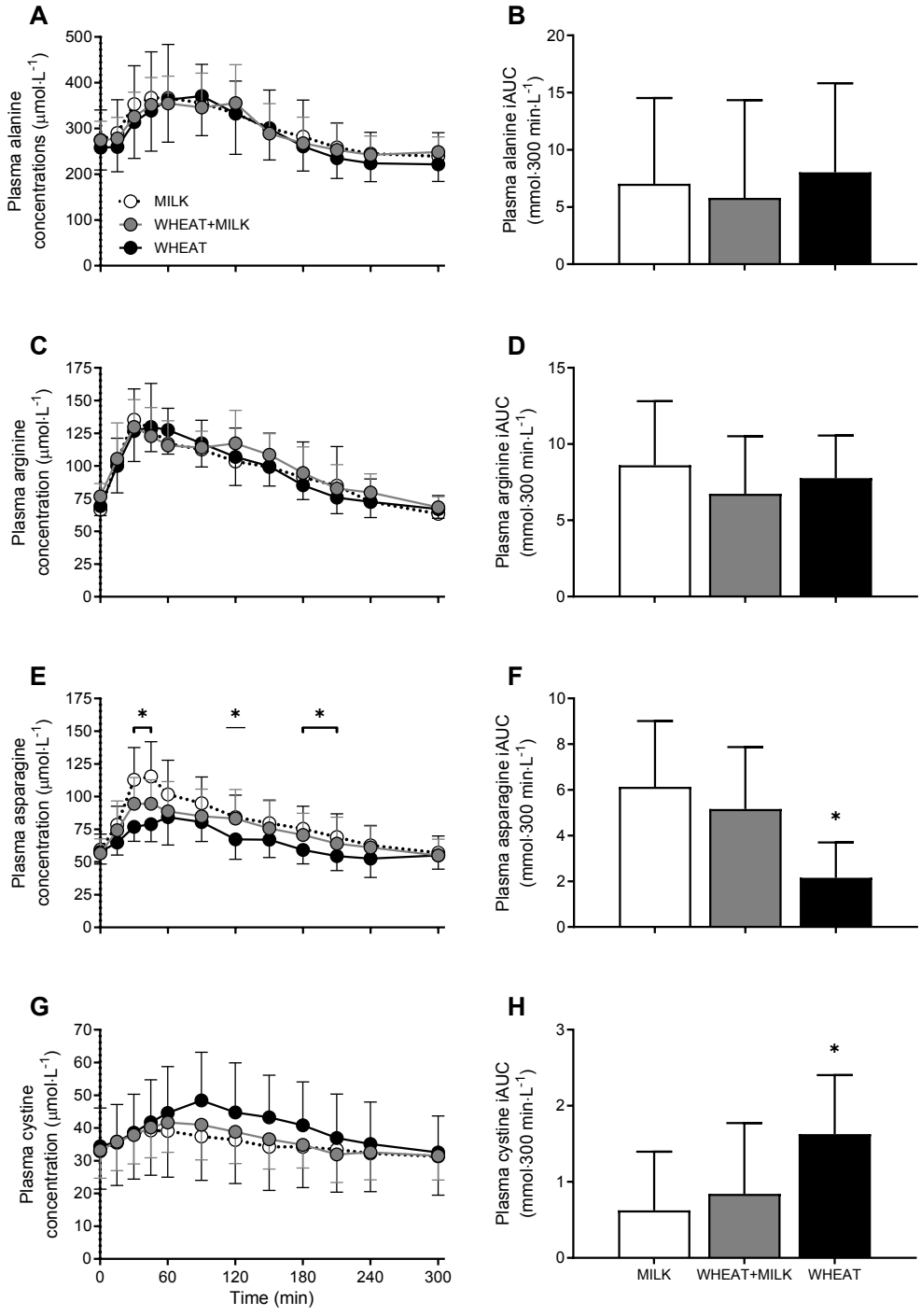
SUPPLEMENTAL MATERIAL



SUPPLEMENTAL FIGURE 4.1 CONSORT flow diagram. CONSORT, Consolidated Standards of Reporting Trials. The current study was part of a larger trial with a total of 7 parallel groups (n = 12 per group) as indicated in the flow diagram. MILK (30 g milk protein), WHEAT (30 g wheat protein), WHEAT+MILK (15 g wheat protein + 15 g milk protein)



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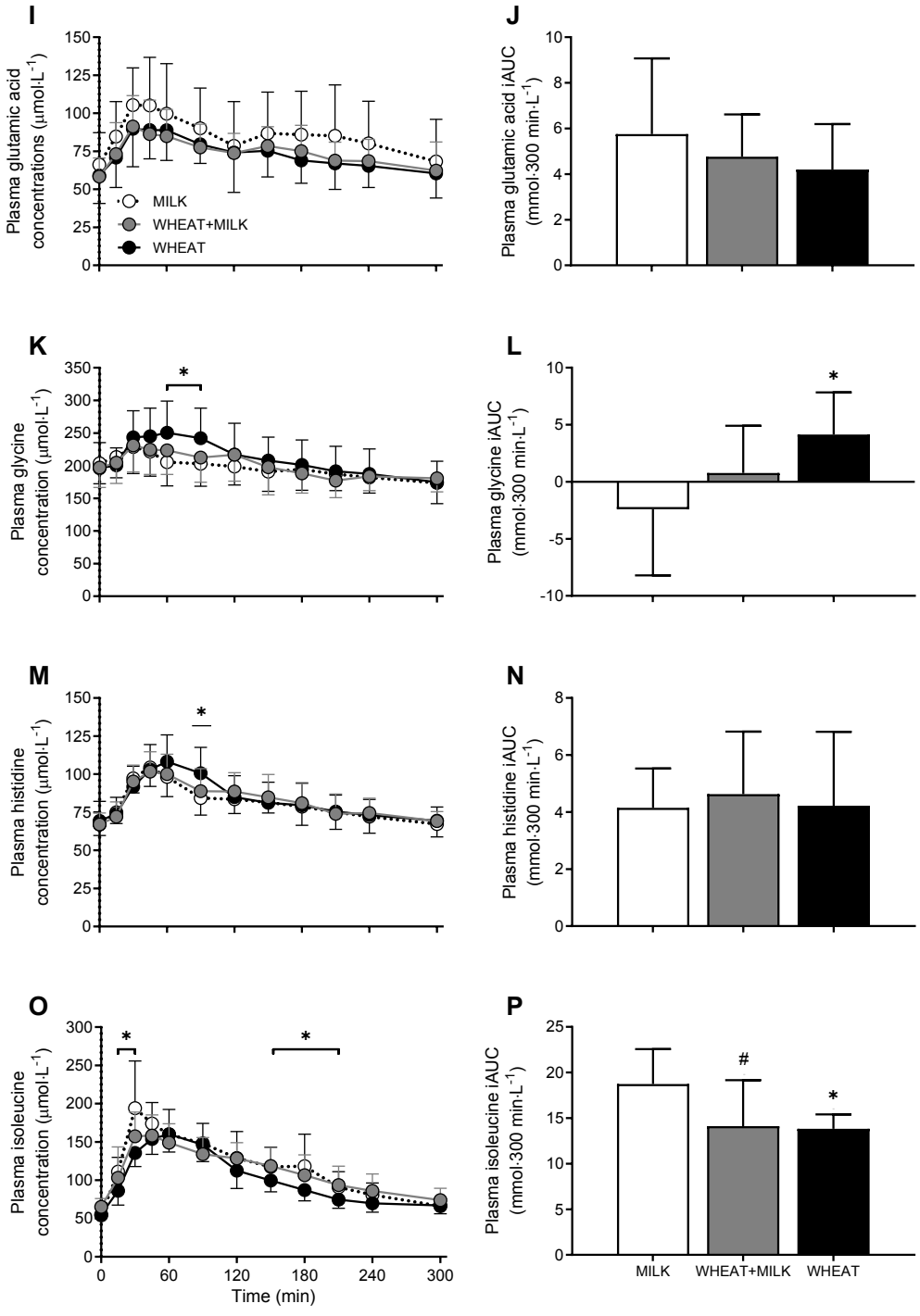

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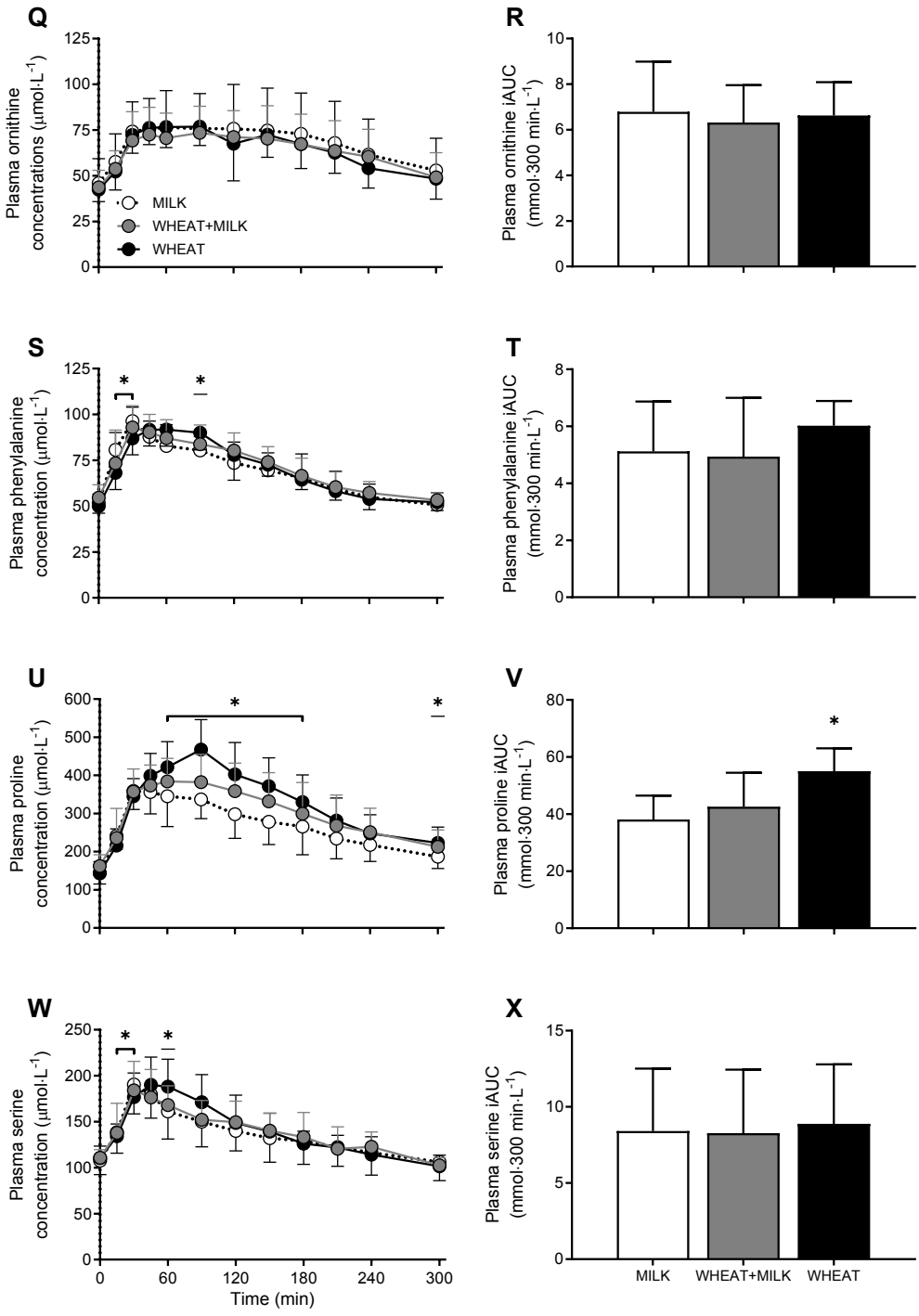

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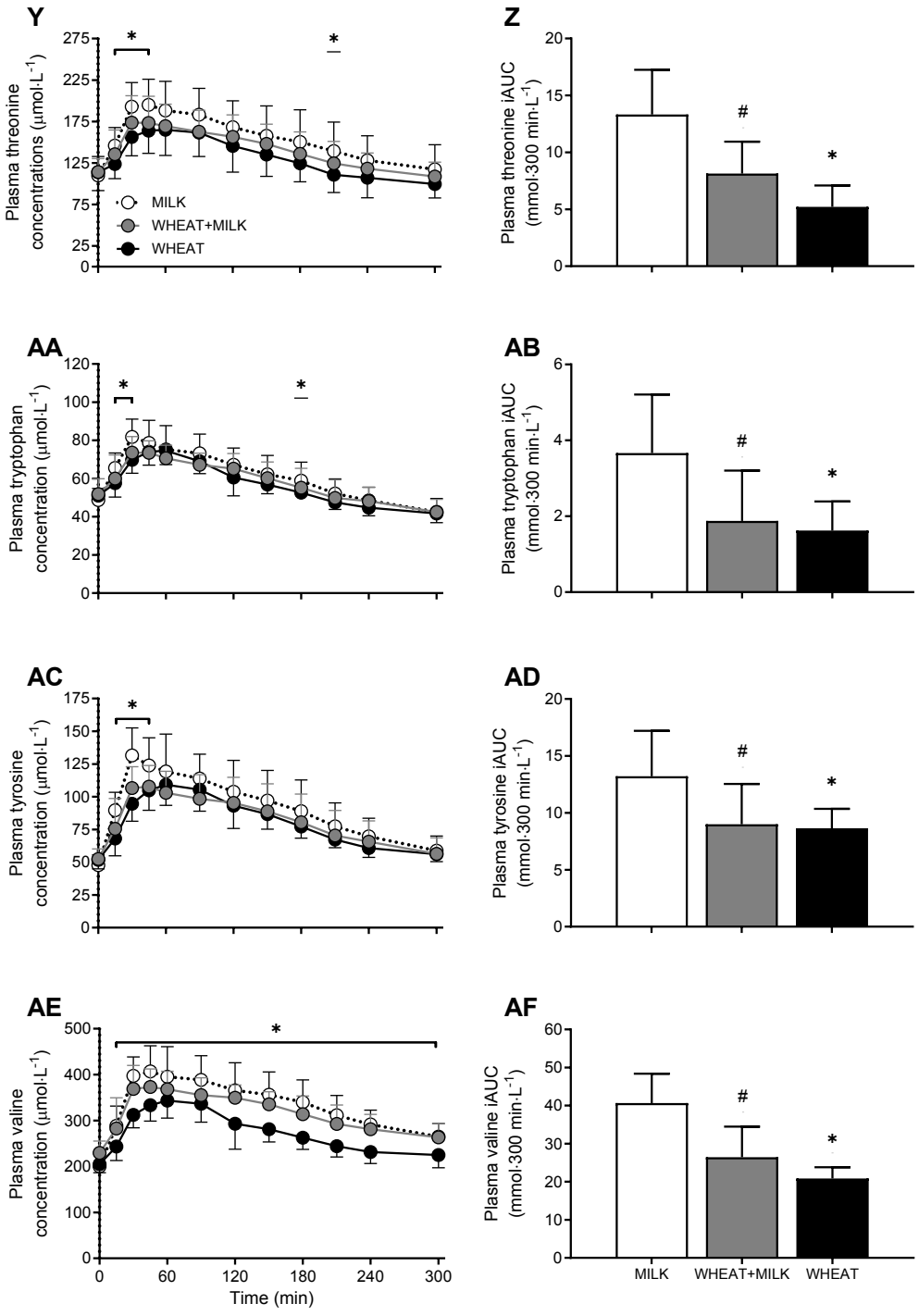

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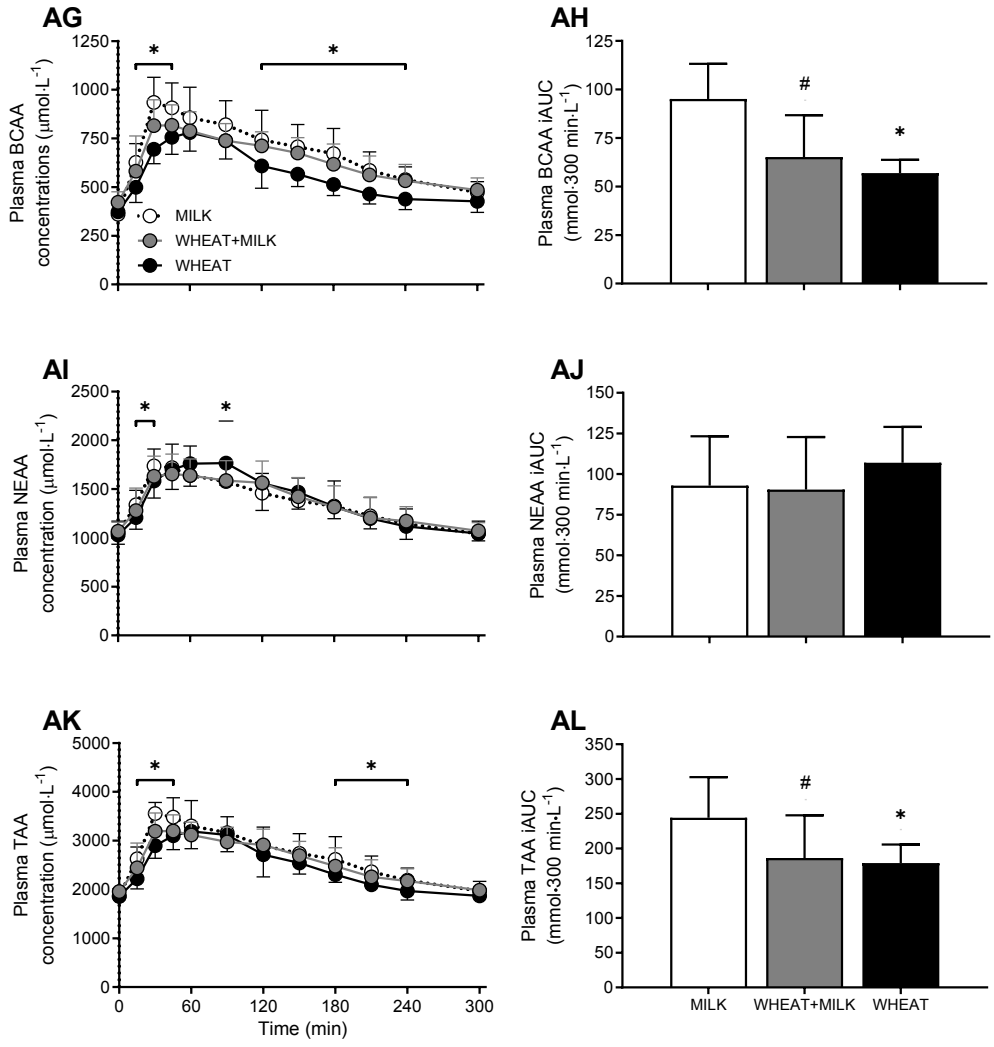
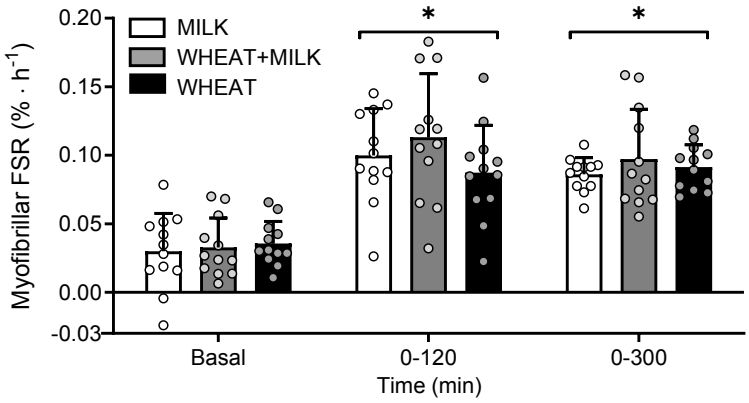


SUPPLEMENTAL FIGURE 4.2 Figure to be continued on next page

SUPPLEMENTAL FIGURE 4.2 Post-prandial plasma amino concentrations during the 300 min post-prandial period following the ingestion of MILK *vs* WHEAT and MILK *vs* WHEAT+MILK. Time 0 min represents time of beverage intake. Panels B, D, F, H, J, L, N, P, R, T, V, X, Z, AB, AD, AF, AH, AJ, AL represent the 0-5 h incremental area under curve (iAUC) following protein ingestion. MILK (30 g milk protein), WHEAT (30 g wheat protein), WHEAT+MILK (15 g wheat protein + 15 g milk protein). Values represent means ± standard deviation; * significantly different for MILK *vs* WHEAT ($P<0.05$), # significantly different for MILK *vs* WHEAT+MILK ($P<0.05$). Repeated measures ANOVA with time as within-subject variable and interventional drink (treatment) as between-subject variable, and independent samples *t*-test were used to determine differences between groups. Values displayed below represent the *P*-values for the different panels.

	2-factor repeated measures ANOVA		Independent samples t-test	
	MILK *vs* WHEAT	MILK *vs* WHEAT+MILK	MILK *vs* WHEAT	MILK *vs* WHEAT+MILK
Alanine	A: 0.27	A: 0.42	B: 0.75	B: 0.71
Arginine	C: 0.32	C: 0.32	D: 0.57	D: 0.27
Asparagine	E: <0.001	E: 0.14	F: <0.001	F: 0.40
Cystine	G: <0.001	G: 0.09	H: <0.01	H: 0.54
Glutamic acid	I: 0.23	I: 0.37	J: 0.18	J: 0.37
Glycine	K: <0.001	K: 0.08	L: <0.01	L: 0.14
Histidine	M: <0.01	M: 0.68	N: 0.93	N: 0.53
Isoleucine	O: 0.01	O: 0.13	P: <0.001	P: 0.02
Ornithine	Q: 0.36	Q: 0.92	R: 0.83	R: 0.56
Phenylalanine	S: <0.001	S: 0.29	T: 0.12	T: 0.82
Proline	U: <0.001	U: 0.27	V: <0.001	V: 0.29
Serine	W: <0.01	W: 0.70	X: 0.77	X: 0.94
Threonine	Y: 0.02	Y: 0.32	Z: <0.001	Z: 0.001
Tryptophane	AA: 0.04	AA: 0.14	AB: <0.001	AB: <0.01
Tyrosine	AC: <0.01	AC: 0.11	AD: 0.001	AD: 0.01
Valine	AE: 0.001	AE: 0.1	AF: <0.001	AF: <0.001
BCAA	AG: <0.01	AG: 0.12	AH: <0.001	AH: 0.001
NEAA	AI: <0.01	AI: 0.36	AJ: 0.21	AJ: 0.85
TAA	AK: <0.01	AK: 0.22	AL: <0.01	AL: 0.03



SUPPLEMENTAL FIGURE 4.3 Myofibrillar fractional synthetic rate (FSR) determined with intra-cellular enrichments as precursor pool at different time points following ingestion of MILK *vs* WHEAT and MILK *vs* WHEAT+MILK in healthy, young males (*n*=12 per group). MILK: 30 g milk protein, WHEAT: 30 g wheat protein, WHEAT+MILK: 15 g wheat protein + 15 g milk protein. Bars represent means ± standard deviation, dots represent individual values. *significantly different from basal; *P*<0.05. Independent samples *t*-test: MILK *vs* WHEAT *P*=0.55, *P*=0.38, and *P*=0.38 for basal, 0-120, and 0-300 min, respectively. MILK *vs* WHEAT+MILK *P*=0.78, *P*=0.43, and *P*=0.33 for basal, 0-120, and 0-300 min, respectively.

SUPPLEMENTAL TABLE 4.1 Average 3 day dietary intake of study participants

	MILK	WHEAT+MILK	WHEAT
Energy (MJ·d^{-1})	9.3* ± 2.2	9.2 ± 2.2	7.4 ± 2.0
Carbohydrate (g·d^{-1})	267* ± 63	274 ± 70	220 ± 46
Fat (g·d^{-1})	78 ± 27	79 ± 29	65 ± 29
Protein (g·d^{-1})	97* ± 29	87 ± 30	72 ± 25
Energy (kJ·kg^{-1}·d^{-1})	131 ± 26	127 ± 33	109 ± 39
Carbohydrate (g·kg^{-1}·d^{-1})	3.8 ± 0.9	3.8 ± 1.0	3.2 ± 0.9
Fat (g·kg^{-1}·d^{-1})	1.1 ± 0.3	1.1 ± 0.4	1.0 ± 0.5
Protein (g·kg^{-1}·d^{-1})	1.3 ± 0.4	1.2 ± 0.4	1.0 ± 0.4
Carbohydrate (% total energy)	50 ± 7	51 ± 9	52 ± 6
Fat (% total energy)	33 ± 8	33 ± 8	32 ± 6
Protein (% total energy)	18 ± 3	16 ± 3	16 ± 4

Values represent mean ± standard deviation. *n*=12 per nutritional intervention group. MILK: 30 g milk protein, WHEAT+MILK: 15 g wheat protein plus 15 g milk protein, WHEAT: 30 g wheat protein. Independent samples *t*-test for MILK *vs* WHEAT and MILK *vs* WHEAT+MILK. *significantly different for MILK *vs* WHEAT (*P*<0.05). 3 Day food records were analyzed using "Mijn Eetmeter" (https://mijn.voedingscentrum.nl/nl/eetmeter/), online software available from the Netherlands Nutrition Centre.

CHAPTER 5

THE MUSCLE PROTEIN SYNTHETIC RESPONSE FOLLOWING CORN PROTEIN INGESTION DOES NOT DIFFER FROM MILK PROTEIN IN HEALTHY, YOUNG ADULTS

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Amino Acids (Published online: February 5, 2024)

ABSTRACT

Background

Plant derived proteins are generally believed to possess lesser anabolic properties when compared with animal derived proteins. This is, at least partly, attributed to the lower leucine content of most plant derived proteins. Corn protein has a leucine content that is highest among most plant derived proteins and it even exceeds the levels observed in animal derived proteins such as whey protein.

Objective

To compare muscle protein synthesis rates following the ingestion of 30 g corn protein and a 30 g blend of corn plus milk protein with 30 g milk protein.

Methods

In a randomized, double blind, parallel-group design, 36 healthy young males (26±4 y) received primed continuous L-[ring-$^{13}C_6$]-phenylalanine infusions and ingested 30 g corn protein (CORN), 30 g milk protein (MILK), or a 30 g protein blend with 15 g corn plus 15 g milk protein (CORN+MILK). Blood and muscle biopsies were collected for 5 hours following protein ingestion to assess post-prandial plasma amino acid profiles and myofibrillar protein synthesis rates. This trial was registered at: Nederlands Trial Register on 27-06-2017: NTR6548 (https://www.trialregister.nl/).

Results

Ingestion of protein increased myofibrillar protein synthesis rates from basal post-absorptive values in all treatments (P<0.001). Post-prandial myofibrillar protein synthesis rates did not differ between CORN *vs* MILK (0.053±0.013 *vs* 0.053±0.013 %·h^{-1}, respectively; *t*-test P=0.90), or between CORN+MILK *vs* MILK (0.052±0.024 *vs* 0.053±0.013 %·h^{-1}, respectively; *t*-test P=0.92).

Conclusion

Ingestion of 30 g corn protein, 30 g milk protein, or a blend of 15 g corn plus 15 g milk protein robustly increases muscle protein synthesis rates in young males. The muscle protein synthetic response to the ingestion of 30 g corn derived protein does not differ from the ingestion of an equivalent amount of milk protein in healthy, young males.

INTRODUCTION

Protein ingestion increases muscle protein synthesis rates [1, 2]. The increase in muscle protein synthesis rate is driven by the post-prandial rise in circulating plasma essential amino acid (EAA) concentrations [3], with the increase in plasma leucine concentration being of particular relevance [4-8]. It has been hypothesized that the skeletal muscle anabolic properties of different types of protein are largely determined by their protein digestion and amino acid absorption kinetics and their specific amino acid profile [9-11]. More specifically, it has been suggested that the ingestion of protein sources with lower EAA content and/or digestibility result in an attenuated anabolic response when compared with the ingestion of the same amount of protein derived from a higher quality protein source [12-14]. In this regard, plant based protein sources are suggested to possess lesser anabolic properties when compared to animal based protein sources. In accordance, we have previously shown that the ingestion of 35 g wheat derived protein, which is relatively low in leucine, lysine and methionine, fails to stimulate muscle protein synthesis rates when compared with the ingestion of an equivalent amount of casein protein in older adults [15]. Soy protein, on the other hand, contains sufficient amounts of all EAAs according to the WHO/FAO/UNU amino acid requirements [16], and has been shown to result in lower [14, 17, 18] or similar [19, 20] post-prandial muscle protein synthesis rates when compared to the ingestion of milk or whey protein in both young and older adults. However, to define the extent to which plant based proteins can stimulate post-prandial muscle protein synthesis rates, more plant derived proteins, besides wheat and soy, should be investigated.

Corn is a widely consumed cereal grain, providing mainly carbohydrates. However, it is also a plant based protein source. While plant derived proteins generally contain less leucine when compared to animal derived proteins, we previously showed that the leucine content of corn derived protein concentrates is surprisingly high with leucine levels as high as ~13% of the total protein content [21]. In comparison, this is higher than the leucine content of whey protein (~11%) which is regarded as one of the most anabolic animal derived protein sources [21]. So far, no studies have assessed the muscle protein synthetic response to the ingestion of corn derived protein *in vivo* in humans.

Although leucine is regarded as the most important EAA to stimulate muscle protein synthesis [8, 22, 23], the presence of other EAAs also plays an important role in providing the required building blocks to support the post-prandial rise in muscle protein synthesis rates. Insufficient post-prandial availability of one (or more) of the EAA may attenuate the post-prandial rise in muscle protein synthesis rates and, as such, compromise the post-prandial anabolic response. It has been suggested that plant derived proteins are less likely to increase muscle protein synthesis due to deficiencies in specific amino acids such as lysine and methionine [24]. In this regard, corn protein has a very high leucine content, but a very low lysine content. However, combining various protein sources in a blend may represent an effective strategy to provide a more balanced amino acid profile [25, 26] and as such prevent any amino acid deficiencies. Since more than half of the worldwide protein consumption originates from

plants [27], blends of both plant and animal based protein may represent an effective and practical strategy to improve the overall quality, anabolic properties, and sustainability of the ingested protein.

We hypothesize that the ingestion of 30 g milk protein results in higher post-prandial muscle protein synthesis rates when compared with the ingestion of the same amount of corn protein. However, when corn and milk protein are combined in a 1/1 ratio, we expect these differences to disappear. To test these hypotheses, we selected 36 healthy young males to partake in a study in which we compared the impact of ingesting 30 g milk protein with the ingestion of 30 g corn protein or a protein blend of 15 g corn plus 15 g milk protein on post-prandial muscle protein synthesis rates *in vivo* in humans.

MATERIALS AND METHODS

Participants

Thirty-six healthy males (26±4 y; 1.78±0.06 m; 72.5±7.5 kg) volunteered to participate in this parallel group, double blind, randomized controlled trial (participants' characteristics are presented in **Table 5.1**). Participants were recreationally active and generally performed between 2-4 exercise sessions per week in various sports (e.g. soccer, basketball, weight lifting, running, cycling, etc.), but were not involved in any structured progressive exercise training regimen. This study was part of a larger trial registered at the Netherlands Trial Register (NTR6548, https://www.trialregister.nl/trial/6364), and was conducted between June 2017 and April 2019 at Maastricht University in Maastricht, The Netherlands (See **Supplemental Figure 5.1** for the CONSORT (Consolidated Standards of Reporting Trials) flow diagram) [28]. All participants were informed about the purpose of the study, the experimental procedures, and possible risks before providing informed written consent to participate. The procedures followed were in accordance with the ethical standards of the medical ethics committee of Maastricht University Medical Centre+ (METC 173001), and in accordance with the Helsinki Declaration of 1975 as revised in October 2013. The study was independently monitored and audited by the Clinical Trial Centre Maastricht.

Preliminary testing

Participants aged 18-35 y, with BMI >18.5 and <27.5 $kg \cdot m^{-2}$ underwent an initial screening session to assess eligibility. Height, weight, blood pressure and body composition (by dual-energy X-ray absorptiometry; Discovery A, Hologic; (National Health and Nutrition Examination Survey - Body composition analysis (NHANES BCA) enabled) were determined. Participants were deemed healthy based on their responses to a medical questionnaire. The screening sessions and experimental trials were separated by at least 3 days.

Study design

Participants were randomly assigned to ingest a 400 mL beverage containing either 30 g corn protein isolate (CORN), 30 g milk protein concentrate (MILK), or 15 g corn protein isolate plus

TABLE 5.1 Participants' characteristics

	MILK	CORN+MILK	CORN
Age (y)	26 ± 4	26 ± 5	27 ± 3
Height (m)	1.76 ± 0.06	1.76 ± 0.05	1.81 ± 0.07
Mass (kg)	71.5 ± 9.0	72.4 ± 6.9	73.7 ± 7.0
BMI ($kg \cdot m^{-2}$)	23.0 ± 2.1	23.4 ± 2.0	22.4 ± 1.7
Systolic blood pressure (mmHg)	19 ± 6	114 ± 7	124 ± 9
Diastolic blood Pressure (mmHg)	71 ± 9	66 ± 7	69 ± 9
Resting heart rate (bpm)	64 ± 10	65 ± 12	61 ± 7
Lean body mass (kg)	53.2 ± 7.9	53.5 ± 4.7	56.9 ± 6.0
Body fat (%)	23.1 ± 3.2	22.5 ± 4.8	20.2 ± 4.7

Values represent mean ± standard deviation. *n* = 12 per nutritional intervention group. MILK: 30 g milk protein, CORN+MILK: 15g corn protein plus 15 g milk protein, CORN: 30 g of corn protein. Independent samples *t*-test for MILK *vs* CORN and MILK *vs* CORN+MILK all *P*>0.05.

15 g milk protein concentrate (CORN+MILK). After beverage ingestion, the bottle was rinsed with 150 mL of water, which was also ingested by the participants. Milk protein concentrate (Refit MPC80) was obtained from FrieslandCampina (Wageningen, the Netherlands) and corn protein isolate was supplied by Cargill (Minneapolis, MN, USA). Participants were allocated to a treatment according to a block randomization list performed using a computerized randomizer (http://www.randomization.com/). An independent researcher was responsible for random assignment (*n*=12 per group) and preparation of the study treatment beverages, which were sequentially numbered according to subject number. The beverages were prepared in non-transparent protein-shakers.

Diet and physical activity

Participants refrained from sports and strenuous physical activities (e.g. lifting heavy weights), and alcohol consumption for 3 days prior to the experimental trial. In addition, all participants were instructed to complete a food and activity record for 3 days prior to the experimental trial. (See **Supplemental Table 5.1** for an overview of participants' habitual food intake in the 3 days prior to the experimental trial). The evening before the trial, all participants consumed a standardized meal containing 2.8 MJ, with 65% energy provided as carbohydrate, 20% as fat, and 15% as protein, before 10:00 PM after which they remained fasted.

Experimental protocol

At ~7:30 AM, participants arrived at the laboratory in an overnight post-absorptive state. A cannula was inserted into an antecubital vein for stable isotope amino acid infusion. A second cannula was inserted into a dorsal hand vein on the contralateral arm for arterialized blood sampling. To obtain arterialized blood samples, the hand was placed in a hot box (60°C) for 10 min prior to blood sample collection. For a schematic representation of the experimental protocol, see **Figure 5.1**.

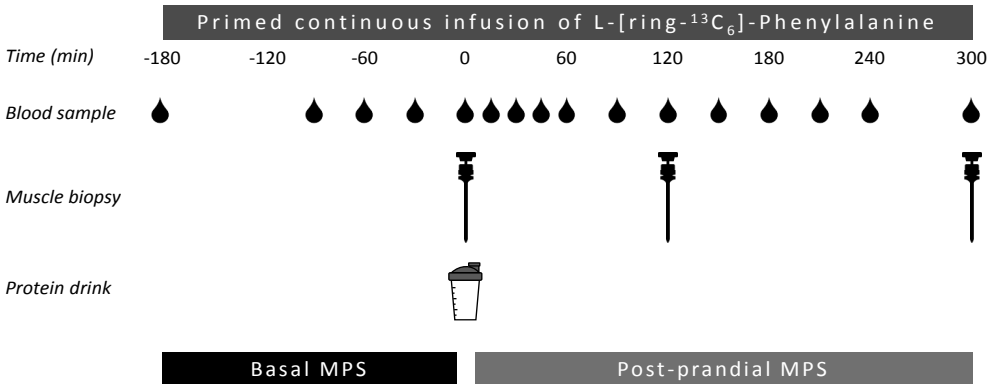

FIGURE 5.1 Schematic representation of the experimental design.

After taking a baseline blood sample (*t*= -180 min), the plasma phenylalanine pool was primed with a single dose of L-[ring-$^{13}C_6$]-phenylalanine (2.25 μmol·kg^{-1}). Thereafter, a continuous intravenous infusion of L-[ring-$^{13}C_6$]-phenylalanine (0.05 μmol·kg^{-1}·min^{-1}) was initiated (*t*= -180 min) using a calibrated IVAC 598 pump (San Diego, CA, USA). Subsequently, arterialized blood samples were collected at *t*=-90, -60 and -30 min. At *t*= 0 min an arterialized blood sample was obtained as well as a muscle biopsy from the *m. vastus lateralis*. Immediately following the muscle biopsy, participants ingested a 400 mL beverage corresponding to their randomized treatment allocation i.e.: CORN (*n*=12), MILK (*n*=12), or CORN+MILK (*n*=12). To minimize dilution of the steady-state plasma L-[ring-$^{13}C_6$]-phenylalanine precursor pool, the phenylalanine content of the protein drink was enriched with 3.85% L-[ring-$^{13}C_6$]-phenylalanine. Arterialized blood samples were then collected at *t*= 15, 30, 45, 60, 90, 120, 150, 180, 210, 240, and 300 min after protein ingestion in the post-prandial period. Blood samples were collected into EDTA-containing tubes and centrifuged at 1200*g* for 10 min at 4°C. Aliquots of plasma were frozen in liquid nitrogen and stored at -80°C. A second and third muscle biopsy from the *m. vastus lateralis* were collected at *t*= 120 and *t*= 300 min to determine post-prandial skeletal muscle protein synthesis rates over the 0-120, 120-300, and 0-300 min post-prandial periods. Muscle biopsy collection was alternated between legs and obtained with the use of a 5 mm Bergström needle [29], custom-adapted for manual suction. Samples were obtained from separate incisions from the middle region of the *m. vastus lateralis*, ~15 cm above the patella and ~3 cm below entry through the fascia. Local anesthetic (1% Xylocaine with adrenaline 1:100,000) was applied to numb the skin and fascia. Muscle samples were freed from any visible non-muscle material, immediately frozen in liquid nitrogen, and stored at -80°C until further processing. When the experimental protocol was complete, cannulae were removed and participants were provided with food and monitored for ~30 min before leaving the laboratory.

Protein powder analysis

Batch specific nitrogen contents of both milk protein concentrate and corn protein isolate were provided by the manufacturer. The protein content of milk protein powder was determined as nitrogen content x 6.38 and the protein content of corn protein powder was determined as nitrogen content x 6.25 [30, 31]. Amino acid contents of the protein powders were determined by acid hydrolysis in triplicate, as previously described [28]. Tryptophan contents were obtained by alkaline hydrolysis, as performed by la Cour et al. [32]. In short, ~10 mg of the protein samples was weighed in glass screw cap vials. Subsequently, a 3 mL solution with 95 mM ascorbic acid and 4 M sodium hydroxide was added to the sample and heated to 110°C for 16 h. Subsequent analysis of the free amino acids was performed using ultra-performance liquid chromatography-mass spectrometry (UPLC-MS; ACQUITY UPLC H-Class with QDa; Waters, Saint-Quentin, France), as previously described [28]. The amino acid composition of the protein powders and protein blend are presented in **Table 5.2**. It is worthwhile to note that the use of a single protein hydrolysis step is suboptimal for a quantitative assessment of all amino acids [33], resulting in the underestimation of the sulphur containing amino acid contents and the inability to assess tryptophan concentrations.

Plasma analysis

Plasma glucose and insulin concentrations were analyzed using commercially available kits (ref. no. A11A01667, Glucose HK CP, ABX Diagnostics, Montpellier, France; and ref. no. HI-14K, Millipore, St. Louis, MO, respectively). Plasma amino acid concentrations were determined by UPLC-MS, as previously described [28].

Plasma L-[ring-$^{13}C_6$]-phenylalanine enrichments were determined by gas chromatography-mass spectrometry (GC-MS; Agilent 7890A GC/5975C MSD; Agilent Technologies), as previously described [28]. In short, the free amino acids from deproteinized plasma samples were purified using cation exchange resin columns (AG 50W-X8, mesh size: 100-200, ionic form: hydrogen (Bio-Rad Laboratories, Hercules, CA, USA)), and subsequently converted to their tert-butyl dimethylsilyl (TBDMS) derivative before analysis by GC-MS.

Basal muscle protein synthesis rates were assessed to confirm that protein ingestion increases muscle protein synthesis rates. The single biopsy approach was applied to assess post-absorptive muscle protein synthesis rates without the need to collect and additional muscle biopsy [34]. In short, plasma protein obtained prior to tracer infusion (t= -180 min) was used to determine background L-[ring-$^{13}C_6$]-phenylalanine enrichments. For this purpose, the plasma sample was precipitated by adding perchloric acid. Subsequently, similarly as for the myofibrillar protein fraction, the denaturized plasma protein pellet was hydrolyzed, passed over a cation exchange resin column (AG 50W-X8, mesh size: 100-200, ionic form: hydrogen (Bio-Rad Laboratories, Hercules, CA, USA)), and the resulting amino acid samples were derivatized to their N(O,S)-ethoxycarbonyl-ethylesters before being measured by gas chromatography-combustion-isotope ratio mass spectrometry (GC-IRMS; Mat 253, Thermo Scientific, Bremen, Germany) using a DB5MS (30m) column (Agilent technologies, Santa Clara, Ca, USA), as previously described [28].

TABLE 5.2 Amino acid composition of the provided proteins

	MILK	CORN+MILK[1]	CORN
Alanine	0.9	1.6	2.3
Arginine	0.8	0.7	0.6
Aspartic acid	1.8	1.6	1.4
Cystine	0.1	0.1	0.2
Glutamic acid	5.1	5.3	5.5
Glycine	0.5	0.6	0.7
Histidine	0.6	0.5	0.4
Isoleucine	0.9	0.7	0.6
Leucine	2.4	3.3	4.1
Lysine	2.0	1.2	0.3
Methionine	0.7	0.7	0.6
Phenylalanine	1.2	1.4	1.5
Proline	2.9	2.8	2.7
Serine	1.2	1.3	1.4
Threonine	0.9	0.9	0.8
Tryptophan	0.4	0.3	0.1
Tyrosine	0.6	0.6	0.6
Valine	1.1	0.9	0.7
TAA	24.2	24.4	24.6
EAA	10.2	9.7	9.2
BCAA	4.4	4.9	5.4
Nitrogen content (%)	13.4	13.8	14.4
Protein content (%)	85.5[3]	87.8	90.0[2]

Values for amino acid contents are in grams per 30 g protein. [1]Values are obtained by averaging the measured values for corn and milk protein. [2]Protein as nitrogen content * 6.38; [3]Protein as nitrogen content * 6.25; MILK: 30 g milk protein, CORN+MILK: 15 g corn protein plus 15 g milk protein, CORN: 30 g of corn protein. BCAA: branched chain amino acids, EAA: essential amino acids, TAA: total amino acids

Muscle analysis

Muscle analysis for the determination of muscle protein bound L-[ring-$^{13}C_6$]-phenylalanine enrichments has previously been explained in detail [28]. In short, a piece of wet muscle (~50-70 mg) was homogenized and a myofibrillar protein-enriched fraction was obtained by removal of the collagen enriched fraction. Subsequently, the amino acids were liberated from the myofibrillar protein-enriched fraction by adding 2 mL of 6 M HCl and heating to 110°C for 16 h. The amino acids from the resulting dried myofibrillar protein-enriched fractions were liberated by adding 2 mL of 6 M HCl and heating to 110°C for 16 h, passed over a cation exchange resin column (AG 50W-X8, mesh size: 100-200, ionic form: hydrogen (Bio-Rad Laboratories, Hercules, CA, USA)), and derivatized to their N(O,S)-ethoxycarbonyl-

ethylesters. The ratio of $^{13}C/^{12}C$ of myofibrillar protein-bound phenylalanine was determined using GC-IRMS. Muscle intra-cellular enrichments were determined from a separate piece of muscle, as described elsewhere [28].

Calculations

Fractional myofibrillar protein synthesis rates (%·h^{-1}) were calculated by the standard precursor-product equation [35]:

$$FSR = \left(\frac{(E_{b2} - E_{b1})}{(E_{precursor} \cdot t)}\right) \cdot 100$$

Where E_b is the increment in myofibrillar protein-bound L-[ring-$^{13}C_6$]-phenylalanine enrichment (mole % excess, MPE) during the tracer incorporation period, and t is the tracer incorporation time in h. Weighted mean plasma L-[ring-$^{13}C_6$]-phenylalanine enrichments were calculated by taking the measured enrichment between consecutive time points and correcting for the time between these sampling time points ($E_{precursor}$). For calculation of post-prandial FSR, skeletal muscle biopsy samples at *t*= 0, 120 and 300 min were used. For the calculation of basal FSR, E_{b2} represented the protein-bound L-[ring-$^{13}C_6$]-phenylalanine enrichments in muscle at *t*= 0 min, and E_{b1} represented the protein bound L-[ring-$^{13}C_6$]-phenylalanine enrichments in plasma protein at *t*= -180 min.

Net incremental area under curve (iAUC) was determined for plasma amino acid concentrations during the 5 h post-prandial period following protein ingestion. The iAUC was calculated using the trapezoid rule, with plasma concentrations before beverage ingestion (*t*= 0 min) serving as baseline.

Outcome measures

Myofibrillar FSR over the entire (i.e. 0 – 300 min) post-prandial period, comparing MILK *vs* CORN and MILK *vs* CORN+MILK was defined as the primary outcome measure. Secondary outcome measures were myofibrillar FSR in the early (i.e. 0 – 120 min) and late (i.e. 120 – 300 min) post-prandial period, plasma glucose, insulin, and amino acid concentrations and plasma amino acid iAUC, comparing MILK *vs* CORN and MILK *vs* CORN+MILK. Plasma glucose, insulin, and amino acid peak concentrations and time to peak were tertiary outcomes, comparing MILK *vs* CORN and MILK *vs* CORN+MILK.

Statistical analysis

A power calculation was performed with differences in postprandial myofibrillar FSRs between 2 treatments as primary outcome measure. Based on previous work in this area, a sample size of 12 participants per treatment, including a 10% dropout rate was calculated using a power of 80%, a significance level of 0.05, a difference in FSR of 0.008 %·h^{-1} (or ~20% when expressed as relative difference, e.g. 0.040 *vs* 0.048 %·h^{-1}) [36], and a within-group standard deviation of 0.0065 %·h^{-1} (or ~16%) [37, 38]. Participants' characteristics were analyzed by independent samples *t*-test for MILK *vs* CORN and MILK *vs* CORN+MILK. Plasma glucose, insulin, and amino acid concentrations and amino acid enrichments over time were compared between

groups using a two-way (*Time x treatment*) repeated measures ANOVA for MILK *vs* CORN and MILK *vs* CORN+MILK, with time as within-subjects factor, and treatment as between-subjects factor. In case a significant *Time x treatment* interaction was observed, independent samples *t*-tests were performed to determine significant differences between treatments for each time point. Plasma glucose, insulin, and amino acid concentrations, expressed as peak values, time to peak and iAUC, were analyzed by independent samples *t*-test for MILK *vs* CORN and MILK *vs* CORN+MILK. Basal post-absorptive, and post-prandial myofibrillar protein synthesis rates during the early (0-120 min) and entire (0-300 min) post-prandial period were analyzed by independent samples *t*-test for MILK *vs* CORN and MILK *vs* CORN+MILK. Similarly, within group post-absorptive *vs* post-prandial myofibrillar protein synthesis rates were analyzed by independent samples *t*-test. Statistical analyses were performed with a software package (IBM SPSS statistics for Windows, version 26.0, IBM Corp., Armonk, NY, USA). Means were considered to be significantly different for P values <0.05. Data are expressed as means±SD.

RESULTS

Plasma glucose and insulin concentrations

Plasma glucose concentrations were maintained following protein ingestion (**Figure 5.2A**). Although a significant *Time x treatment* interaction was observed (*P*=0.02), no differences at individual time points were observed in glucose concentrations between MILK *vs* CORN (*P*>0.05). Plasma glucose concentrations did not change for MILK *vs* CORN+MILK (*Time x treatment*: *P*=0.33; *Time:* *P*<0.001). Plasma insulin concentrations increased following protein ingestion, with MILK reaching higher peak concentrations when compared to CORN (28±8 *vs* 11±4 mU·L^{-1} respectively, *P*<0.001) and compared to CORN+MILK (28±8 *vs* 17±3 mU·L^{-1} respectively, *P*<0.001; **Figure 5.2B**). The insulin iAUC was greater for MILK *vs* CORN (1058±331 *vs* 232±335 mU·$^{-1}$ respectively, *P*<0.001) as well as for MILK *vs* CORN+MILK (1058±331 *vs* 584±575 respectively, *P*=0.03).

Plasma AA concentrations

Plasma EAA concentrations increased following protein ingestion for all treatments (**Figure 5.3A**). This increase was greater for both MILK *vs* CORN and MILK *vs* CORN+MILK (*Time x treatment*: *P*<0.001 and *P*<0.01 respectively). MILK ingestion resulted in higher peak EAA concentrations *vs* CORN (1871±124 *vs* 1355±152 µmol·L^{-1}; *P*<0.001) and *vs* CORN+MILK (1871±124 *vs* 1684±176 µmol·L^{-1}; *P*<0.001). These peak EAA concentrations were reached faster following MILK *vs* CORN (36±10 *vs* 108±27 min; *P*<0.001), and following MILK *vs* CORN+MILK (36±10 *vs* 91±46 min; *P*<0.001). The overall increase in plasma EAA concentrations over the entire 300 min post-prandial period, expressed as iAUC, was 94 % greater for MILK *vs* CORN (151±31 *vs* 78±19 mmol·300 min·L^{-1}; *P*<0.001) and 20 % greater for MILK *vs* CORN+MILK (151±31 *vs* 126±24 mmol·300 min·L^{-1}; *P*=0.04; **Figure 5.3B**).

Plasma leucine concentrations increased over time for all treatments following protein ingestion (**Figure 5.3C**). This increase differed significantly for both MILK *vs* CORN and

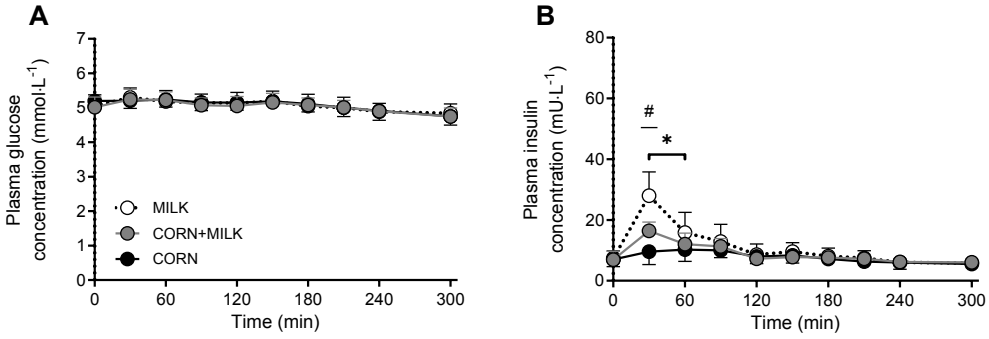

FIGURE 5.2 Post-prandial plasma glucose (Panel A) and insulin (Panel B) concentrations during the 300 min period following the ingestion of MILK *vs* CORN and MILK *vs* CORN+MILK in healthy young males (*n*=12 per group). Time 0 min represents time of beverage intake. MILK: 30 g milk protein, CORN: 30 g corn protein, CORN+MILK: 15 g corn protein + 15 g milk protein. Values represent means ± standard deviation; Repeated measures ANOVA with time as within-subject variable and interventional drink (treatment) as between-subject variable. *Time x treatment*: Panel A: MILK *vs* CORN *P*=0.02, MILK *vs* CORN+MILK *P*=0.33; Panel B: MILK *vs* CORN *P*<0.001, MILK *vs* CORN+MILK *P*<0.001. * Significantly different for MILK *vs* CORN (*P*<0.05), # significantly different for MILK *vs* CORN+MILK (*P*<0.05)

MILK *vs* CORN+MILK (*Time x treatment*, both *P*<0.001). Although peak plasma leucine concentrations did not differ between MILK *vs* CORN (353±45 *vs* 390±66 μmol·L^{-1}; *P*=0.12) and MILK *vs* CORN+MILK (353±45 *vs* 395±62 μmol·L^{-1}; *P*=0.07), MILK reached peak plasma leucine concentrations earlier when compared to CORN (46±43 *vs* 130±35 min, respectively, *P*<0.001) and CORN+MILK (46±43 *vs* 133±45 min, respectively, *P*<0.001). From 90 min onwards, plasma leucine concentrations were higher in both CORN *vs* MILK and CORN+MILK *vs* MILK. The overall increase in plasma leucine concentrations over the entire 300 min post-prandial period, expressed as iAUC, was 45 % greater for CORN *vs* MILK (52±10 *vs* 36±7 mmol·300 min·L^{-1}; *P*<0.001), and 39 % greater for CORN+MILK *vs* MILK (50±9 *vs* 36±7 mmol·300 min·L^{-1}; *P*<0.01; **Figure 5.3D**).

Plasma lysine concentrations increased over time for MILK and CORN+MILK, but not for CORN (**Figure 5.3E**). This increase was greater for MILK *vs* CORN (*Time x treatment*: *P*<0.001), as well as for MILK *vs* CORN+MILK (*Time x treatment*: *P*<0.01). MILK ingestion resulted in higher peak lysine concentrations *vs* CORN (370±29 *vs* 174±25 μmol·L^{-1}; *P*<0.001) and *vs* CORN+MILK (370±29 *vs* 289±46 μmol·L^{-1}; *P*<0.001). Time to reach these peak concentrations was shorter for MILK *vs* CORN (34±7 *vs* 73±36 min, respectively; *P*=0.001), but did not differ for MILK *vs* CORN+MILK: 34±7 *vs* 43±19 min; *P*=0.15). The overall increase in plasma lysine concentrations over the entire 300 min post-prandial period, expressed as iAUC, was much greater for MILK *vs* CORN (25±8 *vs* -3±3 mmol·300 min·L^{-1}; *P*<0.001), and 119 % greater for MILK *vs* CORN+MILK (25±8 *vs* 12±4 mmol·300 min·L^{-1}; *P*<0.001; **Figure 5.3F**).

Plasma methionine concentrations increased over time for all treatments following protein ingestion (**Figure 5.3G**). This increase was greater for MILK *vs* CORN (*Time x treatment*: *P*<0.001), as well as for MILK *vs* CORN+MILK (*Time x treatment*: *P*<0.01). MILK ingestion resulted in higher peak methionine concentrations *vs* CORN (60±5 *vs* 31±4 μmol·L^{-1};

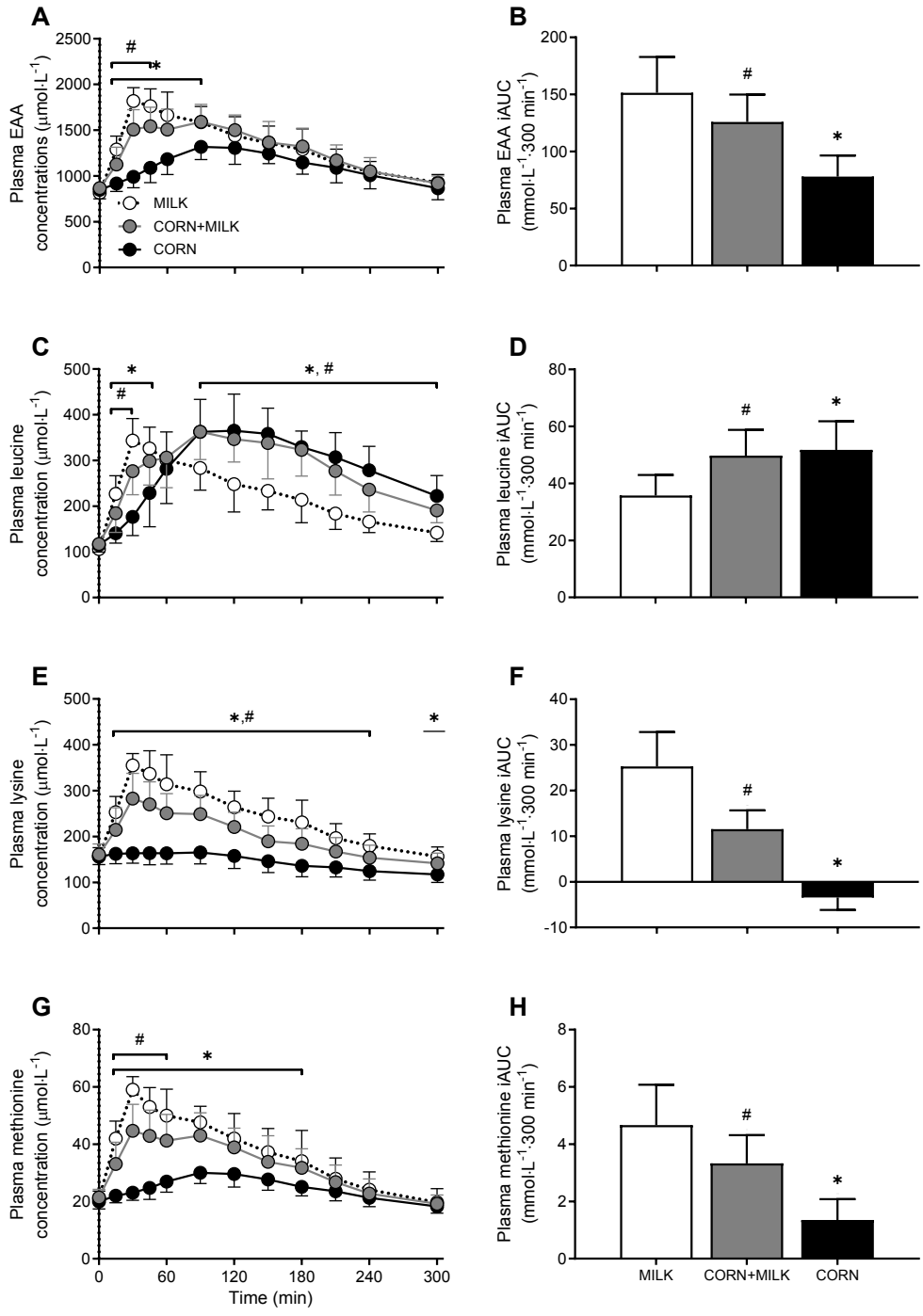

FIGURE 5.3 Post-prandial plasma essential amino acid (EAA, Panel A), leucine (Panel C), lysine (Panel E), and methionine (Panel G) concentrations during the 300 min period following the ingestion of MILK *vs* CORN and MILK *vs* CORN+MILK in healthy, young males (*n*=12 per group). Time 0 min represents time of beverage intake. Panels B, D, F and H represent the 0-5 h incremental area under curve (iAUC)

following protein ingestion. MILK: 30 g milk protein, CORN: 30 g corn protein, CORN+MILK: 15 g corn protein + 15 g milk protein. Values represent means ± standard deviation; * significantly different for MILK *vs* CORN ($P<0.05$), # significantly different for MILK *vs* CORN+MILK ($P<0.05$). Repeated measures ANOVA with time as within-subject variable and interventional drink (treatment) as between-subject variable. *Time x treatment*: Panel **A**: MILK *vs* CORN $P<0.001$, MILK *vs* CORN+MILK $P<0.01$, Panel **C**: MILK *vs* CORN $P<0.001$, MILK *vs* CORN+MILK $P<0.001$, Panel **E**: MILK *vs* CORN $P<0.001$, MILK *vs* CORN+MILK $P<0.01$, Panel **G**: MILK *vs* CORN $P<0.001$, MILK *vs* CORN+MILK $P<0.01$.

$P<0.001$) and *vs* CORN+MILK (60±5 *vs* 47±8 μmol·L^{-1}; $P<0.001$). These peak methionine concentrations were reached faster following MILK ingestion *vs* CORN (34±9 *vs* 103±24 min; $P<0.001$), and *vs* CORN+MILK (34±9 *vs* 53±26 min; $P=0.03$). The overall increase in plasma methionine concentrations over the entire 300 min post-prandial period, expressed as iAUC, was 5 times greater for MILK *vs* CORN (5±1 *vs* 1±1 mmol·300 min·L^{-1}; $P<0.001$), and 40 % greater for MILK *vs* CORN+MILK (5±1 *vs* 3±1 mmol·300 min·L^{-1}; $P<0.001$; **Figure 5.3H**).

In general, increases in plasma amino acid concentrations revealed significant differences over time between MILK and CORN for all measured amino acids (**Supplemental Figure 5.2**, *Time x treatment* all $P<0.001$). For MILK *vs* CORN+MILK, the increase in plasma amino acid concentrations was significantly different for all measured amino acids, except for cystine, glutamic acid, glycine, histidine, ornithine, threonine and valine (**Supplemental Figure 5.2**). The increases in plasma amino acid concentrations over the entire 300 min post-prandial period (iAUC) were greater for glutamic acid, isoleucine, ornithine, proline, serine, threonine, tryptophan, tyrosine valine, BCAA, NEAA, and TAA for MILK *vs* CORN ($P<0.05$). For MILK *vs* CORN+MILK, plasma iAUC were greater for isoleucine, threonine, tryptophan, and valine ($P<0.05$, **Supplemental Figure 5.2**).

Plasma and muscle L-[ring-$^{13}C_6$]-phenylalanine enrichments

Plasma L-phenylalanine concentrations and L-[ring-$^{13}C_6$]-phenylalanine enrichments over time are presented in **Figure 5.4A** and **5.4B**, respectively. Plasma L-[ring-$^{13}C_6$]-phenylalanine enrichments over time were different between MILK *vs* CORN at *t*= 15, 30, 45, 60, 120, 150, 240, and 300 min following protein ingestion (T*ime x treatment: P*<0.001), but not between MILK *vs* CORN+MILK (**Figure 5.4B**; T*ime x treatment: P*=0.15). Mean plasma L-[ring-$^{13}C_6$]-phenylalanine enrichments averaged 7.11±0.65, 7.05±0.43 and 6.78±0.59 MPE during the basal post-absorptive period, and 6.64±0.53, 6.72±0.35, and 6.65±0.33 MPE during the full 300 min post-prandial period for MILK, CORN+MILK, and CORN respectively.

Myofibrillar protein-bound L-[ring-$^{13}C_6$]-phenylalanine enrichments increased following ingestion of MILK, CORN+MILK and CORN from 0.0032±0.0032, 0.0033±0.0032, and 0.0036±0.0026 MPE at *t*=0 min, to 0.0115±0.0041, 0.0116±0.0070, and 0.0124±0.0045 MPE at *t*= 120 min, reaching 0.0214±0.0049, 0.0214±0.0108, and 0.0216±0.0053 MPE, respectively, at 300 min after protein ingestion, with no differences observed between MILK *vs* CORN (all $P>0.65$) and MILK *vs* CORN+MILK (all $P>0.93$) at any time point.

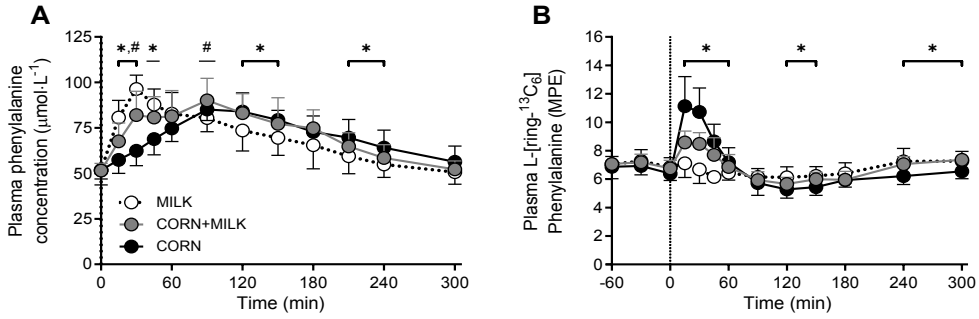

FIGURE 5.4 Post-prandial plasma phenylalanine concentrations (Panel A) and plasma 1-[13C6]-phenylalanine enrichments (Panel B) during the 300 min period following the ingestion of MILK *vs* CORN and MILK *vs* CORN+MILK in healthy, young males (*n*=12 per group). Time 0 min represents time of beverage intake. MILK: 30 g milk protein, CORN: 30 g corn protein, CORN+MILK: 15 g corn protein + 15 g milk protein. Values represent means ± standard deviation; * significantly different for MILK *vs* CORN ($P<0.05$), # significantly different for MILK *vs* CORN+MILK ($P<0.05$). Repeated measures ANOVA with time as within-subject variable and interventional drink (treatment) as between-subject variable. *Time x treatment*: Panel A: MILK *vs* CORN $P<0.001$, MILK *vs* CORN+MILK $P<0.001$, Panel B: MILK *vs* CORN $P<0.001$, MILK *vs* CORN+MILK $P=0.15$.

Muscle protein synthesis rates

Post-absorptive fractional myofibrillar protein synthesis rates averaged 0.014±0.014, 0.015±0.015 and 0.017±0.012 %·h^{-1} in MILK, CORN+MILK, and CORN, with no differences between MILK *vs* CORN ($P=0.61$) and MILK *vs* CORN+MILK ($P=0.88$). Protein ingestion increased myofibrillar protein synthesis rates to 0.059±0.024, 0.054±0.031 and 0.052±0.017 %·h^{-1} during the early post-prandial period (0-120 min) and to 0.049±0.017, 0.051±0.032, 0.052±0.021 %·h^{-1} during the late post-prandial period (120-300 min). Post-prandial muscle protein FSR averaged 0.053±0.013, 0.052±0.024 and 0.052±0.013 %·h^{-1} assessed over the entire 300 min post-prandial period after protein ingestion (**Figure 5.5**). Post-prandial myofibrillar protein synthesis rates did not differ between MILK *vs* CORN, for the early (0-120 min; $P=0.46$), late (120-300 min; $P=0.73$), and entire (0-300 min; $P=0.90$) post-prandial period. Similarly, post-prandial myofibrillar protein synthesis rates did not differ between MILK *vs* CORN+MILK, for the early (0-120 min; $P=0.66$), late (120-300 min; $P=0.87$), and entire (0-300 min; $P=0.92$) post-prandial period (**Figure 5.5**). Myofibrillar protein synthesis rates determined with the intra-cellular L-[ring-$^{13}C_6$]-phenylalanine enrichments used as precursor pool resulted in similar findings with no differences in FSR values between Milk *vs* CORN and MILK *vs* CORN+MILK at any time point (**Supplemental Figure 5.3**).

FIGURE 5.5 Myofibrillar fractional synthetic rate (FSR) at different time points following ingestion of CORN *vs* MILK and MILK *vs* CORN+MILK in healthy, young males (*n*=12 per group). MILK: 30 g milk protein, CORN: 30 g corn protein, CORN+MILK: 15 g corn protein + 15 g milk protein. Bars represent means ± standard deviation, dots represent individual values. *significantly different from basal; *P*<0.05. Independent samples *t*-test: MILK *vs* CORN *P*=0.61, *P*=0.46, and *P*=0.90 for basal, 0-120, and 0-300 min, respectively. MILK *vs* CORN+MILK *P*=0.88, *P*=0.66, and *P*=0.92 for basal, 0-120, and 0-300 min, respectively.

DISCUSSION

The present study shows that ingestion of 30 g corn protein or a blend of corn plus milk protein blend robustly increases muscle protein synthesis rates in healthy, young males. Despite the observation of faster and greater post-prandial plasma EAA availability following milk when compared to corn or corn plus milk protein blend ingestion, post-prandial myofibrillar protein synthesis rates did not differ between treatments.

Corn is a plant-based protein source with an exceptionally high leucine content of ~13% of total protein content, when compared to ~8% in milk protein. As leucine availability represents a key factor for stimulating post-prandial muscle protein synthesis [4, 8, 39, 40], corn derived protein may represent a plant derived protein that can robustly stimulate skeletal muscle protein synthesis rates. However, although corn derived protein holds a very high leucine content, lysine content (1.5%) falls well below the WHO/FAO/UNU requirements of 4.5% [41, 42]. The insufficient provision of one (or more) EAA could be restrictive and, as such, attenuate the post-prandial rise in muscle protein synthesis rates when compared to higher quality animal derived proteins [24, 41]. Whether the relative high leucine content may be offset by the lysine deficiency in corn derived protein remains speculative. In the present study, we confirmed that the leucine content was substantially higher (4.1 *vs* 2.4 g, respectively) and the lysine content was considerably lower (0.3 *vs* 2.0 g, respectively) in the corn derived protein compared with the milk derived protein (**Table 5.2**). Ingestion of the proteins robustly increased circulating amino acid concentrations (**Figure 5.3**). Although the increase in plasma leucine concentrations was more rapid following ingestion of milk when compared with the ingestion of corn derived protein, the latter showed a more sustained

increase resulting in an overall 45% greater plasma leucine availability over the entire 5h post-prandial period (**Figure 5.3D**). In line with the amino acid composition of the proteins, plasma lysine concentrations increased significantly following milk protein ingestion, whereas no increase above post-absorptive values was observed following ingestion of the corn derived protein. In accordance, post-prandial plasma lysine availability was significantly greater following milk when compared with corn protein ingestion, for which no increase in lysine concentration was detected. These results tend to be in line with previous reports on post-prandial amino acid responses following wheat protein ingestion, which is also particular low in lysine content, showing no significant changes in post-prandial plasma lysine availability following ingestion of respectively 30 and 35 g of wheat protein hydrolysate [15, 28]. Though post-prandial plasma amino acid responses seem to follow differences in amino acid profiles of the ingested proteins, it is evident that these differences are certainly not proportional. This discrepancy is likely attributed to various differences in protein digestion, amino acid absorption, and/or amino acid retention in splanchnic tissues.

Previous work suggests that post-prandial plasma amino acid availability may be predictive for the anabolic response following protein ingestion [14, 43]. In the present study, we observed a strong increase in muscle protein synthesis rates above basal post-absorptive rates following the ingestion of corn derived protein. A response that did not differ from the response observed after ingesting an equivalent amount of milk protein (**Figure 5.5**). Clearly, the provided corn derived protein is capable of robustly stimulating muscle protein synthesis rates *in vivo* in humans. We can only speculate whether this is attributed to high(er) leucine content, as we recently also observed no differences in muscle protein synthesis rates following the ingestion of similar amounts of wheat (lower leucine content, [28]) and potato (equal leucine content [44]) derived protein when compared to an equivalent amount of dairy protein. Our data do indicate that the low lysine content provided in corn protein is not restrictive for the acute *in vivo* skeletal muscle anabolic response when ingesting an ample amount of protein. This seems also in line with our previous findings on the post-prandial response to wheat ingestion in young healthy adults, where we failed to detect any differences in muscle protein synthesis rates following ingestion of a sufficient amount (e.g. 30 g) of wheat when compared with milk derived protein, despite its low lysine and methionine contents [28]. These unexpected findings may be attributed to the selection of healthy young volunteers in the present study, in contrast to the older individuals selected in the previous studies [15, 17] who may have been suffering from some level of anabolic resistance. Alternatively, previous studies have provided ~20 g protein to evaluate differences in post-prandial muscle protein synthesis rates [14, 18], which is in line with the estimated ~0.25 $g \cdot kg^{-1}$ body mass animal-derived protein needed to maximize post-prandial muscle protein synthesis. The optimal amount of plant-derived protein to be ingested for stimulating muscle protein synthesis remains to be determined. However, the 30 g protein dose (which represented ~0.40 $g \cdot kg^{-1}$ body mass), provided in the current study, may represent a more than adequate amount to maximize muscle protein synthesis rates, regardless the animal or plant derived origin of the protein. Furthermore, in this acute setting, the high leucine content of corn-derived protein [41] may have been the main driver of the post-prandial

muscle protein synthetic response. Obviously, the lower plasma lysine availability following ingestion of corn-derived when compared to milk-derived protein ingestion, did not restrict the acute post-prandial rise in muscle protein synthesis rate.

We anticipated that the low lysine content in corn derived protein would compromise the anabolic response following protein ingestion and, as such, we added a third treatment group with a blend of 50% corn derived and 50% milk protein. While for the comparison of milk *vs* corn protein, the protein lysine content amounted ~6.5 *vs* ~1% respectively, for the milk *vs* corn plus milk protein comparison, the lysine content was ~6.5 *vs* ~4% respectively. This resulted in the lysine content of the protein blend to approach the lysine requirements for adults of 4.5% as indicated by the WHO/FAO/UNU [42]. In line with the comparison with corn protein only, no differences were observed in post-prandial muscle protein synthesis rates following the ingestion of milk and the corn plus milk protein blend. The finding that both the corn protein and corn plus milk protein blend did not differ in their capacity to stimulate muscle protein synthesis rates when compared with milk protein, provides additional evidence that lysine deficiency in a plant derived protein does not seem to compromise the acute post-prandial muscle protein synthetic response when compared to the ingestion of a higher-quality, animal derived protein, under conditions where a sufficient amount of protein is ingested by healthy, young adults.

There are only few studies that have assessed the capacity of plant-derived protein to directly stimulate post-prandial muscle protein synthesis rates [14, 17, 18, 20, 28]. The present study is the first to evaluate the anabolic properties of corn derived protein as well as a blend of corn plus milk protein in vivo in humans. We clearly show that a deficiency in a specific EAA does not restrict the acute muscle protein synthetic response following protein ingestion. The available free EAA pool in the body resulting from protein breakdown, may be sufficient to compensate a specific EAA deficiency when protein is ingested acutely in a rested state. Especially in a real-life dietary setting where multiple plant and animal based proteins are ingested in mixed meals, the deficiency of a single amino acid in a specific protein source, is not likely to compromise the overall muscle anabolic response to plant based protein ingestion in young individuals. However, whether this would also hold true for other populations such as elderly and clinical populations suffering from anabolic resistance to protein intake, remains questionable. In this regard, we have previously shown that the ingestion of 35 g wheat protein hydrolysate did not stimulate muscle protein synthesis rates in older men [15]. Therefore, more research is warranted to establish whether the lack of specific EAA in plant based proteins would limit its anabolic response in older compared to younger individuals, and more long term *vs* acute settings. In addition, it is important to take into consideration that the present study investigated protein isolates. Although the protein isolates have various applications (e.g.: milk formula, enriching food products with protein), corn and milk both contain only ~3 - 3.5 g protein per 100 g of food product. Evaluating the anabolic response to protein isolates is the first step to determine the anabolic potential of different protein sources. However, it is important to consider that protein quality can also be affected by other nutrients that contribute to the whole foods matrix, or processed food

products. Therefore, future research will need to evaluate the anabolic response to food products containing these protein isolates, as well as whole foods and complex meals.

In conclusion, ingestion of 30 g milk protein, 30 g corn protein, or a blend of 15 g corn plus 15 g milk protein increases muscle protein synthesis rates in young, healthy males. Post-prandial muscle protein synthesis rates following the ingestion of 30 g milk protein do not differ from rates observed after ingesting 30 g corn protein or a blend providing 15 g milk plus 15 g corn protein in healthy, young males. Ingestion of a meal-like (30 g) dose of plant-derived protein can be as effective as ingesting the same amount of animal-derived protein to increase muscle protein synthesis rates *in vivo* in healthy, young males.

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The author contributions were as follows: LJCvL, TS, LBV, LCPGMdG, and PJMP designed research; PJMP, MEGW, LHPH, AHZ, and IWKK conducted research; PJMP, TS and LJCvL analyzed data; PJMP and LJCvL wrote paper; PJMP, TS and LJCvL had primary responsibility for final content. All authors read and approved the final manuscript.

Declarations

PJMP, IWKK, LCPGMdG, LBV, TS and LJCvL have the following interests: This study was funded by TiFN, Wageningen, The Netherlands. The sponsors Tereos Syral (Marckolsheim, France), Cargill (Minneapolis, MN, USA), and Kellogg (Battle Creek, MI, USA) partly financed the project. The researchers are responsible for the study design, data collection and analysis, decision to publish, and preparation of the manuscript. The industrial partners have contributed to the project through regular discussion, and were involved in the study design. More specifically: the choice of interventional products which were produced by these sponsors. The funders had no role in data collection and analysis, decision to publish, or preparation of the manuscript. In addition: LJCvL and LBV have received research grants, consulting fees, speaking honoraria, or a combination of these from Friesland Campina, Tereos Syral, and Pepsico. The other authors report no conflicts of interest.

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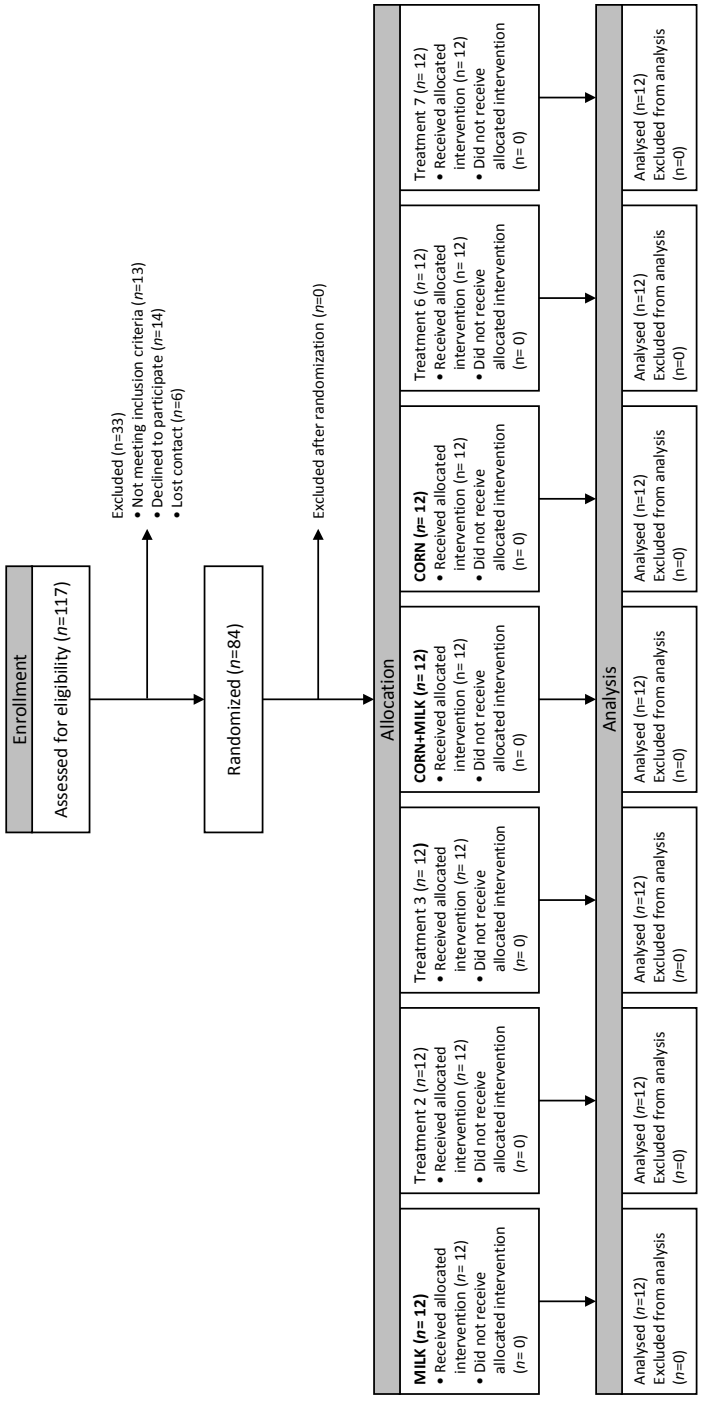
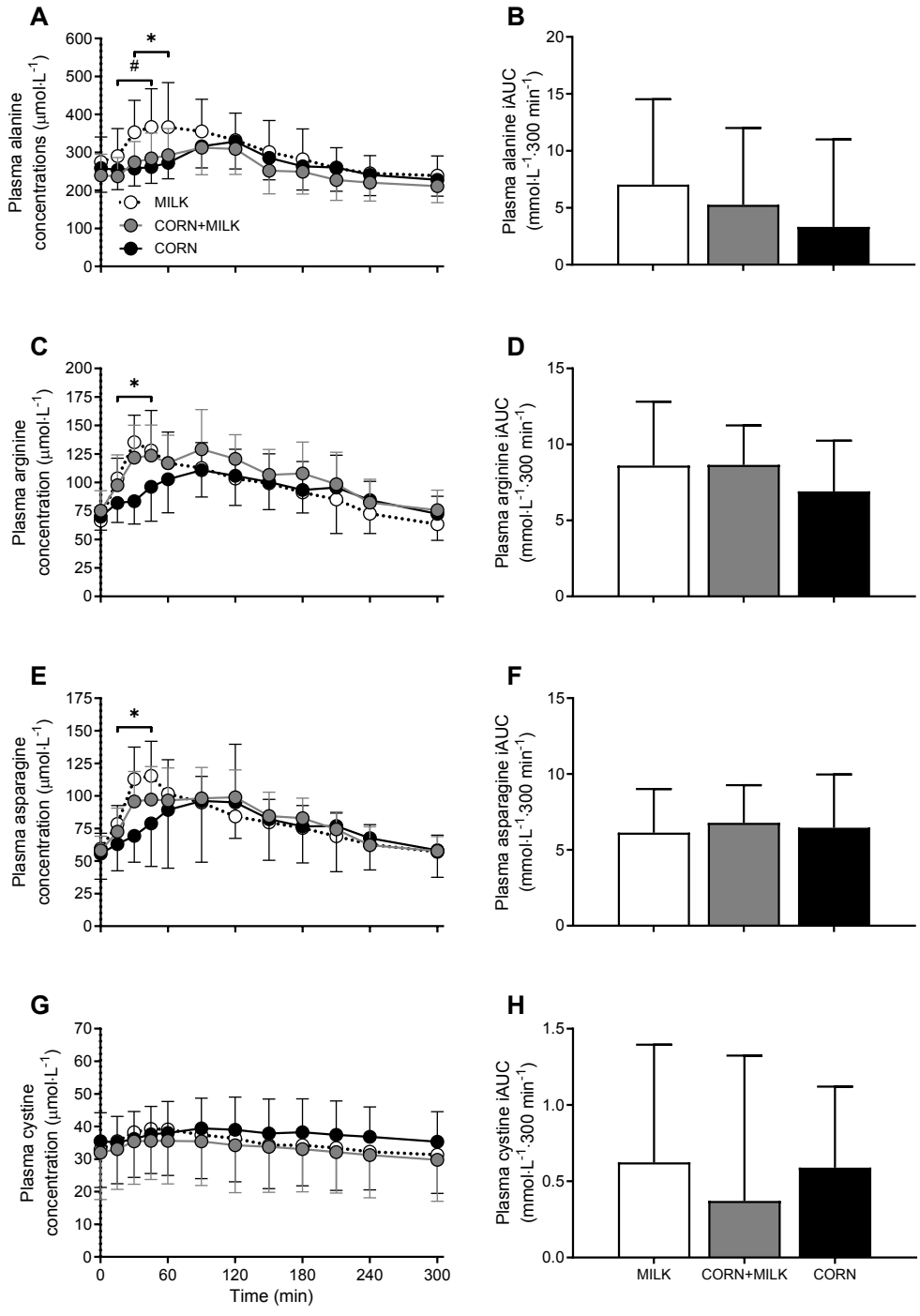
SUPPLEMENTAL MATERIAL



SUPPLEMENTAL FIGURE 5.1 CONSORT flow diagram. CONSORT, Consolidated Standards of Reporting Trials. The current study was part of a larger trial with a total of 7 parallel groups (n = 12 per group) as indicated in the flow diagram. MILK (30 g milk protein), CORN (30 g corn protein), CORN+MILK (15 g corn protein + 15 g milk protein)



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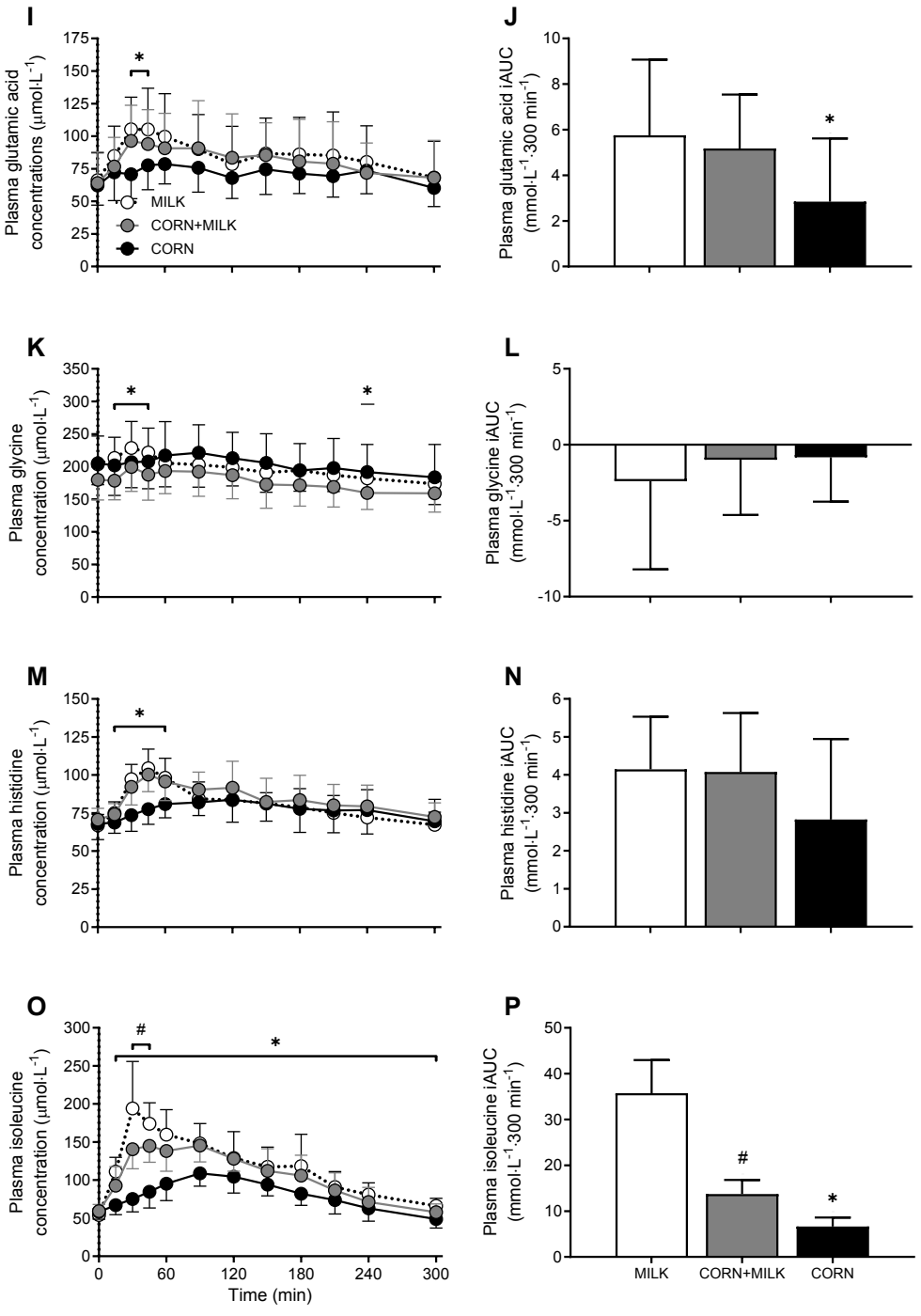

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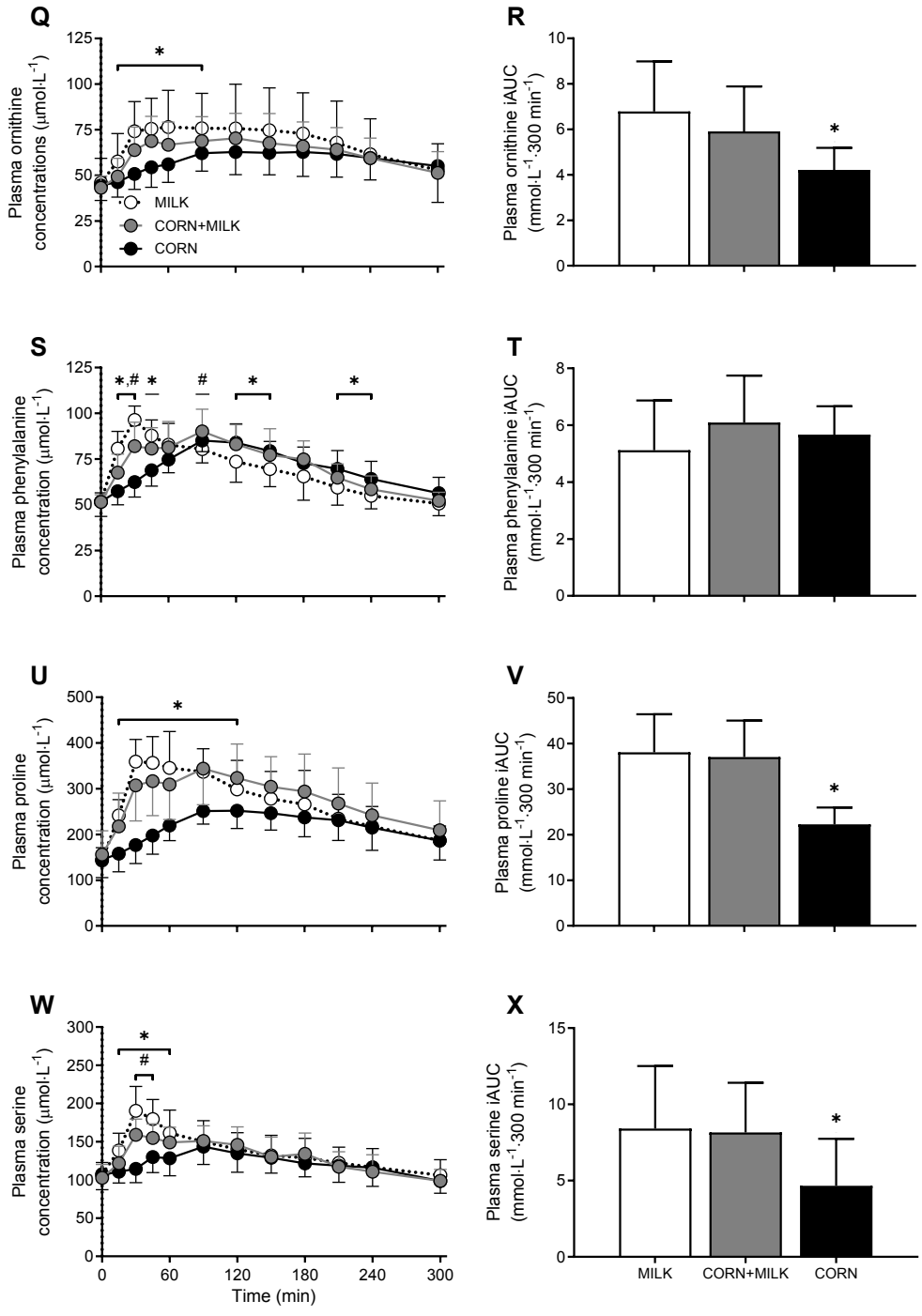

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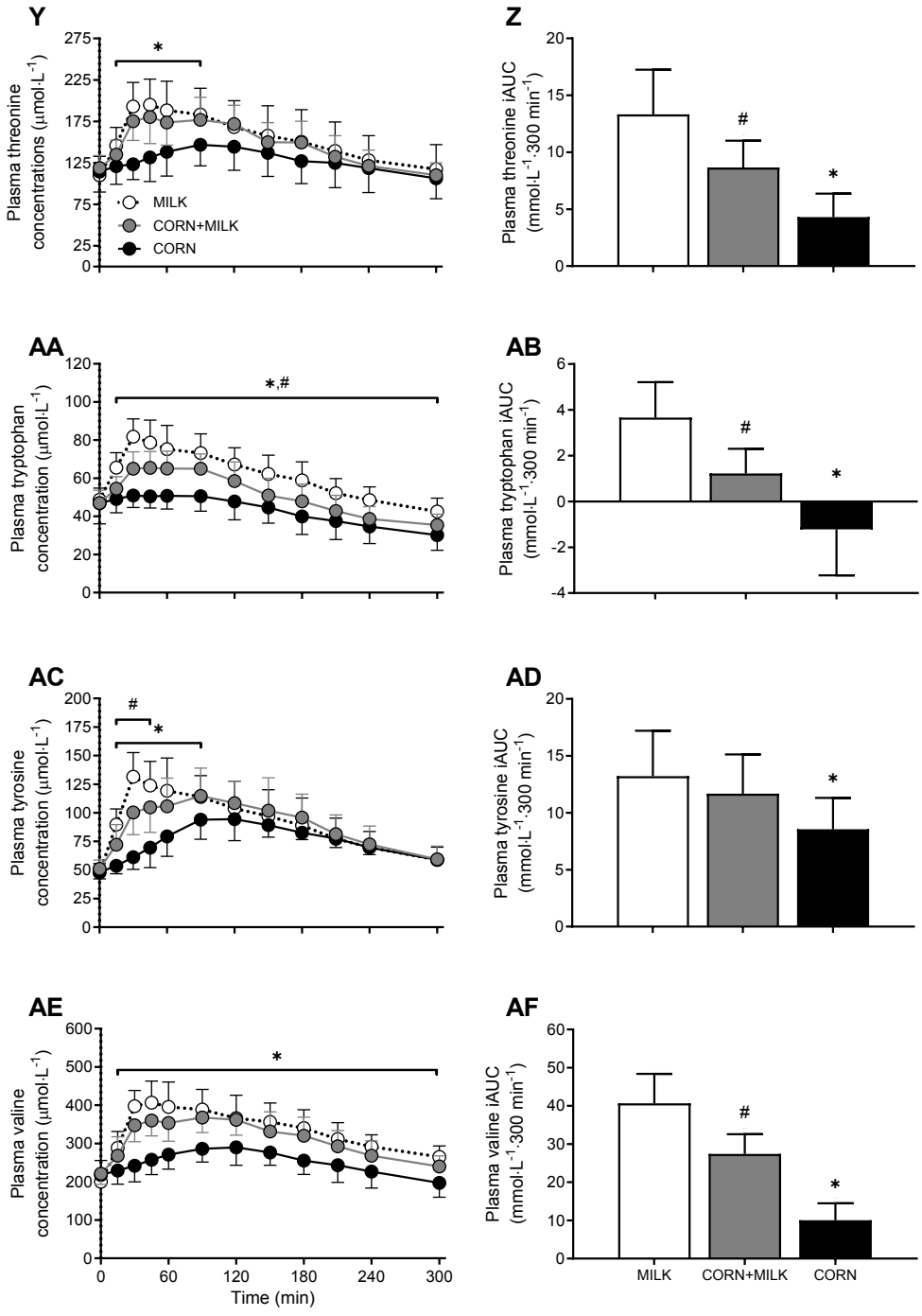

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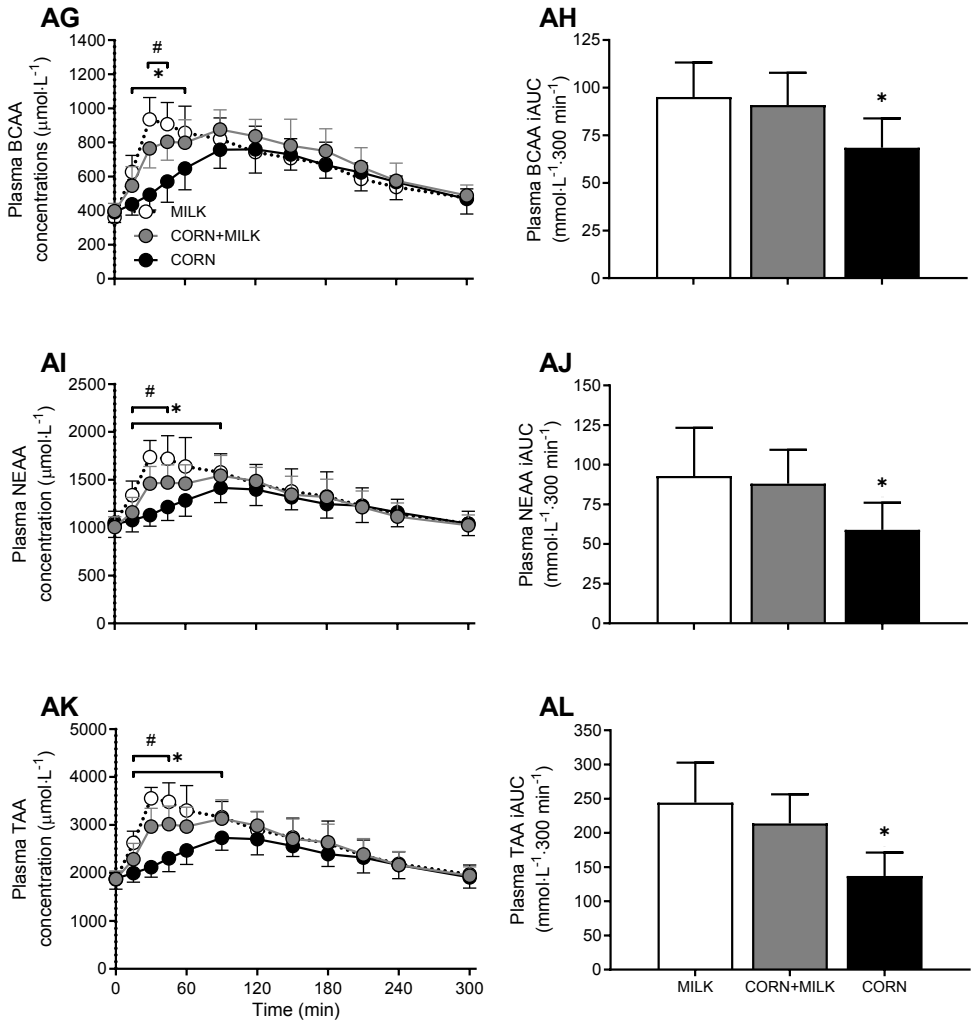

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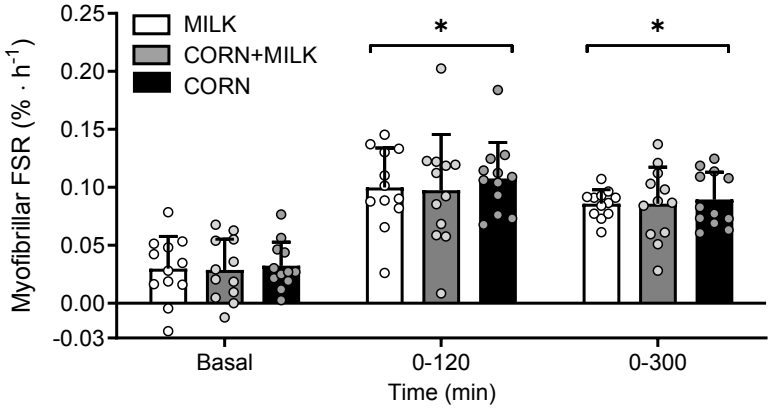
SUPPLEMENTAL FIGURE 5.2 Post-prandial plasma amino concentrations during the 300 min post-prandial period following the ingestion of MILK *vs* CORN and MILK *vs* CORN+MILK. Time 0 min represents time of beverage intake. Panels B, D, F, H, J, L, N, P, R, T, V, X, Z, AB, AD, AF, AH, AJ, AL represent the 0-5 h incremental area under curve (iAUC) following protein ingestion. MILK (30 g milk protein), CORN (30 g corn protein), CORN+MILK (15 g corn protein + 15 g milk protein). Values represent means ± standard deviation; *significantly different for MILK *vs* CORN ($P<0.05$), # significantly different for MILK *vs* CORN+MILK ($P<0.05$). Repeated measures ANOVA with time as within-subject variable and interventional drink (treatment) as between-subject variable, and independent samples *t*-test were used to determine differences between groups. Values displayed below represent the P-values for the different panels.

	2-factor repeated measures ANOVA		Independent samples t-test	
	MILK *vs* CORN	MILK *vs* CORN+MILK	MILK *vs* CORN	MILK *vs* CORN+MILK
Alanine	A: <0.001	A: 0.02	B: 0.25	B: 0.55
Arginine	C: <0.001	C: <0.01	D: 0.29	D: 0.97
Asparagine	E: <0.001	E: <0.01	F: 0.81	F: 0.56
Cystine	G: <0.001	G: 0.30	H: 0.90	H: 0.48
Glutamic acid	I: <0.001	I: 0.15	J: 0.03	J: 0.63
Glycine	K: <0.001	K: 0.05	L: 0.41	L: 0.48
Histidine	M: <0.001	M: 0.10	N: 0.08	N: 0.91
Isoleucine	O: <0.001	O: 0.04	P: <0.001	P: <0.01
Ornithine	Q: <0.001	Q: 0.30	R: 0.001	R: 0.31
Phenylalanine	S: <0.001	S: <0.001	T: 0.35	T: 0.17
Proline	U: <0.001	U: <0.01	V: <0.001	V: 0.76
Serine	W: <0.001	W: <0.01	X: 0.02	X: 0.87
Threonine	Y: <0.001	Y: 0.20	Z: <0.001	Z: <0.01
Tryptophane	AA: <0.001	AA: 0.04	AB: <0.001	AB: <0.001
Tyrosine	AC: <0.001	AC: <0.01	AD: <0.01	AD: 0.32
Valine	AE: <0.001	AE: 0.05	AF: <0.001	AF: <0.001
BCAA	AG: <0.001	AG: 0.001	AH: 0.001	AH: 0.56
NEAA	AI: <0.001	AI: 0.001	AJ: <0.01	AJ: 0.65
TAA	AK: <0.001	AK: <0.01	AL: <0.001	AL: 0.16

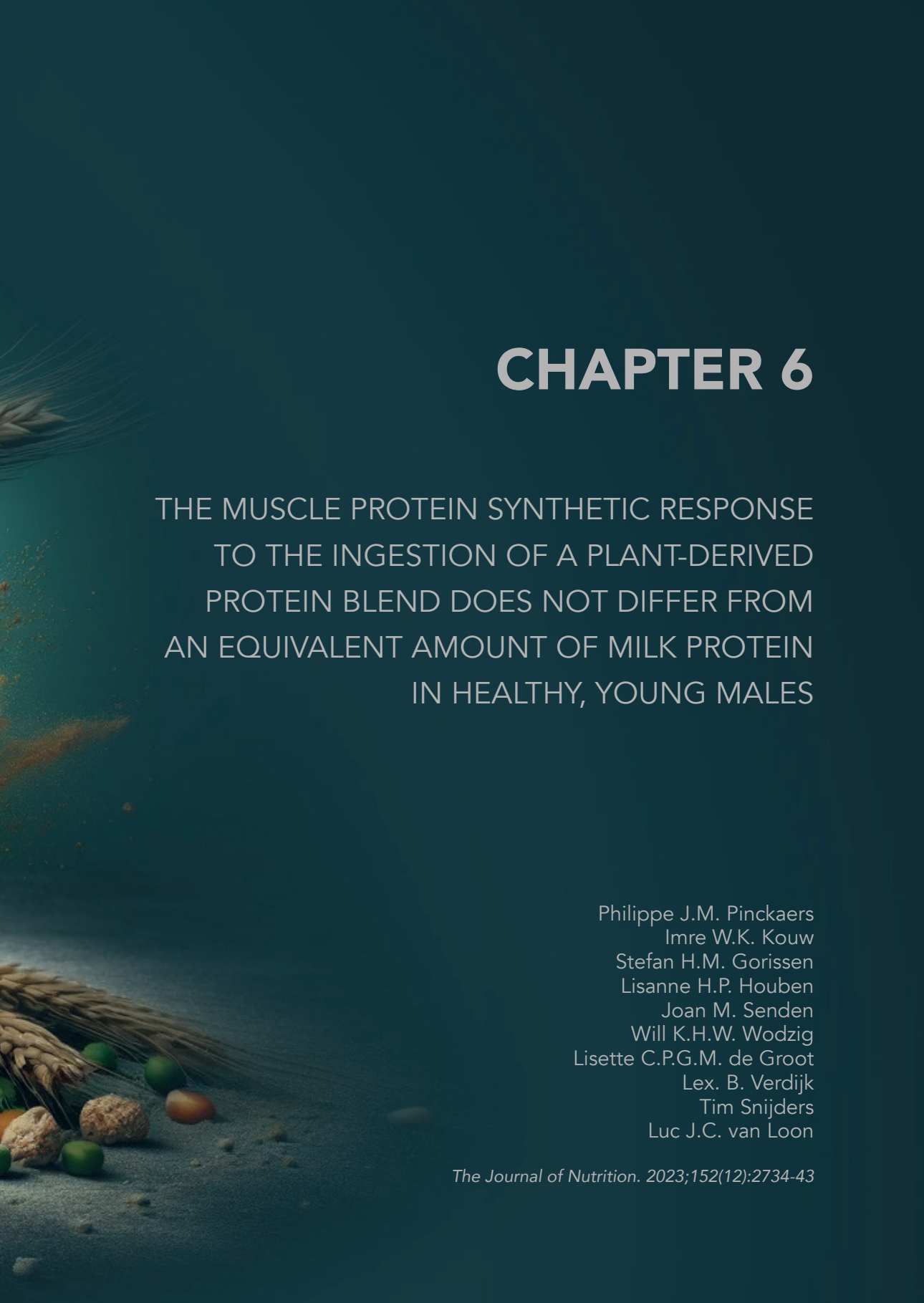


SUPPLEMENTAL FIGURE 5.3 Myofibrillar fractional synthetic rate (FSR) determined with intra-cellular enrichments as precursor pool at different time points following ingestion of MILK *vs* CORN and MILK *vs* CORN+MILK in healthy, young males (*n*=12 per group). MILK: 30 g milk protein, CORN: 30 g corn protein, CORN+MILK: 15 g corn protein + 15 g milk protein. Bars represent means ± standard deviation, dots represent individual values. *significantly different from basal; *P*<0.05. Independent samples *t*-test: MILK *vs* CORN *P*=0.81, *P*=0.56, and *P*=0.64 for basal, 0-120, and 0-300 min, respectively. MILK *vs* CORN+MILK *P*=0.92, *P*=0.89, and *P*=0.99 for basal, 0-120, and 0-300 min, respectively.

SUPPLEMENTAL TABLE 5.1 Average 3 day dietary intake of study participants

	MILK	CORN+MILK	CORN
Energy (MJ·d^{-1})	9.3 ± 2.2	7.9 ± 1.7	10.2 ± 2.5
Carbohydrate (g·d^{-1})	267 ± 63	216 ± 63	277 ± 84
Fat (g·d^{-1})	78 ± 27	69 ± 27	91 ± 31
Protein (g·d^{-1})	97 ± 29	80 ± 29	103 ± 37
Energy (kJ·kg^{-1}·d^{-1})	131 ± 26	104 ± 18	133 ± 30
Carbohydrate (g·kg^{-1}·d^{-1})	3.8$^{\#}$ ± 0.9	3.0 ± 0.9	3.8 ± 1.1
Fat (g·kg^{-1}·d^{-1})	1.1 ± 0.3	0.9 ± 0.3	1.2 ± 0.4
Protein (g·kg^{-1}·d^{-1})	1.3 ± 0.4	1.1 ± 0.4	1.4 ± 0.4
Carbohydrate (% total energy)	50 ± 7	48 ± 7	48 ± 10
Fat (% total energy)	33 ± 8	35 ± 8	35 ± 10
Protein (% total energy)	18 ± 3	18 ± 3	17 ± 3

Values represent mean ± standard deviation. *n*=12 per nutritional intervention group. MILK: 30 g milk protein, CORN+MILK: 15 g corn protein plus 15 g milk protein, CORN: 30 g corn protein. Independent samples *t*-test for MILK *vs* CORN and MILK *vs* CORN+MILK. $^{\#}$significantly different for MILK *vs* CORN+MILK (*P*<0.05). 3 Day food records were analyzed using "Mijn Eetmeter" (https://mijn.voedingscentrum.nl/nl/eetmeter/), online software available from the Netherlands Nutrition Centre.



CHAPTER 6

THE MUSCLE PROTEIN SYNTHETIC RESPONSE TO THE INGESTION OF A PLANT-DERIVED PROTEIN BLEND DOES NOT DIFFER FROM AN EQUIVALENT AMOUNT OF MILK PROTEIN IN HEALTHY, YOUNG MALES

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ABSTRACT

Background

Plant-derived proteins are considered to have lesser anabolic properties when compared with animal-derived proteins. The attenuated rise in muscle protein synthesis rates following ingestion of plant compared with animal-derived protein has been, at least partly, attributed to deficiencies in specific amino acids such as leucine, lysine, and/or methionine. Combining different plant-derived proteins may provide plant-derived protein blends with a more balanced amino acid profile.

Objective

This study aimed to compare post-prandial muscle protein synthesis rates following the ingestion of 30 g milk protein with a 30 g blend combining wheat, corn, and pea protein in healthy, young males.

Methods

In a randomized, double blind, parallel-group design, 24 young males (24±4 y) received a primed continuous L-[ring-$^{13}C_6$]-phenylalanine infusion after which they ingested 30 g milk protein (MILK) or a 30 g plant-derived protein blend combining 15 g wheat, 7.5 g corn, and 7.5 g pea protein (PLANT-BLEND). Blood and muscle biopsies were collected frequently for 5 h to assess post-prandial plasma amino acid profiles (secondary outcome) and subsequent muscle protein synthesis rates (primary outcome). Data were analyzed by two way-repeated measures ANOVA and two-samples *t*-tests.

Results

MILK increased plasma essential amino acid concentrations more than PLANT-BLEND over the 5 h postprandial period (incremental area under curve 151±31 *vs* 79±12 mmol·300 min·L^{-1} respectively; $P<0.001$). Ingestion of both MILK and PLANT-BLEND increased myofibrillar protein synthesis rates ($P<0.001$), with no significant differences between treatments (0.053±0.013 and 0.064±0.016 %·h^{-1}, respectively; $P=0.08$).

Conclusion

Ingestion of 30 g of a plant-derived protein blend combining wheat, corn, and pea-derived protein increases muscle protein synthesis rates in healthy, young males. The muscle protein synthetic response to the ingestion of 30 g of this plant-derived protein blend does not differ from the ingestion of an equivalent amount of a high quality animal-derived protein.

INTRODUCTION

Protein ingestion increases muscle protein synthesis rates [1, 2]. The increase in muscle protein synthesis rate is driven by the post-prandial increase in circulating plasma essential amino acid concentrations [3], with the rise in circulating leucine being of particular relevance [4-8]. The anabolic properties of different proteins or protein sources seem to be largely determined by their protein digestion and amino acid absorption kinetics, essential amino acid content, and amino acid profile [9-11]. Consequently, post-prandial muscle protein synthesis rates can differ substantially following ingestion of the same amount of protein derived from different sources [12-14].

Plant-based proteins comprise a large part of our daily protein intake [15] and are likely to become more important with respect to the global transition towards the consumption of a more plant-based protein diet [16, 17]. However, plant-derived proteins are believed to have lesser anabolic properties when compared to animal-derived proteins due to their lower digestibility and/or incomplete amino acid profile [17, 18]. Most plant-derived proteins are relatively low in essential amino acid content and often show deficiencies in one or more specific amino acids, such as leucine, lysine, and/or methionine [19]. Combining different plant-derived proteins within a single blend represents one of the strategies to compose a bolus of plant-derived proteins with a more balanced amino acid profile, with less apparent amino acid deficiencies [17-22]. Whereas some plant-based proteins are particularly deficient in lysine, others are deficient in methionine [19]. Combining corn, hemp, or brown rice-derived protein (low lysine and high methionine content) with soy or pea-derived protein (low methionine and high lysine content) provides us with the opportunity to compose blends of proteins that complement each other for their amino acid deficiencies [18-22]. As such, plant-based protein blends may provide amino acid profiles that closely resemble high quality animal derived proteins, with less amino acid deficiencies when compared to individual plant-based proteins.

Previous work has shown that blends of animal and plant-derived proteins can be as effective as high quality animal-derived proteins to increase muscle protein synthesis during recovery from exercise [21, 23]. As far as we know there are no studies that have compared the anabolic properties of an exclusively plant-derived protein blend to a high quality animal-derived protein when ingested in a resting condition. Therefore, we composed a plant-derived protein blend with an amino acid composition that is similar to most animal-derived proteins, combining a high leucine content and no apparent amino acid deficiencies. By combining wheat and corn protein (with lysine contents below WHO/FAO/UNU requirements [24]) with pea protein (with lysine content being amongst the highest for plant-derived proteins) we composed a protein blend with no apparent lysine deficiency. Furthermore, whereas wheat and pea-derived proteins fall short for the WHO/FAO/UNU methionine requirements [25], corn protein can compensate for this with its high methionine content. Finally, the leucine content of corn exceeds even the levels observed in whey protein and, as such, can be used to create a plant-derived protein blend with a high leucine content [19].

We hypothesize that the ingestion of a plant-based protein blend consisting of wheat, corn, and pea derived protein, can strongly increase muscle protein synthesis rates. Furthermore, we hypothesized that the muscle protein synthetic response to the ingestion of this protein blend is not inferior when compared with a high quality animal-derived protein such as milk protein. To test our hypotheses, we selected 24 healthy young males to partake in this study in which we compared the impact of ingesting 30 g milk protein with the ingestion of an equivalent amount of a plant-based protein blend (providing 15 g wheat protein, 7.5 g corn protein, 7.5 g pea protein) on *in vivo* post-prandial muscle protein synthesis rates.

MATERIALS AND METHODS

Participants

Healthy, recreationally active males aged 18-35 y inclusive were eligible to participate in this parallel group, double blind, randomized controlled trial (participants' characteristics are presented in **Table 6.1**). Participants were recreationally active and generally performed between 2-4 exercise sessions per week in various sports (e.g. soccer, basketball, weight lifting, running, cycling, etc.), but were not involved in any structured progressive exercise training regimen. This study was part of a larger trial registered at the Netherlands Trial Register (NTR6548), and was conducted between June 2017 and April 2019 at Maastricht University in Maastricht, The Netherlands (See **Supplemental Figure 6.1** for the CONSORT (Consolidated Standards of Reporting Trials) flow diagram, indicating the specific comparison that the current study was based on). The data of the milk protein group have been published previously, as well as the procedures applied in this trial [26]. All participants were informed about the purpose of the study, the experimental procedures, and possible risks before providing written informed consent to participate. The procedures followed were in accordance with the ethical standards of the medical ethics committee of Maastricht University Medical Centre+ (METC 173001), and in accordance with the Helsinki Declaration of 1975 as revised in October 2013. The study was independently monitored and audited by the Clinical Trial Centre Maastricht.

Preliminary testing

Participants aged 18-35 y, with BMI >18.5 and <27.5 $kg \cdot m^{-2}$ underwent an initial screening session to assess eligibility. Height, weight, blood pressure and body composition (by dual-energy X-ray absorptiometry; Discovery A, Hologic; (National Health and Nutrition Examination Survey - Body composition analysis (NHANES BCA) enabled) were determined. Participants were deemed healthy based on their responses to a medical questionnaire. The screening sessions and experimental trials were separated by at least 3 days.

Study design

Participants were randomly assigned to ingest a 400 mL beverage containing either 30 g milk protein concentrate (MILK) or a 30 g plant-protein blend consisting of 15 g wheat protein hydrolysate, 7.5 g corn protein isolate, and 7.5 g pea protein concentrate (PLANT-

TABLE 6.1 Participants' characteristics

	MILK	PLANT-BLEND
Age (y)	26 ± 4	22 ± 4
Height (m)	1.76 ± 0.06	1.80 ± 0.06
Mass (kg)	71.5 ± 9.0	70.8 ± 7.9
BMI ($kg \cdot m^{-2}$)	23.0 ± 2.1	21.9 ± 2.2
Systolic blood pressure (mmHg)	119 ± 6	129 ± 7
Diastolic blood Pressure (mmHg)	71 ± 9	68 ± 8
Resting heart rate (bpm)	64 ± 10	64 ± 8
Lean body mass (kg)	53.2 ± 7.9	54.0 ± 5.3
Body fat (%)	23.1 ± 3.2	20.9 ± 4.4

Values represent mean ± standard deviation. n = 12 per nutritional intervention group. MILK: 30 g milk protein, PLANT-BLEND: 15 g wheat protein, 7.5 g corn protein, and 7.5 g pea protein. Two-samples t-test all $P>0.05$.

BLEND). After beverage ingestion, the bottle was rinsed with 150 mL of water, which was also ingested by the participants. Milk protein concentrate (Refit MPC80) was obtained from FrieslandCampina (Wageningen, the Netherlands), wheat protein hydrolysate (Meripro 500) was supplied by Tereos Syral (Marckolsheim, France), corn protein isolate was supplied by Cargill (Minneapolis, MN, USA), and pea protein concentrate (Nutralys S85F) was supplied by Kellogg (Battle Creek, MI, USA). Participants were allocated to a treatment according to a block randomization list performed using a computerized randomizer (http://www.randomization.com/). An independent researcher was responsible for random assignment (n=12 per group) and preparation of the study treatment beverages, which were sequentially numbered according to subject number. The beverages were prepared in non-transparent protein shakers.

Diet and physical activity

Participants refrained from sports and strenuous physical activities (e.g. lifting heavy weights), and alcohol consumption for 3 days prior to the experimental trial. In addition, all participants were instructed to complete a food and activity record for 3 days prior to the experimental trial. (See **Supplemental Table 6.1** for an overview of participants' habitual food intake in the 3 days prior to the experimental trial). The evening before the trial, all participants consumed a standardized dinner containing 2.8 MJ, with 65% energy provided as carbohydrate, 20% as fat, and 15% as protein, before 10:00 PM after which they remained fasted.

Experimental protocol

The procedures applied in this trial, have previously been described elsewhere [26]. At ~7:30 AM, participants arrived at the laboratory in an overnight post-absorptive state. A cannula was inserted into an antecubital vein for stable isotope amino acid infusion. A second cannula was inserted retrogradely into a dorsal hand vein on the contralateral arm for arterialized

blood sampling. To obtain arterialized blood samples, the hand was placed in a hot box (60°C) for 10 min prior to blood sample collection.

After taking a baseline blood sample (*t*= -180 min), the plasma phenylalanine pool was primed with a single dose of L-[ring-$^{13}C_6$]c-phenylalanine (2.25 μmol·kg^{-1}). Thereafter, a continuous intravenous infusion of L-[ring-$^{13}C_6$]-phenylalanine (0.05 μmol·kg^{-1}·min^{-1}) was initiated (*t*= -180 min) using a calibrated IVAC 598 pump (San Diego, CA, USA). Subsequently, arterialized blood samples were collected at *t*=-90, -60 and -30 min relative to beverage ingestion. At *t*= 0 min an arterialized blood sample was obtained and a biopsy was collected from the *M. vastus lateralis*. Immediately following the muscle biopsy, participants ingested a 400 mL beverage corresponding to their randomized treatment allocation, i.e. MILK (*n*=12), or PLANT-BLEND (*n*=12). To minimize dilution of the steady-state plasma L-[ring-$^{13}C_6$]-phenylalanine precursor pool, the phenylalanine content of the protein drink was enriched with 3.85% L-[ring-$^{13}C_6$]-phenylalanine. Frequent arterialized blood samples were then collected for 300 min after protein ingestion. A second and third biopsy from the *M. vastus lateralis* were collected at *t*= 120 and *t*= 300 min to determine post-prandial skeletal muscle protein synthesis rates over the 0-120, 120-300, and 0-300 min post-prandial periods. Muscle biopsies were obtained with the use of a 5 mm Bergström needle [27], custom-adapted for manual suction and blood samples were collected into EDTA-containing tubes, according to the procedures described previously [26]. For a schematic representation of the infusion protocol, see **Supplemental Figure 6.2**.

Protein powder analysis

Batch specific nitrogen contents for milk protein concentrate, wheat protein hydrolysate, corn protein isolate and pea protein concentrate were provided by the manufacturer. The protein content of the milk protein was determined as nitrogen content x 6.38, the protein content of wheat protein powder was determined as nitrogen content x 5.7 [28, 29], and the protein content of corn and pea protein were determined as nitrogen x 6.25. Amino acid contents of the protein powders were determined by acid hydrolysis in triplicate, and subsequent analysis of the free amino acids using ultra-performance liquid chromatography-mass spectrometry (UPLC-MS; ACQUITY UPLC H-Class with QDa; Waters, Saint-Quentin, France), as previously described [26]. The amino acid composition of the protein powders and the protein blend are presented in **Table 6.2**.

Plasma analysis

Plasma glucose and insulin concentrations were analyzed using commercially available kits (ref. no. A11A01667, Glucose HK CP, ABX Diagnostics, Montpellier, France; and ref. no. HI-14K, Millipore, St. Louis, MO, respectively). Plasma amino acid concentrations were determined by UPLC-MS, as previously described [26]. Plasma L-[ring-$^{13}C_6$]-phenylalanine enrichments were determined by gas chromatography-mass spectrometry (GC-MS; Agilent 7890A GC/5975C MSD; Agilent Technologies), as previously described [26].

Basal muscle protein synthesis rates were assessed to confirm that protein ingestion increases muscle protein synthesis rates. The single biopsy approach was applied to assess post-absorptive muscle protein synthesis rates without the need to collect an additional muscle biopsy, as previously described [26, 30].

TABLE 6.2 Amino acid composition of protein or protein blend consumed[1]

	MILK	PLANT-BLEND[2]
Alanine	0.9	1.2
Arginine	0.8	1.0
Aspartic acid	1.8	1.4
Cystine	0.1	0.2
Glutamic acid	5.1	7.6
Glycine	0.5	1.0
Histidine	0.6	0.5
Isoleucine	0.9	0.6
Leucine	2.4	2.4
Lysine	2.0	0.7
Methionine	0.7	0.4
Phenylalanine	1.2	1.4
Proline	2.9	3.0
Serine	1.2	1.4
Threonine	0.9	0.7
Tyrosine	0.6	0.5
Valine	1.1	0.7
TAA	23.8	24.7
EAA	9.8	7.4
BCAA	4.4	3.7
Nitrogen content (%)	13.4	13.9
Protein content (%)	85.5[3]	83.2[4]

[1]Values for amino acid contents are in grams per 30 g protein. [2]Values are obtained by averaging the measured values for wheat, corn and pea protein in a 2:1:1 ratio. [3]Nitrogen-to-protein conversion factor: 6.38; [4]Nitrogen-to-protein conversion factor: 5.7 for wheat and 6.25 for corn and pea protein; MILK: 30 g milk protein, PLANT-BLEND: 15 g wheat protein + 7.5 g corn protein + 7.5 g pea protein. BCAA: branched chain amino acids, EAA: essential amino acids, TAA: total amino acids.

Muscle analysis

Muscle analysis for the determination of muscle protein-bound L-[ring-$^{13}C_6$]-phenylalanine enrichments by GC-IRMS has previously been explained in detail [26]. In short, a piece of wet muscle (~50-70 mg) was homogenized and prepared and a myofibrillar protein-enriched fraction was obtained by removal of the collagen-enriched fraction. Subsequently, the amino

acids were liberated from the myofibrillar protein-enriched fraction by adding 2 mL of 6 M HCl and heating to 110°C for 16 h. The amino acids from the resulting dried myofibrillar protein-enriched fractions were liberated by adding 2 mL of 6 M HCl and heating to 110°C for 16 h, passed over a cation exchange resin column (AG 50W-X8, mesh size: 100-200, ionic form: hydrogen (Bio-Rad Laboratories, Hercules, CA, USA)), and derivatized to their N(O,S)-ethoxycarbonyl-ethylesters. The ratio of $^{13}C/^{12}C$ of myofibrillar protein-bound phenylalanine was determined using GC-IRMS.

Calculations

Net incremental area under curve (iAUC) was determined for plasma amino acid concentrations during the 5 h post-prandial period following protein ingestion. The iAUC was calculated using the trapezoid rule, with plasma concentrations before beverage ingestion (*t*= 0 min) serving as baseline.

Myofibrillar protein fractional synthetic rates (FSR, $\%\cdot h^{-1}$) were calculated by the standard precursor-product equation [31], as previously described [26].

Outcome measures

Myofibrillar FSR over the entire (i.e. 0 – 300 min) post-prandial period, comparing MILK *vs* PLANT-BLEND was defined as the primary outcome measure. Secondary outcome measures included myofibrillar FSR in the early (i.e. 0 – 120 min) and late (i.e. 120 – 300 min) post-prandial period, plasma glucose, insulin, and amino acid concentrations over time and plasma amino acid iAUC. Plasma glucose, insulin, and amino acid peak concentrations and time to peak were tertiary outcomes.

Statistical analysis

A power calculation was performed with differences in postprandial myofibrillar FSRs between the 2 treatments as primary outcome measure. Based on previous work in this area, a sample size of 12 participants per treatment, including a 10% dropout rate was calculated using a power of 80%, a significance level of 0.05, a difference in FSR of 0.008 %/h (or ~20% when expressed as relative difference, e.g. 0.040 *vs* 0.048 %/h) [25], and a within-group standard deviation of 0.0065 $\%\cdot h^{-1}$ (or ~16%) [32, 33]. Participants' characteristics were analyzed by two-samples *t*-test. Plasma glucose, insulin, and amino acid concentrations and amino acid enrichments over time were compared between groups using a two-way (*time x treatment*) repeated measures ANOVA, with time as within-subjects factor, and treatment as between-subjects factor. In case a significant *time x treatment* interaction was observed, post-hoc analyses were performed to determine significant differences between treatments for each time point. Within treatments, repeated measures analyses were performed to evaluate which time points were increased above baseline (before protein intake). Plasma glucose, insulin, and amino acid concentrations, expressed as peak values, time to peak and iAUC, were analyzed by two-samples *t*-test to locate differences between groups. Basal post-absorptive (-180 – 0 min), and post-prandial myofibrillar protein synthesis rates during the early (0-120 min), late (120-300 min), and entire (0-300 min) post-prandial period were analyzed by

two-samples *t*-test. Statistical analyses were performed with a software package (IBM SPSS statistics for Windows, version 26.0, IBM Corp., Armonk, NY, USA). Means were considered to be significantly different for P values <0.05. Data are expressed as means±SD, additionally, for the main outcome parameter (post-prandial muscle protein FSR), and aggregate EAA, leucine, lysine, and methionine iAUC, the estimated differences±SD with 95% confidence intervals (CI) are provided. Except for plasma insulin concentrations (*n*=11 for MILK), no missing values were present for any of the outcome parameters.

RESULTS

Participants' characteristics

Twenty-four healthy, recreationally active males (24±4 y; 1.78±0.07 m; 71.2±8.7 kg) volunteered to participate in this parallel group, double blind, randomized controlled trial (Table 6.1).

Plasma glucose and insulin concentrations

No significant changes or differences between treatments in plasma glucose concentrations were observed following protein ingestion (*time x treatment P*=0.92; Figure 6.1A). Plasma insulin concentrations increased following protein ingestion, with a greater initial rise following MILK compared with PLANT-BLEND ingestion (*time x treatment P*=0.03; 1 missing value MILK *n*=11; Figure 6.1B). However, peak plasma insulin concentrations did not differ between treatments (28±8 *vs* 19±11 mU·L^{-1} respectively; two-sample *t*-test: *P*=0.15). Post-prandial plasma insulin availability (iAUC) was greater following MILK *vs* PLANT-BLEND ingestion (1.06±0.33 *vs* 0.50±57 mU·300 min·L^{-1} respectively; two-sample *t*-test: *P*<0.05).

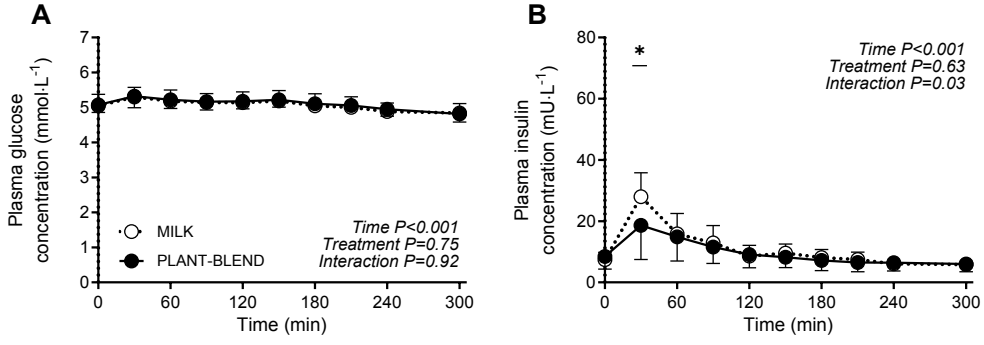

FIGURE 6.1 Post-prandial plasma glucose (Panel A) and insulin (Panel B) concentrations during the 5 hour period following the ingestion of MILK *vs* PLANT-BLEND in healthy, young males (*n*=12 per group). Time 0 min represents time of beverage intake. MILK: 30 g milk protein, PLANT-BLEND: 15 g wheat + 7.5 g corn + 7.5 g pea protein. Values represent means ± standard deviation; Two-way repeated measures ANOVA with time as within-subjects variable and interventional drink (treatment) as between-subjects variable.

Plasma AA concentrations

Plasma EAA concentrations increased following protein ingestion, with a more rapid and greater rise in circulating EAA concentrations following MILK *vs* PLANT-BLEND ingestion (*time x treatment* $P<0.001$; **Figure 6.2A**). Plasma EAA concentrations increased for respectively 300 and 240 min after MILK and PLANT-BLEND ingestion. In line with the significant *time x treatment* interaction, peak plasma EAA concentrations were reached at an earlier point in time following MILK *vs* PLANT-BLEND ingestion (at 36±10 and 75±26 min after protein ingestion, respectively; two-sample *t*-test: $P<0.001$) reaching levels of 1870±124 and 1370±93 μmol·L^{-1}, respectively (two-sample *t*-test: $P<0.001$). The overall increase in plasma EAA availability over the entire 300 min post-prandial period, expressed as iAUC, was ~2 fold greater for MILK *vs* PLANT-BLEND (151±31 *vs* 79±12 mmol·300 min·L^{-1}; two-sample *t*-test: $P<0.001$; estimated mean difference: 72.6±33.5 (95% CI: 52.6 – 92.7) mmol·300 min·L^{-1}; **Figure 6.2B**).

The post-prandial increase in plasma leucine concentrations following protein ingestion (**Figure 6.2C**) differed between MILK *vs* PLANT-BLEND (*time x treatment* $P<0.001$). Plasma leucine concentrations increased for the entire 300 min post-prandial period following ingestion of both protein drinks. In line with the significant *time x treatment* interaction, peak plasma leucine concentrations were ~25% greater for MILK *vs* PLANT-BLEND (353±45 *vs* 283±22 μmol·L^{-1}, respectively; two-sample *t*-test: $P<0.001$) and were reached ~1h earlier (46±43 and 113±46 min after protein ingestion, respectively; two-sample *t*-test: $P=0.001$). The overall increase in plasma leucine availability over the entire 300 min post-prandial period, expressed as iAUC, was ~16 % greater for MILK *vs* PLANT-BLEND (36±7 *vs* 31±4 mmol·300 min·L^{-1}; two-sample *t*-test: $P=0.046$; estimated mean difference: 5.1±8.3 (95% CI: 0.1 – 10.1) mmol·300 min·L^{-1}; **Figure 6.2D**).

The post-prandial increase in plasma lysine concentrations following protein ingestion was significantly greater following MILK *vs* PLANT-BLEND ingestion (*time x treatment* $P<0.001$; **Figure 6.2E**). Plasma lysine concentrations increased for respectively 240 and 120 min after MILK and PLANT-BLEND consumption. In line with the significant *time x treatment* interaction, peak plasma lysine concentrations were 85% greater following MILK *vs* PLANT-BLEND ingestion (370±29 *vs* 201±24 μmol·L^{-1}, respectively; two-sample *t*-test: $P<0.001$), and were reached earlier (34±7 *vs* 60±34 min after protein ingestion, two-sample *t*-test: $P=0.02$). Peak plasma lysine concentrations increased ~137% above baseline values for MILK, but only increased ~38% above baseline for PLANT-BLEND. Consequently, the overall increase in plasma lysine availability over the entire 300 min post-prandial period, expressed as iAUC, was much greater for MILK *vs* PLANT-BLEND (25±8 *vs* 2±2 mmol·300 min·L^{-1}; two-sample *t*-test: $P<0.001$; estimated mean difference: 23.2±7.9 (95% CI: 18.5 – 28.0) mmol·300 min·L^{-1}; **Figure 6.2F**).

The post-prandial increase in plasma methionine concentrations following protein ingestion was significantly greater following MILK *vs* PLANT-BLEND ingestion (*time x treatment* $P<0.001$; **Figure 6.2G**). Plasma methionine concentrations increased for respectively 240

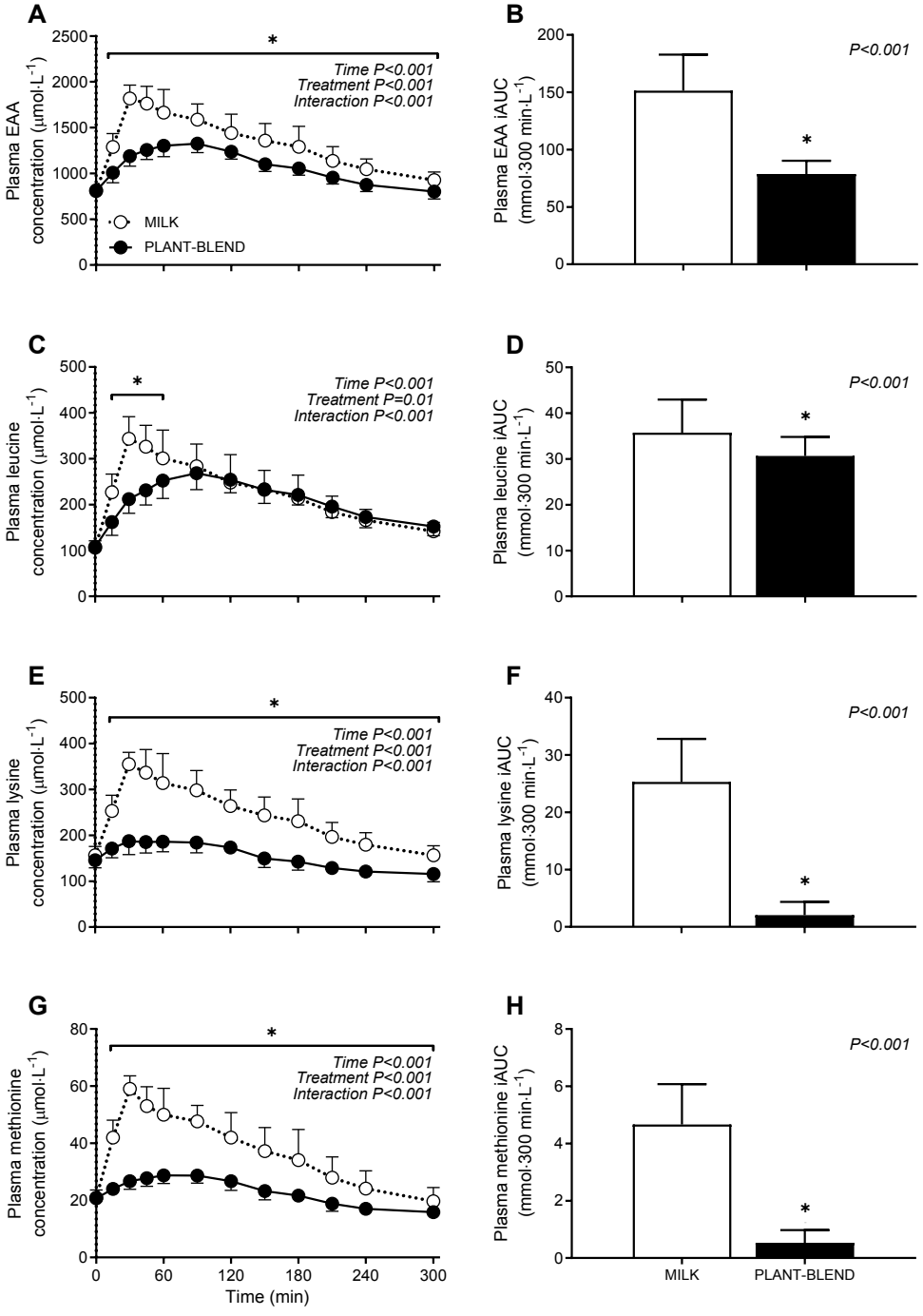

FIGURE 6.2 Post-prandial plasma essential amino acid (EAA, Panel A), leucine (Panel C), lysine (Panel E), and methionine (Panel G) concentrations during the 5 hour post-prandial period following the ingestion of MILK *vs* PLANT-BLEND in healthy, young males (*n*=12 per group). Time 0 min represents time of

beverage intake. Panels **B**, **D**, **F** and **H** represent the 0-5 h incremental area under curve (iAUC) following protein ingestion. MILK: 30 g milk protein, PLANT-BLEND: 15 g wheat + 7.5 g corn + and 7.5 g pea protein. Values represent means ± standard deviation; * significantly different between intervention groups (P<0.05). Two-way repeated measures ANOVA with time as within-subject variable and interventional drink (treatment) as between-subject variable.

and 150 min after MILK and PLANT-BLEND consumption. In line with the significant *time x treatment* interaction, peak plasma methionine concentrations were ~104 % greater for MILK *vs* PLANT-BLEND (60±5 and 30±2 μmol·L^{-1}, two-sample *t*-test: *P*<0.001) and were reached earlier (34±9 *vs* 69±24 min after protein ingestion; two-sample *t*-test: *P*<0.001). As a result, peak plasma methionine concentrations increased ~190 % above baseline values for MILK, but only increased ~42 % above baseline values for PLANT-BLEND. The overall increase in plasma methionine availability over the entire 5 h post-prandial period, expressed as iAUC, was severalfold greater for MILK *vs* PLANT-BLEND (4.7±1.4 *vs* 0.5±0.4 mmol·300 min·L^{-1}; two-sample *t*-test: *P*<0.001; estimated mean difference: 4.1±1.5 (95% CI: 3.3 – 5.0) mmol·300 min·L^{-1}; **Figure 6.2H**).

In general, post-prandial increases in plasma amino acid concentrations revealed significant differences over time following MILK *vs* PLANT-BLEND ingestion for most amino acids (**Supplemental Figure 6.3**; *time x treatment P*<0.05). The post-prandial increases in plasma isoleucine, threonine, tryptophane, tyrosine, and valine availability over the entire 5 h post-prandial period (iAUC) were greater for MILK *vs* PLANT-BLEND (two-sample *t*-test: *P*<0.05), whereas only for glycine, plasma availability which was lower for MILK *vs* PLANT-BLEND (two-sample *t*-test: *P*<0.01, **Supplemental Figure 6.3**).

Plasma free and muscle protein-bound L-[ring-$^{13}C_6$]-phenylalanine enrichments

Plasma phenylalanine concentrations and L-[ring-$^{13}C_6$]-phenylalanine enrichments over time are presented in **Figure 6.3A** and **6.3B**, respectively. Plasma L-[ring-$^{13}C_6$]-phenylalanine enrichments were lower following MILK *vs* PLANT-BLEND ingestion during the early post-prandial period (*time x treatment P*<0.001). Weighted mean plasma L-[ring-$^{13}C_6$]-phenylalanine enrichments averaged 7.11±0.65 and 6.48±0.70 MPE during the basal post-absorptive period (two-sample *t*-test: *P*=0.04), and 6.64±0.53 and 6.32±0.55 MPE throughout the 5 h post-prandial period (two-sample *t*-test: *P*=0.16) following MILK and PLANT-BLEND ingestion, respectively.

Myofibrillar protein-bound L-[ring-$^{13}C_6$]-phenylalanine enrichments were higher following ingestion of MILK and PLANT-BLEND from 0.0032±0.0031 and 0.0045±0.0045 MPE at *t*=0 min, to 0.0115±0.0041 and 0.0145±0.0076 MPE at *t*= 120 min, reaching 0.0214±0.0049 and 0.0250±0.0083 MPE at *t*= 300 min after protein ingestion, respectively. The plasma free and muscle protein-bound L-[ring-$^{13}C_6$]-phenylalanine enrichments were subsequently used to calculate muscle protein synthesis rates.

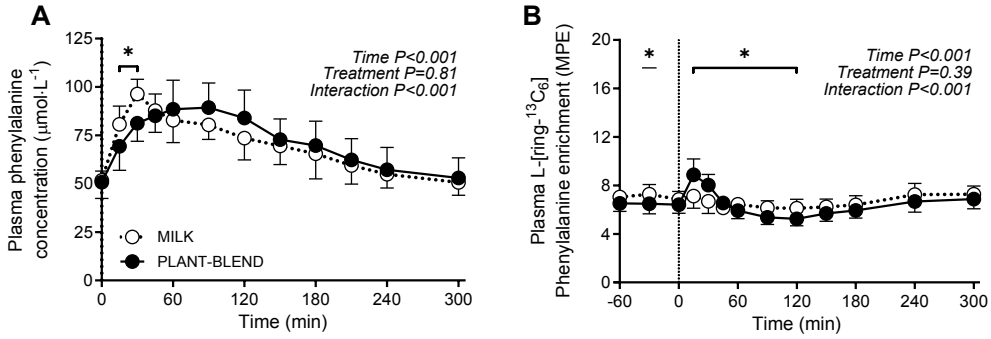

FIGURE 6.3 Post-prandial plasma phenylalanine concentrations (Panel A) and plasma L-[ring-$^{13}C_6$]-phenylalanine enrichments (Panel B) during the 5 h post-prandial period following the ingestion of MILK *vs* PLANT-BLEND in healthy, young males (*n*=12 per group). Time 0 min represents time of beverage intake. MILK: 30 g milk protein, PLANT-BLEND: 15 g wheat + 7.5 g corn + 7.5 g pea protein. Values represent means ± standard deviation; * significantly different between intervention groups (*P*<0.05). Two-way repeated measures ANOVA with time as within-subject variable and interventional drink (treatment) as between-subject variable.

Muscle protein synthesis rates

Post-absorptive myofibrillar protein fractional synthetic rates averaged 0.014±0.014 and 0.021±0.021 %·h^{-1} in the MILK and PLANT-BLEND group, with no differences between treatments (two-sample *t*-test: *P*=0.39). Protein ingestion increased myofibrillar protein synthesis rates to 0.059±0.024 and 0.071±0.031 %·h^{-1} during the early post-prandial period (0-120 min) and to 0.049±0.017 and 0.058±0.015 %·h^{-1} during the late post-prandial period (120-300 min) in MILK and PLANT-BLEND, respectively (two-sample *t*-test: *P*<0.05). Post-prandial muscle protein synthesis rates averaged 0.053±0.013 and 0.064±0.016 %·h^{-1} assessed over the entire 5 h post-prandial period (**Figure 6.4**). Post-prandial myofibrillar protein synthesis rates did not differ between MILK *vs* PLANT-BLEND for the early (0-120 min; two-sample *t*-test: *P*=0.58), late (120-300 min; two-sample *t*-test: *P*=0.20), and entire (0-300 min; two-sample *t*-test: *P*=0.08) post-prandial period. The estimated differences ± standard deviation and 95% CI for the muscle protein synthesis rates were respectively: -0.0125±0.0393 (-0.03609 – 0.0110) %·h^{-1} for the early, -0.0086±0.0223 (-0.0220 – 0.0048) %·h^{-1} for the late, and -0.0107±0.0202 (-0.2284 – 0.0014) %·h^{-1} for the entire post-prandial period.

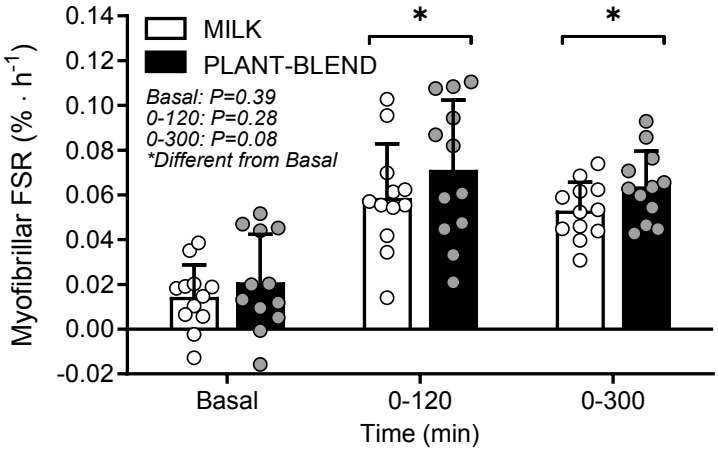

FIGURE 6.4 Myofibrillar protein fractional synthetic rates (FSR) at different time periods prior to and following ingestion of MILK *vs* PLANT-BLEND in healthy, young males (*n*=12 per group). MILK: 30 g milk protein, PLANT-BLEND: 15 g wheat + 7.5 g corn + 7.5 g pea protein. Values represent means ± standard deviation. *significantly different from basal; two-samples *t*-test: *P*<0.05. two-samples *t*-test: *P*=0.39, *P*=0.28, and *P*=0.08 for basal, 0-120, and 0-300 min, respectively. No differences were observed between treatments.

DISCUSSION

The present study shows that ingestion of a wheat, corn, and pea protein blend strongly increases muscle protein synthesis rates in healthy, young males. The muscle protein synthetic response to the ingestion of 30 g of this plant-derived protein blend did not differ from the ingestion of an equivalent amount of milk protein, despite an attenuated post-prandial rise in circulating plasma essential amino acid concentrations.

Plant-derived proteins are known to have specific amino acid deficiencies according to the WHO/FAO/UNU requirements [24], and are generally low in essential amino acid content and leucine in particular [19]. Combining different plant-derived proteins allows us to compose a protein blend with a more balanced amino acid profile, with no apparent amino acid deficiencies. We combined wheat, corn, and pea derived protein in a 2:1:1 ratio to provide a plant-derived protein blend with an amino acid profile that resembles high quality animal-derived proteins, such as milk protein (Table 6.2). With leucine being one of the key amino acids driving the anabolic response to protein ingestion [4-8], we included an ample amount of corn protein to compose a plant-derived protein blend with a leucine content (8%) well above the WHO/FAO/UNU leucine requirements (5.9%) [24]. Although we were not able to provide essential amino acid (27%), lysine (4.5%) and methionine (1.6%) contents fully compliant with the WHO/FAO/UNU requirements [29], the protein blend did provide an essential amino acid content of no less than 25%, a lysine content ~2 fold higher than wheat and corn protein on their own, and a methionine content that was ~3 fold higher than pea protein on its own. This demonstrates that blending different plant-derived proteins

can effectively improve the amino acid composition far beyond the composition of their individual proteins.

In the present study, the leucine content of the plant-derived protein blend was matched with milk protein (2.4 g), but the essential amino acid (7.4 *vs* 9.8 g), lysine (0.7 *vs* 2.0 g), and methionine (0.4 *vs* 0.7 g) contents remained below the levels observed in the milk protein (**Table 6.2**). These differences in amino acid composition translated into lower peak plasma essential amino acid, lysine and methionine concentrations and a lower post-prandial plasma amino acid availability (**Figure 6.2**). Despite the matching leucine contents, peak plasma leucine concentrations and iAUC were lower following ingestion of the blend when compared with the milk protein (**Figure 6.2**). The observed differences in post-prandial plasma amino acid profiles tend to agree with previous work showing an attenuated post-prandial rise in circulating plasma amino acids following ingestion of plant-derived protein isolates and concentrates when compared with the ingestion of an equivalent amount of animal-based protein [26, 34]. Though we can only speculate on the mechanisms responsible, there are ample reports suggesting that differences in protein structure and function and the presence of anti-nutritional factors may compromise protein digestion and amino acid absorption, and/or modulate splanchnic extraction of protein-derived amino acids [35-37].

To assess the impact of these differences in post-prandial plasma amino acid responses on the post-prandial stimulation of muscle protein synthesis, we combined a primed continuous L-[ring-$^{13}C_6$]-phenylalanine infusion with the collection of muscle biopsies. The post-prandial rise in circulating plasma essential amino acids following ingestion of the plant-derived protein blend strongly increased muscle protein synthesis rates when compared to basal, post-absorptive values (**Figure 6.4**). The response tended to be of a similar magnitude when compared to previous responses observed following ingestion of similar amounts of high quality animal-derived proteins [25, 38, 39]. In the present study, we included a control treatment in which 30 g of high quality milk protein concentrate was ingested. Interestingly, despite the lower post-prandial plasma amino acid availability following ingestion of the plant-derived protein blend when compared with the milk protein ingestion, we observed no differences in post-prandial muscle protein synthesis rates. In fact, there was a trend for post-prandial muscle protein synthesis rates to increase to a greater extent following the ingestion of the plant-derived protein blend when compared with milk protein (P=0.08; **Figure 6.4**). The present study extends on previous studies comparing the anabolic properties of dairy plus plant-based protein blends with dairy protein [21, 23, 26], by showing that even an exclusively plant-derived protein blend can be as effective as a high quality animal protein in stimulating muscle protein synthesis *in vivo* in healthy, young adults.

There has been a growing interest in the consumption of a more plant-based diet and the application of plant-derived proteins in our food as a means to replace animal based food products. However, individual plant-derived foods are regarded as a lesser quality protein source because of their lower digestibility, low essential amino content, and/or specific amino acid deficiencies [17, 19, 22]. However, these deficiencies can be overcome by composing

blends of complementary plant-based protein sources or plant-derived protein isolates and concentrates, making the overall protein quality comparable to a high quality animal based protein source [22]. Therefore, plant-derived protein blends can be effectively applied in the development of high quality plant-based products, or in composing high quality plant-based protein meals. Here, we show that ingestion of a plant-derived protein blend does not compromise the post-prandial muscle protein synthetic response when compared to the consumption of an equivalent amount of a high quality animal-derived reference protein (**Figure 6.4**). We provided our participants with 30 g protein, containing no less than 2.4 g leucine in both protein groups. Consequently, we provided a similar amount of leucine as what has been shown to maximally stimulate resting post-prandial muscle protein synthesis rates in young adults when providing 20 g whey protein (2.2 g) [25]. This allowed us to evaluate the true anabolic potential of plant-derived protein sources, which are usually low in leucine. Thereby, 30 g protein is still a feasible amount of protein to ingest in a meal, while ingestion of much higher dosages of protein may become challenging. Therefore, if differences in the muscle protein synthetic response would already have been apparent with a protein intake of 30 g, while providing a sufficient amount of leucine, than the feasibility of this protein blend for stimulating muscle protein synthesis would have been questionable. Consequently, given the amount of protein and leucine provided, we might have maximally stimulated the muscle protein synthetic response for both intervention groups. In line, the lower plasma aminoacidemia following ingestion of the plant-blend compared to milk protein, may already have been sufficient to maximally stimulate muscle protein synthesis. We can only speculate whether differences in the muscle protein synthetic response to the consumption of plant-derived proteins [26, 34] and plant-derived protein blends when compared with animal-derived protein may become apparent when (much) lower amounts of protein are ingested. Providing less protein may result in lower post-prandial plasma amino acid availability and, as such, may lead to the detection of differences between plant and animal derived proteins in the capacity to stimulate muscle protein synthesis. The latter may be attributed to specific amino acid deficiencies in the plant derived proteins. However, the use of protein blends compensates for specific amino acid deficiencies, so we speculate that lower amounts would still show no differences in anabolic properties of this protein blend when compared with milk protein. This rationale seems to be supported by the fact that we even observed a trend for higher post-prandial protein synthesis rates following ingestion of the plant derived protein blend versus the animal derived protein source. More work will be needed to assess the anabolic responses to the ingestion of plant-derived protein blends in older and more clinically compromised populations who typically consume less protein per serving and/or have a lower anabolic response to protein ingestion [40]. Furthermore, it should be highlighted that this work focusses specifically on protein concentrates/isolates in order to provide insight in the potential of the proteins themselves. However, these findings may not directly translate towards plant based whole foods and food products. These products contain many other nutrients and anti-nutritional factors that may strongly impact protein digestion and amino acid absorption kinetics and, as such, are likely to restrict the post-prandial anabolic response. Furthermore, protein extraction and the associated processing of plant derived proteins may also affect bioavailability as well as biofunctionality of these

proteins. Therefore, more research is needed to translate the current findings in a meal based setting. Lastly, our data were performed in healthy men and as there may [41, 42] or may not [43, 44] be differences in post-prandial protein handling between men and women, the presented data do not necessarily apply to females. Future research should include both male and female participants.

We conclude that ingestion of 30 g of a wheat (50%), corn (25%), and pea (25%) derived protein blend increases muscle protein synthesis rates in healthy, young males. The muscle protein synthetic response to the ingestion of a well composed plant-derived protein blend can be as robust as an equivalent amount of a high quality animal-derived protein. Balanced plant-derived protein blends can have anabolic properties that do not differ from high quality animal-derived proteins.

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Conflicts of Interest

PJMP, IWKK, JMS, LCPGMdG, LBV, TS and LJCvL have the following interests: This study was funded by TiFN, Wageningen, The Netherlands. The sponsors Tereos Syral (Marckolsheim, France), Cargill (Minneapolis, MN, USA), and Kellogg (Battle Creek, MI, USA) partly financed the project. The researchers are responsible for the study design, data collection and analysis, decision to publish, and preparation of the manuscript. The industrial partners have contributed to the project through regular discussion, and were involved in the study design. More specifically: the choice of interventional products which were produced by these sponsors. The funders had no role in data collection and analysis, decision to publish, or preparation of the manuscript. In addition: LJCvL and LBV have received research grants, consulting fees, speaking honoraria, or a combination of these from Friesland Campina, Tereos Syral, and Pepsico. The other authors report no conflicts of interest.

Authorship

The author contributions were as follows: LJCvL, TS, LBV, LCPGMdG, and PJMP designed research; PJMP, IWKK, SHMG, LHPH, and JMS conducted research; PJMP, TS and LJCvL analyzed data; PJMP and LJCvL wrote paper; PJMP, TS and LJCvL had primary responsibility for final content. All authors read and approved the final manuscript.

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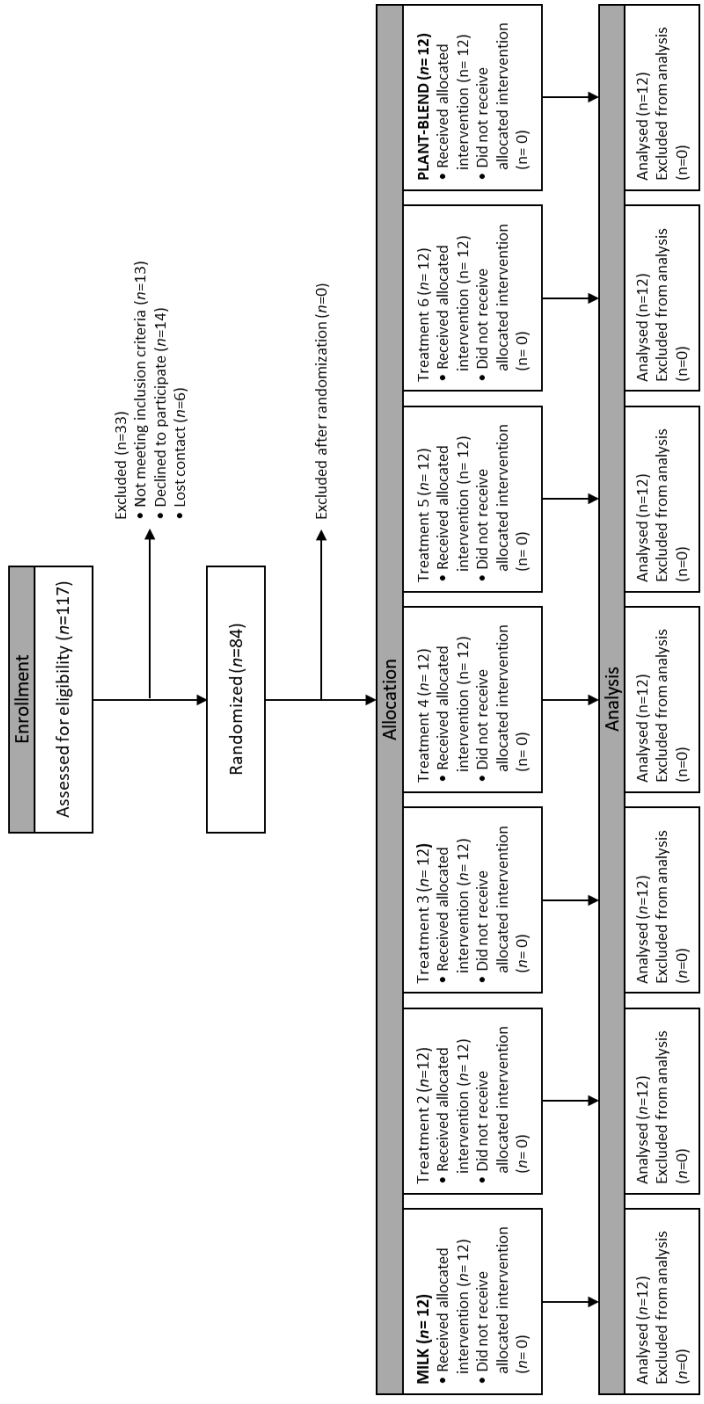
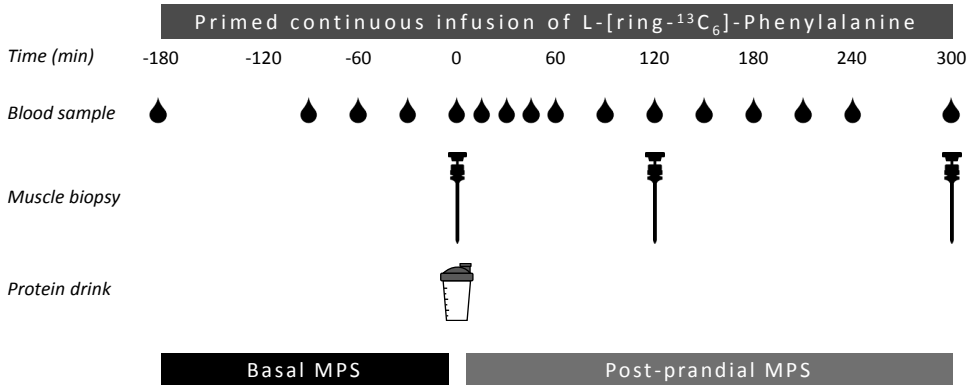
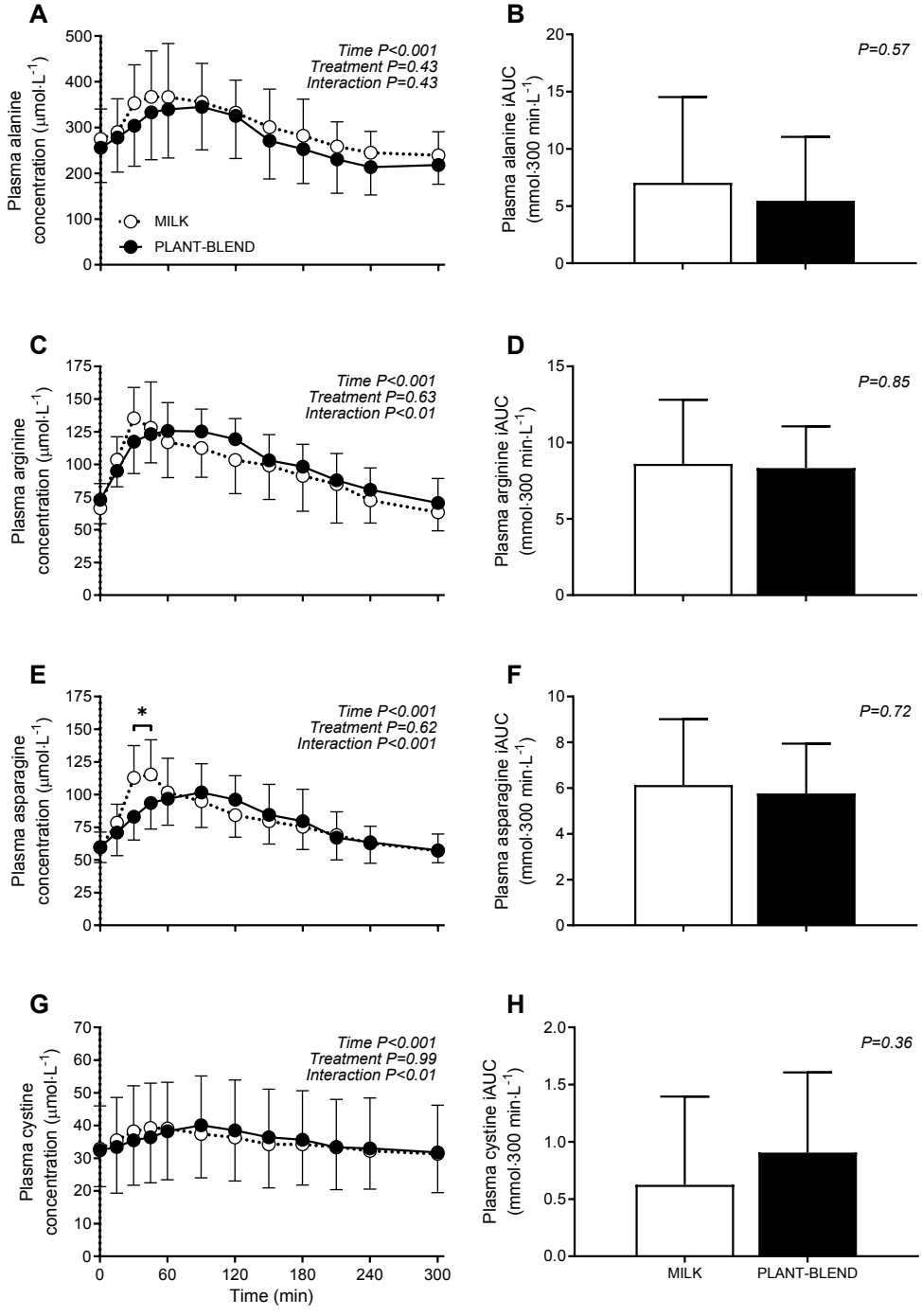
SUPPLEMENTAL MATERIAL



SUPPLEMENTAL FIGURE 6.1 CONSORT flow diagram. CONSORT, Consolidated Standards of Reporting Trials. The current study was part of a larger trial with a total of 7 parallel groups (*n* = 12 per group) as indicated in the flow diagram. MILK (30 g milk protein), PLANT-BLEND (15 g wheat protein + 7.5 g corn protein + 7.5 g pea protein).



SUPPLEMENTAL FIGURE 6.2 Schematic representation of the experimental design.



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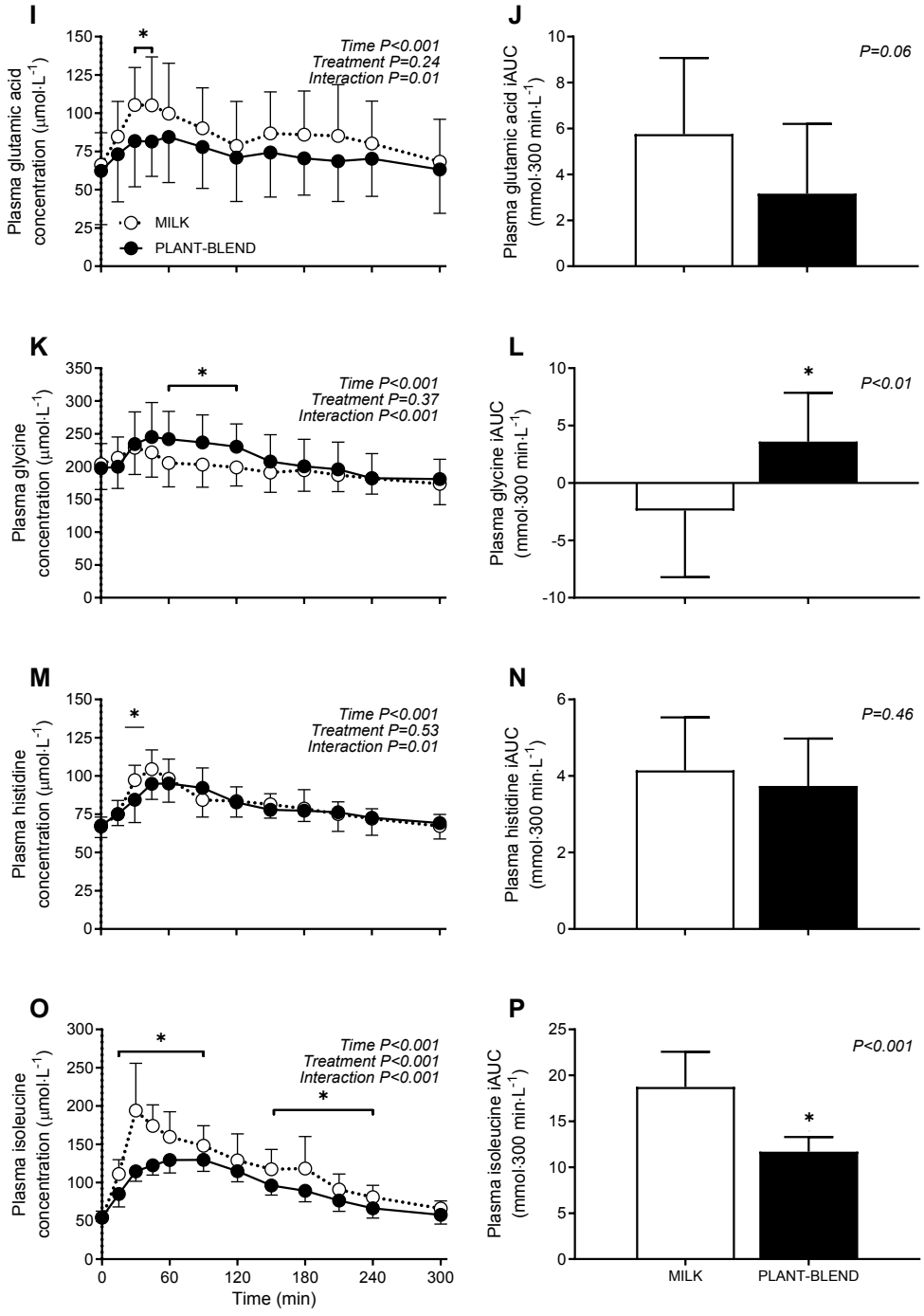

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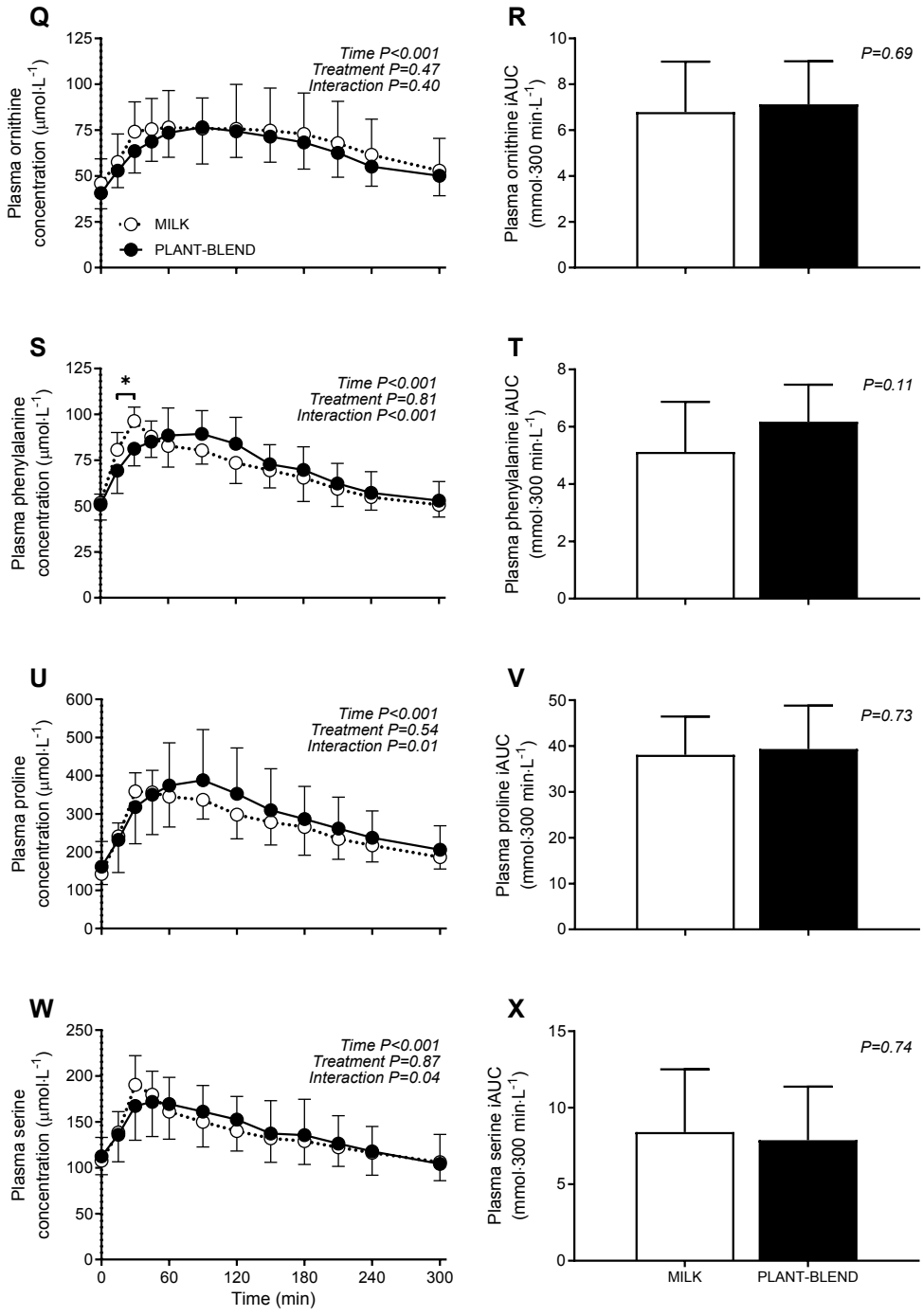

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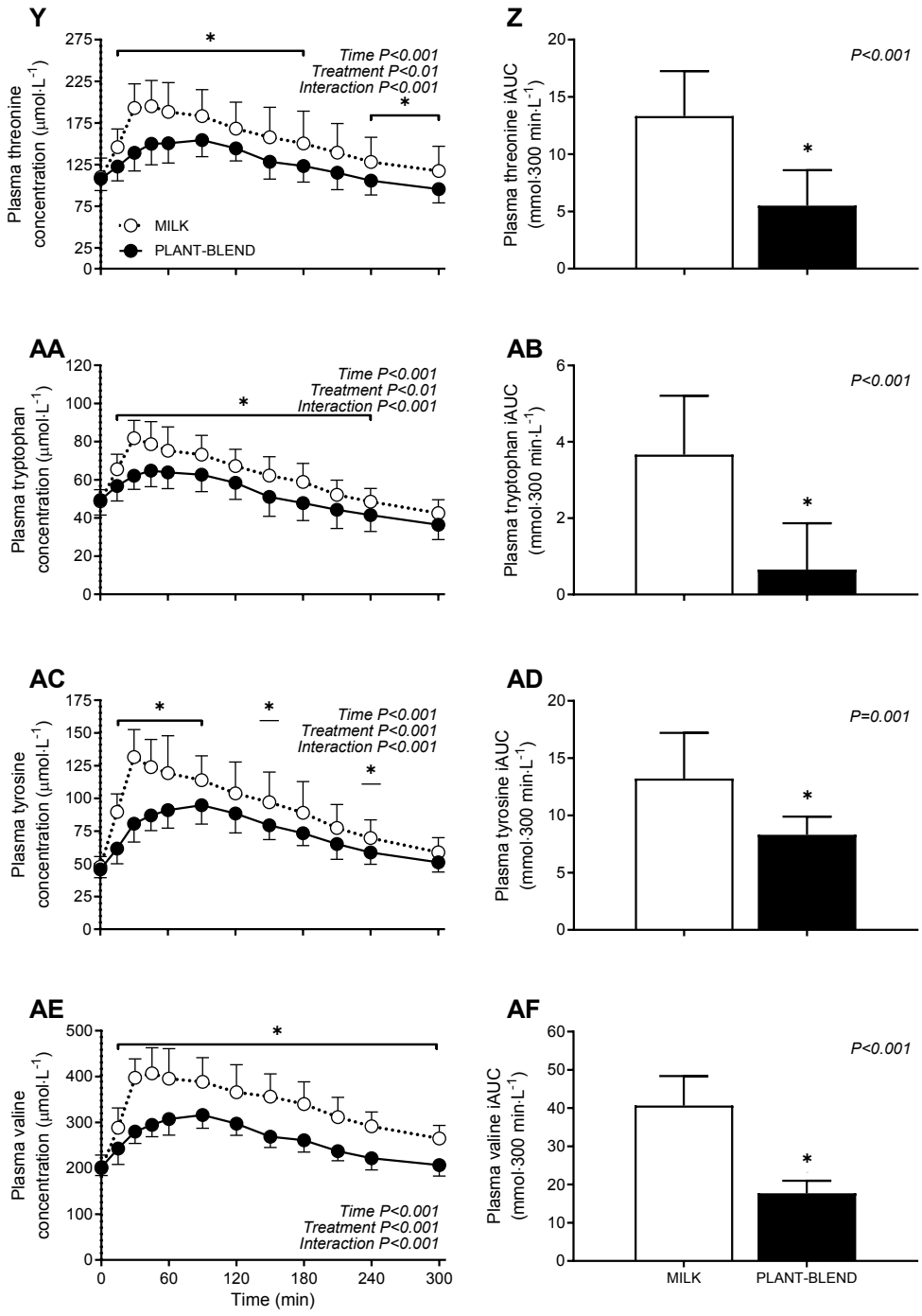

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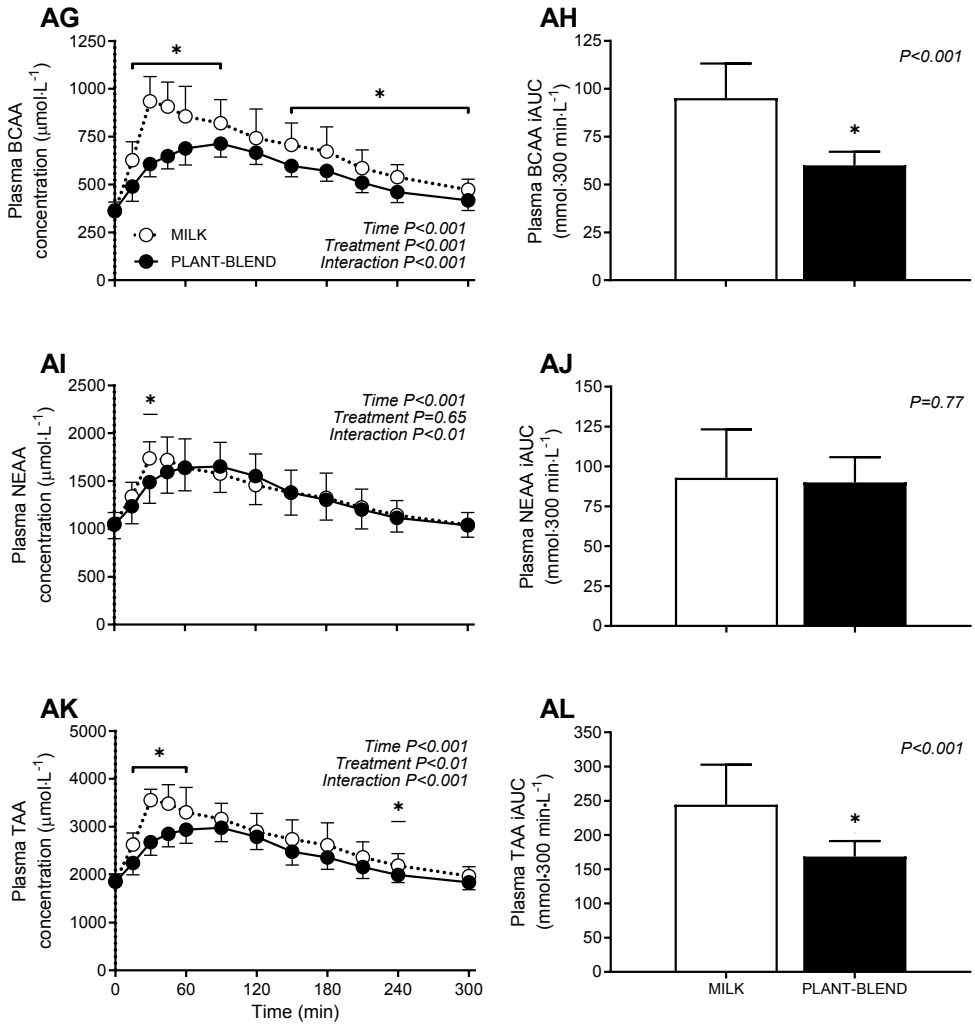

SUPPLEMENTAL FIGURE 6.3 Post-prandial plasma amino concentrations during the 300 min post-prandial period following the ingestion of MILK *vs* PLANT-BLEND (*n*=12 per group). Time 0 min represents time of beverage intake. Panels B, D, F, H, J, L, N, P, R, T, V, X, Z, AB, AD, AF, AH, AJ, AL represent the 0-5 h incremental area under curve (iAUC) following protein ingestion. MILK (30 g milk protein), PLANT-BLEND (15 g wheat protein + 7.5 g corn protein + 7.5 g pea protein). Values represent means ± standard deviation; *significantly different between interventions (*P*<0.05). Repeated measures ANOVA with time as within-subject variable and interventional drink (treatment) as between-subject variable, and two samples *t*-test were used to determine differences between groups.

SUPPLEMENTAL TABLE 6.1 Average 3 day dietary intake of study participants

	MILK	PLANT-BLEND
Energy ($MJ \cdot d^{-1}$)	9.3 ± 2.2	9.6 ± 2.2
Carbohydrate ($g \cdot d^{-1}$)	267 ± 63	274 ± 56
Fat ($g \cdot d^{-1}$)	78 ± 27	88 ± 27
Protein ($g \cdot d^{-1}$)	97 ± 29	94 ± 13
Energy ($kJ \cdot kg^{-1} \cdot d^{-1}$)	131 ± 26	136 ± 32
Carbohydrate ($g \cdot kg^{-1} \cdot d^{-1}$)	3.8 ± 0.9	3.9 ± 0.9
Fat ($g \cdot kg^{-1} \cdot d^{-1}$)	1.1 ± 0.3	1.2 ± 0.4
Protein ($g \cdot kg^{-1} \cdot d^{-1}$)	1.3 ± 0.4	1.3 ± 0.2
Carbohydrate (% total energy)	50 ± 7	49 ± 4
Fat (% total energy)	33 ± 8	34 ± 6
Protein (% total energy)	18 ± 3	17 ± 4

Values represent mean ± standard deviation. *n*=12 per nutritional intervention group. MILK: 30 g milk protein, WHEAT+MILK: 15 g wheat protein plus 15 g milk protein, WHEAT: 30 g wheat protein. Independent samples *t*-test for MILK *vs* WHEAT and MILK *vs* WHEAT+MILK. *significantly different for MILK *vs* WHEAT ($P<0.05$). 3 Day food records were analyzed using "Mijn Eetmeter" (https://mijn.voedingscentrum.nl/nl/eetmeter/), online software available from the Netherlands Nutrition Centre.

CHAPTER 7

POTATO PROTEIN INGESTION INCREASES MUSCLE PROTEIN SYNTHESIS RATES AT REST AND DURING RECOVERY FROM EXERCISE IN HUMANS

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ABSTRACT

Background

Plant-derived proteins have received considerable attention as an alternative to animal based proteins and are now frequently used in both plant-based diets and sports nutrition products. However, little information is available on the anabolic properties of potato-derived protein.

Objective

This study compares muscle protein synthesis rates following the ingestion of 30g potato protein versus 30g milk protein at rest and during recovery from a single bout of resistance exercise in healthy, young males.

Methods

In a randomized, double blind, parallel-group design, 24 healthy young males (24±4y) received primed continuous L-[*ring*-$^{13}C_6$]-phenylalanine infusions while ingesting 30g potato derived protein or 30g milk protein following a single bout of unilateral resistance exercise. Blood and muscle biopsies were collected for 5 hours following protein ingestion to assess post-prandial plasma amino acid profiles and mixed muscle protein synthesis rates at rest and during recovery from exercise.

Results

Ingestion of both potato and milk protein increased mixed muscle protein synthesis rates when compared to basal post-absorptive values (from 0.020±0.011 to 0.053±0.017 %·h^{-1} and from 0.021±0.014 to 0.050±0.012 %·h^{-1}, respectively (P<0.001)), with no differences between treatments (P=0.54). In the exercised leg, mixed muscle protein synthesis rates increased to 0.069±0.019 and 0.064±0.015 %·h^{-1} after ingesting potato and milk protein, respectively (P<0.001), with no differences between treatments (P=0.52). The muscle protein synthetic response was greater in the exercised compared with the resting leg (P<0.05).

Conclusion

Ingestion of 30g potato protein concentrate increases muscle protein synthesis rates at rest and during recovery from exercise in healthy, young males. Muscle protein synthesis rates following the ingestion of 30g potato protein do not differ from rates observed after ingesting an equivalent amount of milk protein.

INTRODUCTION

Protein ingestion [1-3] and physical activity [4] stimulate muscle protein synthesis and are essential for the maintenance and accretion of skeletal muscle mass. Protein ingested during recovery from exercise further augments muscle protein synthesis rates [5-7] and supports the skeletal muscle adaptive response to more prolonged exercise training [8]. The muscle protein synthetic response to protein ingestion is driven by the post-prandial increase in circulating essential amino acids (EAA) concentrations [9], with plasma leucine being of particular relevance [10-12]. Post-prandial muscle protein synthesis rates at rest and during recovery from exercise have been reported to differ substantially following ingestion of different protein sources [13-15]. The anabolic properties of a protein source is largely determined by its protein digestion and amino acid absorption kinetics, as well as the amino acid composition of the protein [9, 16-18].

Our habitual protein intake originates from both animal- and plant-based sources [19, 20]. In general, plant-based proteins are considered to provide a lesser anabolic stimulus following ingestion when compared to animal based proteins. This is mainly attributed to their lower digestibility and incomplete amino acid (AA) profile, characterized by low leucine, lysine, and/or methionine contents [19, 20]. Plant-based proteins already comprise a large part of our daily protein intake, but their contribution will become much greater due to the growing interest in consuming more plant-based diets and plant-based proteins [21]. The trend of consuming a more plant-based diet has also reached the athletic community, where sports supplements containing whey or egg protein are now frequently traded in for supplements providing plant-derived protein isolates or concentrates. Despite their popularity, only few studies have actually compared the anabolic properties of animal *vs* plant-derived proteins [15, 22-24]. Lesser anabolic properties have been reported following soy [15, 23, 24] and wheat [25] protein ingestion when compared to dairy protein both at rest and/or during recovery from exercise. However, these differences are not always apparent [15, 26, 27]. As there is a large variety in plant-derived protein characteristics [28], more plant-derived proteins should be evaluated for their anabolic properties both at rest and during recovery from exercise.

Potatoes are the third most consumed crop worldwide [29, 30]. Potatoes contain a mere ~1.5% protein based on their fresh weight [30]. However, when potatoes are used for starch extraction, a residue remains (potato fruit juice) which is generally used for feed production or discarded as a waste product. From this residue a potato protein concentrate can be extracted [31]. We previously identified the amino acid profile of potato derived protein along with various other plant-based protein sources [28]. The analysis revealed an amino acid composition of potato protein that closely resembles milk protein. In contrast to most other plant-derived proteins, potato protein provides sufficient amounts of all individual essential amino acids according to the WHO/FAO/UNU amino acid requirements, with no apparent deficiencies [28]. However, whether this favorable amino acid profile of potato

derived protein also translates to strong anabolic properties upon ingestion remains to be established.

We hypothesize that ingestion of 30 g potato protein concentrate increases muscle protein synthesis rates both at rest and during recovery from exercise in healthy, young men. Furthermore, we hypothesize that the muscle protein synthetic response following the ingestion of 30 g potato protein does not differ from the ingestion of 30 g milk protein. To test our hypotheses, we assessed post-absorptive and post-prandial muscle protein synthesis rates following ingestion of either 30 g potato or milk derived protein concentrate following a single bout of unilateral resistance exercise in 24 healthy, young males.

MATERIALS AND METHODS

Participants

Twenty-four healthy, recreationally active males (24±4 y; 1.79±0.07 m; 72.4±7.1 kg) volunteered to participate in this parallel group, double blind, randomized, controlled trial (subjects' characteristics are presented in **Table 7.1**). The trial was registered at the Netherlands Trial Register (NTR7152), and was conducted between April 2018 and February 2020 at Maastricht University in Maastricht, The Netherlands (See **Supplemental Figure 7.1** for the CONSORT (Consolidated Standards of Reporting Trials) flow diagram). All participants were informed on the purpose of the study, the experimental procedures, and possible risks before providing informed written consent to participate. The procedures followed were in accordance with the ethical standards of the medical ethics committee of the Maastricht University Medical Centre+ (METC 173053) on research involving human participants, and in accordance with the Helsinki Declaration of 1975 as revised in October 2013. The study was independently monitored by the Clinical Trial Centre Maastricht (CTCM).

Preliminary screening

Participants aged 18-35 y, with BMI >18.5 and <27.5 $kg \cdot m^{-2}$ underwent an initial screening session to assess eligibility. For this purpose, height, weight, blood pressure and body composition (by dual-energy X-ray absorptiometry; Discovery A, Hologic) were determined. Participants were deemed healthy based on their responses to a medical questionnaire, and were excluded from participation when smoking, performing progressive resistance exercise training, were using medication that affected protein metabolism, or were intolerant to the investigated protein products. Following initial screening, the participants were familiarized with the exercise testing protocol and the exercise equipment. Unilateral 1 repetition maximum (1-RM) strength was assessed for both legs separately, on the supine leg press (Technogym BV, Capelle aan den Ijssel, the Netherlands) and seated knee extension (Technogym BV) exercise using the multiple repetition testing procedure [32]. Before testing, participants performed a unilateral warm-up at low resistance for 20 repetitions to become familiarized with the equipment and to have exercise technique assessed and adjusted. Working sets of 8 repetitions were then performed with progressively increased loads until failure, to perform

TABLE 7.1 Participants' characteristics

	POTATO	MILK
Age (y)	23 ± 3	25 ± 4
Height (m)	1.81 ± 0.04	1.77 ± 0.08
Body mass (kg)	73.7 ± 6.4	71.2 ± 7.9
BMI ($kg \cdot m^{-2}$)	22.7 ± 1.4	22.7 ± 1.7
Systolic blood pressure (mmHg)	119 ± 11	119 ± 12
Diastolic blood Pressure (mmHg)	63 ± 8	68 ± 11
Resting heart rate (bpm)	63 ± 10	62 ± 8
Lean body mass (kg)	57.7 ± 6.1	52.6 ± 5.7
Body fat (%)	19.7 ± 3.1	22.8 ± 4.3
Leg press 1-RM (kg)	113 ± 26	98 ± 22
Knee extension 1-RM (kg)	61 ± 10	54 ± 9

Values represent mean ± standard deviation. *n* = 12 per nutritional intervention group. POTATO: 30 g of potato derived protein, MILK: 30 g milk protein. 1-RM: 1 repetition maximum of the exercised leg. Independent samples T-test for POTATO *vs* MILK all $P \geq 0.05$.

a valid estimation within 1-8 repetitions of the set. A repetition was considered valid if the subject was able to complete it in a controlled manner. A 2-min rest period was allowed between successive sets. In between the screening session and the experimental trial, subjects reported to the laboratory for an additional visit to perform a true 1-RM strength test.

1-RM strength test

During this visit, the participant's unilateral 1-RM strength was determined for each leg separately, starting with the supine leg press, followed by the seated knee extension. The estimated 1-RM obtained during the screening visit was used to determine the initial load for the actual 1-RM test [33]. Before testing each exercise, participants performed 2 sets of unilateral warm-up at low weight. First 20 repetitions at 25% of the estimated 1-RM followed by 8 repetitions at 50% of the estimated 1-RM. During these sets, the exercise technique was again closely assessed and adjusted when necessary. Following warm-up, the 1-RM was determined based on the protocol described by Kraemer and Fry [34]. In short, for the first attempt, the load was set at 90% of the estimated 1-RM and was increased by 2.5-5% after each successful lift until failure. A 2-min rest period was allowed between successive attempts. A lift was deemed successful when performed in a controlled manner, without assistance, and for the full range of motion. The range of motion for the supine leg press started at a knee angle of 70° until full extension (without locking the knee), for the seated knee extension, the knee angle was set from 70° to 160°. The 1-RM testing and experimental trials were separated by at least 3 days.

Study design

Participants were randomly assigned to ingest a 400 mL beverage containing 30 g potato protein (POTATO) or 30 g milk protein (MILK). After beverage ingestion, the bottle was rinsed with 150 mL of water. Potato protein concentrate (Solanic 100) was supplied by AVEBE (Veendam, the Netherlands) and milk protein concentrate (MPC80) was obtained from FrieslandCampina (Wageningen, the Netherlands). Participants were allocated to a treatment according to a block randomization list performed using a computerized randomizer (http://www.randomization.com/). An independent researcher was responsible for random assignment (*n*=12 per group) and preparation of the study treatment beverages, which were sequentially numbered according to subject number. The beverages were prepared in non-transparent protein-shakers.

Diet and physical activity

Participants refrained from sports and strenuous physical activities (such as heavy lifting), and alcohol consumption for 3 days prior to the experimental trial. In addition, all participants filled out a food and activity diary for 3 days prior to the experimental trial. For the evening before the trial, all participants consumed the same standardized meal containing 2.3 MJ, with 20% energy provided as carbohydrate, 65% as fat, and 15% as protein, before 22:00 after which they remained fasted.

Experimental protocol

At ~7:30 AM, participants arrived at the laboratory in the overnight fasted state. A catheter was inserted into an antecubital vein for stable isotope amino acid infusion, while a second catheter was inserted retrogradely into a dorsal hand vein of the contralateral arm for arterialized blood sampling. To obtain arterialized blood samples, the hand was placed in a hot box (60°C) for 10 min prior to each blood sample collection [35].

After taking a baseline blood sample (*t*= -180 min), the plasma phenylalanine pool was primed with a single dose of L-[*ring*-$^{13}C_6$]-phenylalanine (2.25 μmol/kg). Thereafter, a continuous intravenous infusion of L-[*ring*-$^{13}C_6$]-phenylalanine (0.05 μmol/kg/min) was initiated (*t*= -180 min) using a calibrated IVAC 598 pump (San Diego, CA). While resting in a supine position, arterialized blood samples were collected in EDTA containing tubes 60 and 120 min (*t*= -120 and *t*= -60 respectively) following initiation of the tracer infusion. At 130 min (*t*= -50) the unilateral exercise session commenced. Following the exercise session (*t*= -10 min) the participants returned to the resting position. At *t*= 0 min an arterialized blood sample was obtained as well as bilateral muscle biopsy samples from the *M. vastus lateralis* of the rested and exercised leg. Immediately following the muscle biopsy, participants ingested the beverage corresponding to their randomized treatment allocation i.e.: POTATO (*n*=12) or MILK (*n*=12). To minimize dilution of the steady-state plasma L-[*ring*-$^{13}C_6$]-phenylalanine precursor pool, 3.85% L-[*ring*-$^{13}C_6$]-phenylalanine was added to the drinks. Arterialized blood samples were then collected at *t*= 15, 30, 45, 60, 90, 120, 150, 180, 210, 240 and 300 min into the postprandial period. A second and third muscle biopsy were collected at *t*= 120 and 300 min to determine postprandial muscle protein synthesis rates from 0-120, 120-300, and

0-300 min. Muscle biopsy collection was performed from both the rested and exercised leg during each time point, starting with the exercised leg. Blood samples were collected into EDTA-containing tubes and centrifuged at 1200*g* for 10 min at 4°C. Aliquots of plasma were frozen in liquid nitrogen and stored at -80°C. Biopsy samples were collected with the use of a 5 mm Bergström needle [36] custom-adapted for manual suction. Samples were obtained from separate incisions from the middle region of the *M. vastus lateralis*, ~15 cm above the patella and ~3 cm below entry through the fascia. Local anesthetic (1% Xylocaine with adrenaline 1:100,000) was applied to numb the skin and fascia. Muscle samples were freed from any visible non-muscle material, immediately frozen in liquid nitrogen, and stored at -80°C until further processing. When the experimental protocol was complete, cannulae were removed and participants were fed and assessed for ~30 min before leaving the laboratory. For a schematic representation of the infusion protocol, see Figure 7.1.

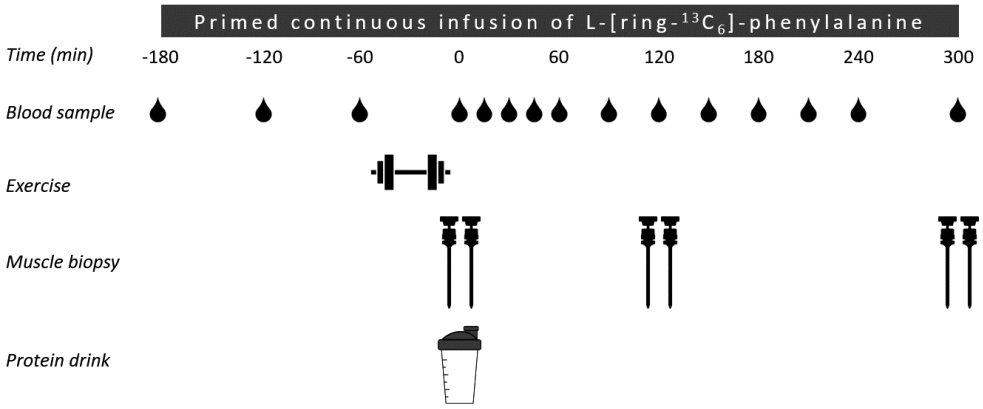

FIGURE 7.1 Schematic representation of the experimental design.

Exercise protocol

Participants began with a standardized warm-up on the supine leg press (20 repetitions at 25% 1-RM followed by 8 repetitions at 50% 1-RM) followed by 3 sets of 8 repetitions at ~80% 1-RM. For the 4th set, participants were instructed to perform as many repetitions as possible. Participants then carried out the same exercise protocol (i.e., same warm-up, number of sets and repetitions at percentage of estimated 1-RM) on the seated knee-extension machine. Each set was separated by 2 min of passive recovery during which the participant remained seated. Strong verbal encouragement was provided by 1 of the study investigators during each set. Participants were randomly allocated to perform the exercise session with their dominant or non-dominant leg. The randomization scheme ensured an equal amount of participants performed the exercise with the dominant (*n*=6) as well as non-dominant (*n*=6) leg within each interventional group (*n*=12).

Dietary protein analysis

Batch specific nitrogen contents of both potato and milk were provided by the manufacturer. Milk protein content was determined as nitrogen content x6.38 [37, 38] and potato protein content as nitrogen content x6.25 [39]. Amino acid contents in protein were determined by acid hydrolysis in triplicate. Specifically, the amino acids were liberated from the protein powders (~4 mg) by adding 2 mL of 6M HCl and heating to 110°C for 12 h. The hydrolysed proteins were subsequently dried under a nitrogen stream while heated to 120°C. Before analysis, the hydrolysate was dissolved in 5 mL of 0.1 M HCl and 20 µL of AccQ/Tag derivatizing reagent solution (Waters, Saint/Quentin, France) was added as described here below for the plasma amino acid concentration analysis. Amino acid composition of the proteins are presented in Table 7.2.

Plasma analysis

Plasma glucose and insulin concentrations were analyzed using commercially available kits (ref. no. A11A01667, Glucose HK CP, ABX Diagnostics, Montpellier, France; and ref. no. HI-14K, Millipore, St. Louis, MO, respectively). Plasma L-[*ring*-$^{13}C_6$]-phenylalanine, enrichments were determined by gas chromatography-mass spectrometry (GC-MS; Agilent 7890A GC/5975C MSD; Agilent Technologies). Specifically, the plasma was deproteinized on ice with dry 5-sulfosalicyclic acid. Free amino acids were purified using cation exchange AG 50W-X8 resin (mesh size: 100-200, ionic form: hydrogen (Bio-Rad Laboratories, Hercules, CA, USA)) columns. The free amino acids were converted to their tert-butyl dimethylsilyl (TBDMS) derivative before analysis by GC-MS using selected ion monitoring of masses 336 and 342 for unlabeled and labeled L-[*ring*-$^{13}C_6$]-phenylalanine, respectively. Standard regression curves were applied from a series of known standard enrichment values against the measured values to assess the linearity of the mass spectrometer and to account for any isotope fraction which may have occurred during the analysis.

In order to determine basal mixed muscle fractional synthetic rate (FSR), the single biopsy approach was applied as described by Burd *et al.* 2012 [40]. In short, plasma protein obtained prior to tracer infusion (*t*= -180 min) was used to determine baseline L-[*ring*-$^{13}C_6$]-phenylalanine enrichments. For this purpose, the plasma sample was precipitated by adding perchloric acid. Subsequently, similarly as for the mixed muscle protein fraction, the denaturized plasma protein pellet was hydrolyzed, passed over a Dowex exchange resin, and the resulting amino acid samples were derivatized to their N(O,S)-ethoxycarbonyl-ethylesters before being measured by GC-IRMS, as explained below.

Plasma amino acid concentrations were determined by ultra-performance liquid chromatography-mass spectrometry (UPLC-MS; ACQUITY UPLC H-Class with QDa; Waters, Saint-Quentin, France). Specifically, 50 µL blood plasma was deproteinized using 100 µL of 10% SSA with 50 µM of MSK-A2 internal standard (Cambridge Isotope Laboratories, Massachusetts, USA). Subsequently, 50 µL of ultra-pure demineralized water was added and samples were centrifuged. After centrifugation, 10 µL of supernatant was added to 70 µL of Borate reaction buffer (Waters, Saint-Quentin, France). In addition, 20 µL of AccQ/

Tag derivatizing reagent solution (Waters, Saint/Quentin, France) was added after which the solution was heated to 55°C for 10 min. Of this 100 µL derivative 1 µL was injected and measured using UPLC-MS.

TABLE 7.2 Amino acid composition of protein or protein blend consumed

	POTATO	MILK
Alanine	1.4	1.0
Arginine	1.3	0.9
Aspartic acid	2.6	1.8
Cystine	0.2	0.1
Glutamic acid	2.5	5.5
Glycine	1.3	0.5
Histidine	0.5	0.7
Isoleucine	0.9	1.0
Leucine	2.6	2.6
Lysine	1.8	2.1
Methionine	0.6	0.6
Phenylalanine	1.5	1.3
Proline	1.4	2.9
Serine	1.4	1.3
Threonine	1.4	1.1
Tyrosine	0.7	0.7
Valine	1.1	1.2
TAA	23.2	25.3
EAA	10.5	10.7
BCAA	4.7	4.9
Nitrogen content (%)	13.1	12.8
Protein content (%)	81.9[1]	81.5[2]

Values for amino acid contents are in g per 30 g protein. [1]Protein as nitrogen * 6.25; [2]Protein as nitrogen content * 6.38; POTATO: 30 g potato derived protein, MILK: 30 g of milk protein. BCAA: Branched chain amino acids, EAA: Essential amino acids, TAA: Total amino acids.

Muscle analysis

A piece of wet muscle (~50-70 mg) was freeze dried for 48 h. Collagen, excessive blood and other non-muscle materials were subsequently removed from the muscle fibers under a light microscope. The isolated muscle fiber mass was weighed and 35 volumes (7x wet weight of isolated muscle fibers x wet-to-dry ratio 5:1) of ice-cold 2% perchloric acid (PCA) was added. Thereafter, the tissue was homogenized by sonification, and centrifuged to separate the supernatant from the protein pellet. The supernatants containing the muscle tissue free amino acids were purified, and derivatized before analysis by GC-MS, similarly as for the

plasma L-[*ring* $^{13}C_6$]-phenylalanine enrichments. The protein pellet was washed 3 times with 1 mL 2% PCA. The amino acids were liberated from the mixed muscle enriched protein fraction by adding 2 mL of 6M HCl and heating to 110°C for 15.5 h. The hydrolysed mixed muscle protein fractions were dried under a nitrogen stream while heated to 110°C. The dried mixed muscle protein fraction was dissolved in a 50% acetic acid solution. The amino acids from the mixed muscle protein fraction were passed over a Dowex exchange resin (AG 50W-X8, 100-200 mesh hydrogen form; Bio-Rad, Hercules, CA, USA) using 2M NH4OH. Subsequently, the purified amino acid solution was dried under a nitrogen stream at room temperature, followed by derivatization to their N(O,S)-ethoxycarbonyl-ethylesters. The ratio of $^{13}C/^{12}C$ of mixed muscle protein-bound phenylalanine was determined using gas chromatography-combustion-isotope ratio mass spectrometry (GC-IRMS; Delta V, Thermo Scientific, Bremen, Germany) by monitoring ion masses 44, 45 and 46. Standard regression curves were applied from a series of known standard enrichment values against the measured values to assess the linearity of the mass spectrometer and to account for any isotope fractionation which may have occurred during the analysis.

Calculations

The FSR (%·h^{-1}) of mixed muscle protein enriched fractions was calculated by the standard precursor-product equation [41]:

$$FSR = \left(\frac{(E_{b2} - E_{b1})}{(E_{precursor} \cdot t)}\right) \cdot 100$$

Where E_b is the increment in mixed muscle protein bound L-[*ring*-$^{13}C_6$]-phenylalanine enrichment (mole % excess) during the tracer incorporation period, and t is the tracer incorporation time in h. Weighted mean plasma enrichments were calculated by taking the measured enrichment between consecutive time points and correcting for the time between these sampling time points ($E_{precursor}$). For calculation for postprandial FSR, biopsy samples at *t*= 0, 120 and 300 min were used. For the calculation of basal FSR, E_{b2} represented the protein bound L-[*ring*-$^{13}C_6$]-phenylalanine enrichments in the muscle of the rested leg at *t*= 0 min, and E_{b1} represented the protein bound L-[*ring*-$^{13}C_6$]-phenylalanine enrichments in plasma albumin at *t*= -180 min.

Net incremental area under curve (iAUC) was determined for plasma amino acid concentrations during the 5 h post-prandial period following protein ingestion. The iAUC was calculated using the trapezoid rule, with plasma concentrations before beverage ingestion (*t*= 0 min) serving as baseline.

Outcome measures

The primary outcome measure is mixed muscle FSR over the aggregate (i.e. 0-300 min) postprandial period, comparing POTATO *vs* MILK in the rested and exercised leg. Secondary outcome measures were mixed muscle FSR changes from basal (i.e. -180 – 0 min and 0-300 min) and changes from basal to the early and late postprandial period (i.e. -180 – 0 min, 0 – 120 min, and 120 – 300 min), comparing POTATO *vs* MILK in the rested and exercised

leg. Additional secondary outcome measures were plasma glucose, insulin and amino acid concentrations, and plasma amino acid iAUC. Plasma glucose, insulin, and amino acid peak concentrations and time to peak were tertiary outcomes.

Statistical analysis

A power calculation was performed with differences in postprandial muscle FSRs between the two interventional groups as primary outcome measure. A sample size of 12 participants per treatment, including a 10% dropout rate was calculated using a power of 80%, a significance level of 0.05, a standard deviation of 0.0065 %·h^{-1}, and a difference in FSR of 0.008 %·h^{-1} between treatments (or ~20% when expressed as a relative difference). Participants' characteristics, were analyzed by an independent samples T-test. Plasma glucose, insulin, and amino acid concentrations and amino acid enrichments were analyzed by a 2-factor (*treatment x time*) repeated measures ANOVA. Plasma amino acid iAUC as well as plasma glucose, insulin, and amino acid peak concentrations and time to peak were analyzed by an independent samples T-test. Basal post-absorptive mixed muscle protein synthesis rates for the rested leg were analyzed by an independent samples T-test. Similarly, post-prandial mixed muscle protein synthesis rates during the early (0-120 min) and entire (0-300 min) postprandial period were analyzed by independent samples T-test for MILK *vs* POTATO in the rested leg as well as exercised leg. Changes is muscle protein synthesis rates over time (-180 – 0; 0-120; 120-300) were analyzed by a 2-factor repeated measures ANOVA. For the repeated measures ANOVA tests, Bonferroni post hoc analysis were performed whenever a significant F ratio was found to isolate specific differences. Statistical analyses were performed with a software package (IBM SPSS statistics for Windows, version 26.0, IBM Corp., Armonk, NY, USA). Means were considered to be significantly different for *P* values <0.05.

RESULTS

Plasma glucose and insulin concentrations

No significant changes in plasma glucose concentrations were observed following protein ingestion, with no differences between interventions (*time x treatment*: *P*=0.12, **Figure 7.2A**). Plasma insulin concentrations increased following MILK but not following POTATO ingestion (*time x treatment: P*<0.001), with a modest peak value of 26±12 mU·L^{-1} achieved 30 min after MILK ingestion (**Figure 7.2B**).

Plasma AA concentrations

Plasma EAA concentrations increased following protein ingestion (**Figure 7.3A**), with a delayed and smaller post-prandial rise following POTATO compared with MILK ingestion (*time x treatment: P*<0.001). Overall, plasma EAA concentrations were 16% lower following POTATO *vs* MILK protein ingestion (incremental area under curve (iAUC): 108±20 *vs* 129±29 mmol·300 min·L^{-1} respectively, *P*=0.04, **Figure 7.3B**). The lower post-prandial EAA availability was also accompanied by 22% lower peak EAA concentrations (1402±118 *vs* 1788±250

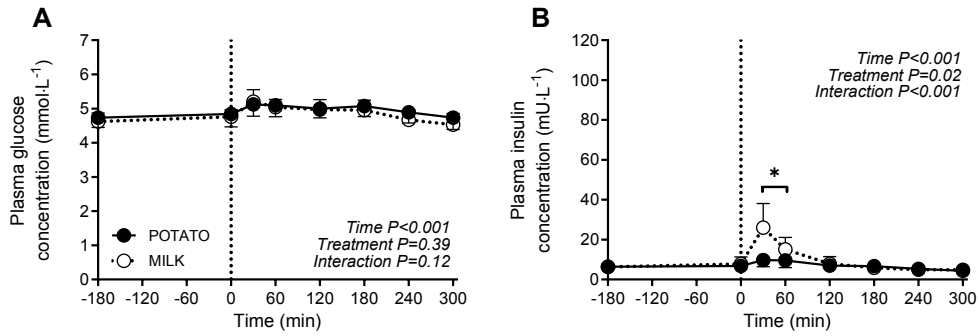

FIGURE 7.2 Post-prandial plasma glucose (Panel A) and insulin (Panel B) concentrations during the 300 min period following the ingestion of POTATO *vs* MILK in 24 healthy young males (*n*=12 per group). Time 0 min represents time of beverage intake. POTATO: 30 g potato derived protein, MILK: 30 g milk protein. Values represent means ± standard deviation; Repeated measures ANOVA with time as within-subject variable and interventional drink (treatment) as between-subject variable.

μmol·L^{-1} respectively, *P*<0.001), that were reached 143±54 and 48±27 min following POTATO *vs* MILK ingestion (*P*<0.001).

The post-prandial rise in circulating plasma leucine (**Figure 7.3C**), lysine (**Figure 7.3E**) and methionine (**Figure 7.3G**) concentrations was delayed and smaller following POTATO when compared with MILK ingestion (*time x treatment:* all *P*<0.001). Post-prandial plasma leucine (**Figure 7.3D**), lysine (**Figure 7.3F**), and methionine (**Figure 7.3H**) availability were respectively 23, 21, and 67% lower for POTATO when compared with MILK (iAUC: 27±4 *vs* 35±8, 15±4 *vs* 19±5, and 1±1 *vs* 3±2 mmol·300 min·L^{-1}, respectively; all *P*<0.05). Peak values were also respectively 26, 29 and 41% lower for POTATO *vs* MILK (252±23 *vs* 341±65, 247±34 *vs* 347±43, and 34±3 *vs* 58±11 μmol·L^{-1}, respectively; all *P*<0.001). Time to reach peak values was significantly longer for POTATO when compared to MILK ingestion (153±50 *vs* 48±27, 100±35 *vs* 40±20, and 103±41 *vs* 40±23 min, respectively; all *P*<0.001).

In general, all post-prandial plasma amino acid concentrations revealed similar differences between treatments (**Supplemental Figure 7.2**, *time x treatment:* all *P*<0.05). The overall proline and valine concentrations (iAUC) were lower for POTATO *vs* MILK, while the overall glycine concentrations were higher for POTATO *vs* MILK (**Supplemental Figure 7.2**). Collectively, when evaluating the total sum of all amino acids (TAA), the post-prandial increase over time differed significantly between protein sources (*time x treatment*: *P*<0.001), with a trend towards overall lower plasma amino acid availability following POTATO *vs* MILK ingestion (iAUC: 115±43 *vs* 147±47 mmol·300 min·L^{-1} respectively; *P*=0.095). In line, peak TAA concentrations were 37% lower for POTATO *vs* MILK (2884±230 *vs* 3626±440 μmol·L^{-1} respectively; *P*<0.001) and were reached 118±56 and 43±24 min after protein ingestion, respectively (*P*<0.001).

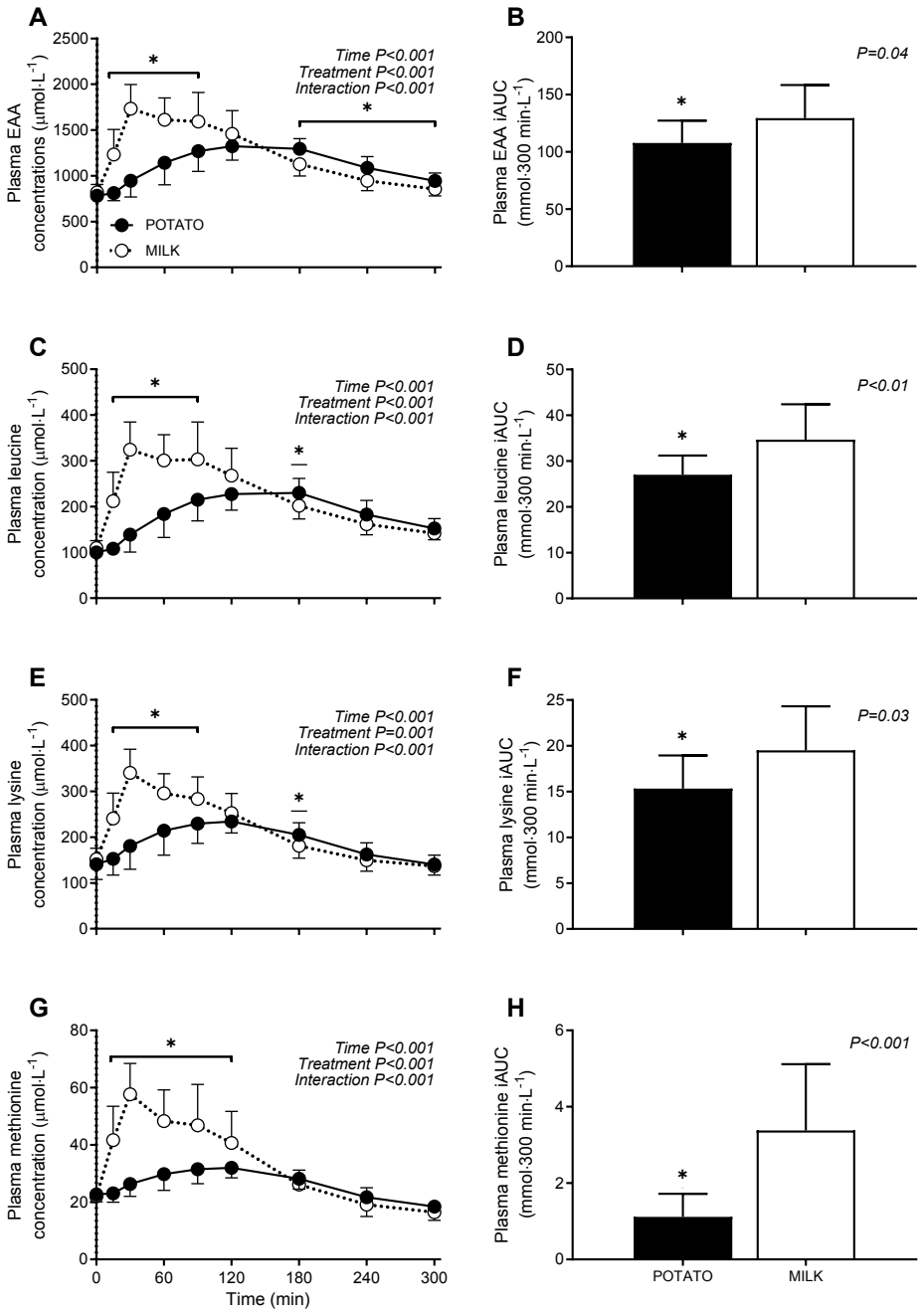

FIGURE 7.3 Post-prandial plasma essential amino acid (EAA, Panel A), leucine (Panel C), lysine (Panel E), and methionine (Panel G) concentrations during the 300 min post-prandial period following the ingestion of POTATO *vs* MILK. Time 0 min represents time of beverage intake. Panels B, D, F and H represent the 0-5 h incremental area under the curve (iAUC) following protein ingestion. POTATO: 30 g potato derived protein, MILK: 30 g milk protein. Values represent means ± standard deviation; *significantly different for POTATO *vs* MILK ($P<0.05$). Repeated measures ANOVA with time as within-subject variable and interventional drink (treatment) as between-subject variable.

Plasma and muscle protein-bound L-[ring-$^{13}C_6$]-phenylalanine enrichments

Plasma phenylalanine concentrations and L-[*ring*-$^{13}C_6$]-phenylalanine enrichments over time are presented in **Figure 7.4A** and **7.4B**, respectively. Plasma L-[*ring*-$^{13}C_6$]-phenylalanine enrichments over time were higher during the first 60 min and lower during the last 150 min following POTATO *vs* MILK ingestion (*time x treatment: P*<0.001). Weighted mean plasma L-[*ring*-$^{13}C_6$]-phenylalanine enrichments averaged 6.63±0.46 and 6.75±0.55 MPE during the basal post-absorptive period (*P*=0.55), and 6.26±0.41 and 6.59±0.48 MPE during the post-prandial period (*P*=0.09) for POTATO and MILK respectively.

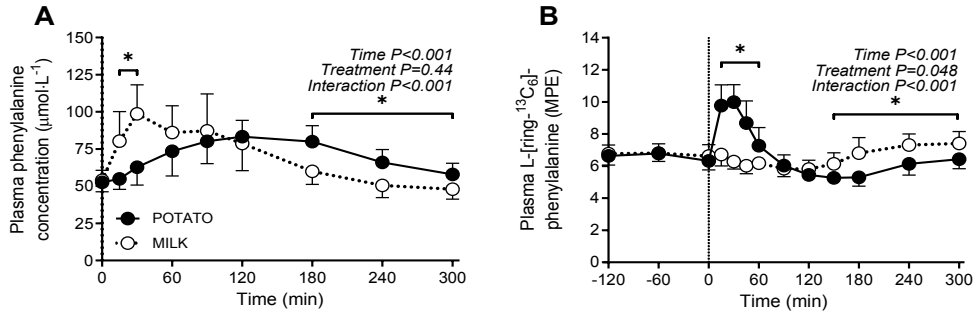

FIGURE 7.4 Post-prandial plasma phenylalanine concentrations (Panel **A**) and plasma L-[*ring*-$^{13}C_6$]-phenylalanine enrichments (Panel **B**) during the 300 min period following the ingestion of POTATO *vs* MILK in healthy, young males (*n*=12 per group). Time 0 min represents time of protein ingestion. POTATO: 30 g potato protein, MILK: 30 g milk protein. Values represent means ± standard deviation; * significantly different for MILK *vs* WHEAT (*P*<0.05). Repeated measures ANOVA with time as within-subject variable and interventional drink (treatment) as between-subject variable.

In the rested leg, mixed muscle protein-bound L-[*ring*-$^{13}C_6$]-phenylalanine enrichments increased following ingestion of POTATO and MILK from 0.0046±0.0028 and 0.0046±0.0029 MPE (at *t*=0 min, *P*=0.99), to 0.0135±0.0058 and 0.0124±0.0041 MPE (at *t*= 120 min, *P*=0.61) reaching 0.0230±0.0076 and 0.0220±0.0052 MPE, respectively, at 300 min after protein ingestion (at *t*=300 min, *P*=0.72; *time x treatment: P*=0.70).

In the exercised leg, mixed muscle protein-bound L-[*ring*-$^{13}C_6$]-phenylalanine enrichments increased following POTATO and MILK ingestion from 0.0050±0.0036 and 0.0044±0.0026 MPE (at *t*=0 min, *P*=0.68), to 0.0156±0.0063 and 0.0128±0.0035 MPE (at *t*= 120 min, *P*=0.20) reaching 0.0280±0.0096 and 0.0262±0.0049 MPE, respectively, at 300 min after protein ingestion (at *t*=300 min, *P*=0.56; *time x treatment: P*=0.50). Collectively, 300 min after protein ingestion, the mixed muscle protein-bound L-[*ring*-$^{13}C_6$]-phenylalanine enrichments were higher in the exercised compared with the rested leg (both treatments; *P*<0.05).

Muscle protein synthesis rates

In the rested leg, post-absorptive fractional mixed muscle protein synthesis rates averaged 0.020±0.011 and 0.021±0.014 %·h^{-1} in the POTATO and MILK trial, respectively, with no differences between groups (*P*=0.88; **Figure 7.5**). POTATO and MILK ingestion both strongly

increased mixed muscle protein synthesis rates (main effect of time $P<0.001$), with no *time x treatment* interaction ($P=0.52$) from the basal post-absorptive to 5 h post-prandial period. No differences in post-prandial mixed muscle protein synthesis rates were observed between POTATO and MILK ingestion during the early (e.g. 0-120 min; 0.051±0.019 and 0.055±0.017 %·h^{-1} respectively; $P=0.55$) late (e.g. 120-300 min; 0.055±0.023 and 0.046±0.017 %·h^{-1} respectively; $P=0.33$) or entire post-prandial period (e.g. 0-300 min; 0.053±0.017 and 0.050±0.012 %·h^{-1} respectively; $P=0.54$).

In the exercised leg, post-absorptive mixed muscle protein synthesis rates averaged 0.023±0.015 and 0.023±0.017 %·h^{-1} for POTATO and MILK respectively, with no differences between groups ($P=0.97$; **Figure 7.5**). POTATO and MILK ingestion both strongly increased mixed muscle protein synthesis rates following exercise (main effect of time $P<0.001$), with no *time x treatment* interaction ($P=0.58$) from the basal post-absorptive to 5 h post-prandial period. No differences in post-prandial muscle protein synthesis rates were observed following POTATO and MILK ingestion during the early (0.060±0.021 and 0.058±0.021 %·h^{-1} respectively; $P=0.74$), late (0.071±0.031 and 0.065±0.021 %·h^{-1} respectively; $P=0.25$), and entire post-prandial period (0.069±0.019 and 0.064±0.015 %·h^{-1} respectively; $P=0.52$). Post-prandial muscle protein synthesis rates over the 5 h period following exercise were significantly higher in the exercised versus rested leg, for both treatments ($P<0.05$).

Mixed muscle protein synthesis rates determined with the intra-cellular L-[*ring*-$^{13}C_6$]-phenylalanine enrichments used as precursor pool (**Supplemental Figure 7.3**) resulted in similar findings with no differences between treatments (**Supplemental Figure 7.4**).

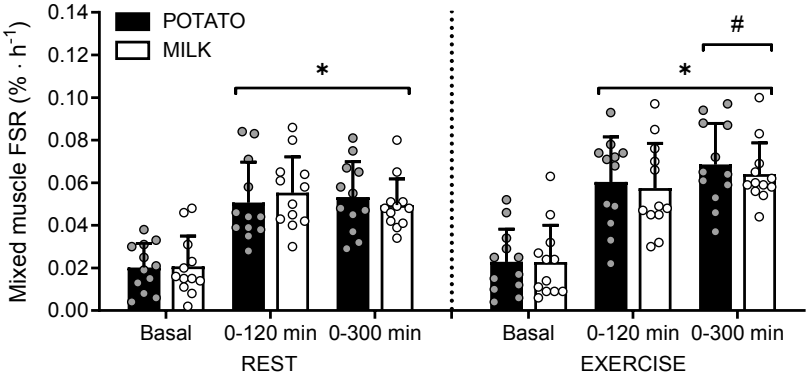

FIGURE 7.5 Mixed muscle fractional synthetic rate (FSR) in the basal post-absorptive and post-prandial period following ingestion of POTATO *vs* MILK in the rested and exercised leg. POTATO: 30 g potato derived protein, MILK: 30 g milk protein, REST: rested leg, EXERCISE: exercised leg. Values represent means ± standard deviation. *significantly different from basal, $P<0.05$. #significantly different from rested leg, $P<0.05$. Independent samples *t*-test POTATO *vs* MILK: REST: $P=0.88$, $P=0.55$, and $P=0.54$ for basal, 0-120, and 0-300 respectively. EXERCISE: $P=0.97$, $P=0.73$, and $P=0.52$ for basal, 0-120, and 0-300 respectively.

DISCUSSION

The present study shows that ingestion of 30 g potato derived protein strongly increases muscle protein synthesis rates at rest and during recovery from exercise in healthy, young males. Despite the observation of a lesser and more delayed post-prandial rise in plasma essential amino acid availability following potato when compared to milk protein ingestion, post-prandial mixed muscle protein synthesis rates did not differ between protein sources at rest or during recovery from exercise.

The anabolic properties of plant-derived proteins are generally considered to be lower when compared to animal-derived proteins [19, 20]. This has been, at least partly, attributed to plant-derived proteins providing overall less essential amino acids and the prevalence of one or more specific amino acid deficiencies in these proteins [19, 20]. In contrast to many plant-derived proteins [28], we observed that potato derived protein provides sufficient amounts of all essential amino acids according to the WHO/FAO guidelines for protein requirements. In fact, 30 g of the applied potato derived protein was shown to provide similar amounts of essential amino acids (10.5 *vs* 10.7 g), leucine (2.6 *vs* 2.6 g), lysine (1.8 *vs* 2.1 g), and methionine (0.6 *vs* 0.6 g) when compared to the equivalent amount of milk protein (**Table 7.2**). Despite similar amino acid composition, the post-prandial rise in circulating (essential) amino acids was attenuated following the ingestion of potato compared with milk protein (**Figure 7.3**), resulting in lower peak essential amino acid, leucine, lysine, and methionine concentrations (-22, -26, -29, and -41%) that were reached at a much later point in time (+200, +221, +150, and +156 min, respectively). Consequently, post-prandial plasma amino acid availability was substantially lower throughout the 5 h post-prandial period following potato when compared with milk protein ingestion (**Figure 7.3**). Based on the phenylalanine tracer kinetics (**Figure 7.4**), we attribute this to a more delayed protein digestion and amino acid absorption, an increased amino acid retention in splanchnic tissues, and/or a less efficient digestion of potato compared with milk protein. As the intrinsically labeled protein approach [42] simply can't be applied in the case of plant-derived proteins, it is impossible to directly quantify the exact amount of potato protein derived amino acids that were released in the circulation, as we have done previously for milk [2] and mealworm derived protein [43].

Despite the attenuated postprandial rise in circulating amino acids following the ingestion of potato derived protein we observed a strong increase in muscle protein synthesis rates (**Figure 7.5**). A response that did not differ from the response observed after ingesting an equivalent amount of milk protein (**Figure 7.5**). Clearly, the provided potato derived protein is capable of strongly stimulating muscle protein synthesis *in vivo* in humans. Whether the absence of any differences in the anabolic response to potato versus milk protein ingestion can be attributed to the favorable amino acid profile of potato protein when compared to other plant-derived proteins remains unclear, as previous work [26, 27] but certainly not all studies [15, 23-25] have reported no differences in the anabolic response to the ingestion of similar amounts of plant- versus animal derived protein. Obviously, the observed post-prandial rise in circulating amino acids following the ingestion of 30 g potato protein concentrate was

sufficient to elevate muscle protein synthesis rates. The more sustained release of amino acids throughout the latter stages of the post-prandial period may have compensated for a potential lesser initial increase in post-prandial plasma amino acid availability, allowing a post-prandial muscle protein synthetic response that did not differ from the ingestion of 30 g of milk protein. However, our data did not show an early attenuated post-prandial increase in muscle protein synthesis rates following potato protein when compared with milk protein ingestion, with FSR values calculated using plasma (**Figure 7.5**) and tissue free enrichments (**Supplemental Figure 7.3**) as precursor pools. As there was some initial disbalance between L-[*ring*-$^{13}C_6$]-phenylalanine release and overall phenylalanine kinetics (**Figure 7.4B**), we cannot exclude that this may have caused a minor overestimation of the early post-prandial FSR in the potato group.

Exercise has previously been shown to sensitize skeletal muscle tissue to the anabolic properties of protein ingestion [44]. In the current study we applied a unilateral exercise design to allow assessment of post-prandial muscle protein synthesis rates both at rest as well as during recovery from exercise. We observed a strong increase in muscle protein synthesis rates in the exercised leg following both potato as well as milk protein ingestion (**Figure 7.5**), with responses that were greater when compared with the rested leg. Again, no differences were observed in post-exercise muscle protein synthesis rates following ingestion of 30 g potato versus 30 g milk protein. These data imply that plant-derived protein concentrates can be applied effectively to support post-exercise muscle conditioning in athletes. These findings are in contrast to some [15, 23, 24] but certainly not all [15, 26] studies comparing post-exercise muscle protein synthesis rates following soy compared with dairy protein ingestion. The apparent discrepancy may be, at least partly, explained by the amount of protein provided. In the present study we provided 30 g potato or milk protein, which is more than the amount of egg or milk protein (20 g) that has previously been suggested to be required to maximize post-exercise muscle protein synthesis rates [45]. Though we can only speculate on the impact of ingesting smaller amounts of potato derived protein on post-prandial muscle protein synthesis rates, our data imply that a maximal post-exercise muscle protein synthetic response can be obtained by ingesting up to 30 g of a high quality plant-derived protein concentrate.

There is an increasing interest in the consumption of food products and sports supplements containing alternative, more sustainable, sources of protein [20]. The present study extends on prior work evaluating the post-prandial and/or post-exercise muscle protein synthetic responses following soy and wheat derived protein ingestion [15, 23-27], showing that potato protein ingestion can strongly increase muscle protein synthesis rates at rest and during recovery from exercise. In support, Oikawa and colleagues [46] observed increases in daily muscle protein synthesis rates following more prolonged potato protein supplementation in females during a period of exercise training. Furthermore, increases in muscle mass and strength gains have been reported following both plant as well as animal derived protein supplementation during prolonged resistance type exercise training [47-50]. The present data clearly show that there are ample opportunities for the use of plant-derived proteins

in sports nutrition, but more research will be needed to evaluate the anabolic properties of the various plant-derived proteins that are currently available and their potential blends [27].

In conclusion, ingestion of 30 g potato derived protein concentrate strongly increases muscle protein synthesis rates at rest and during recovery from exercise *in vivo* in healthy, young males. The post-prandial muscle protein synthetic response following the ingestion of 30 g potato protein does not differ from the response following ingestion of an equivalent amount of milk protein. Plant-derived proteins may be applied effectively in vegan protein products and sports nutrition supplements to support skeletal muscle conditioning during recovery from exercise.

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Conflicts of Interest

LJCvL has received research grants, consulting fees, speaking honoraria, or a combination of these from Friesland Campina, Tereos Syral, and Pepsico. PJMP, FKH, WJHH, JPBG, JMS, JMXvK, WKHW, and TS report no conflicts of interest.

The results of the present study do not constitute endorsement by ACSM.

We declare that the results of the study are presented clearly, honestly, and without fabrication, falsification, or inappropriate data manipulation.

Clinical Trial Registry number: Nederlands Trial Register: NTR7152 https://www.trialregister.nl/

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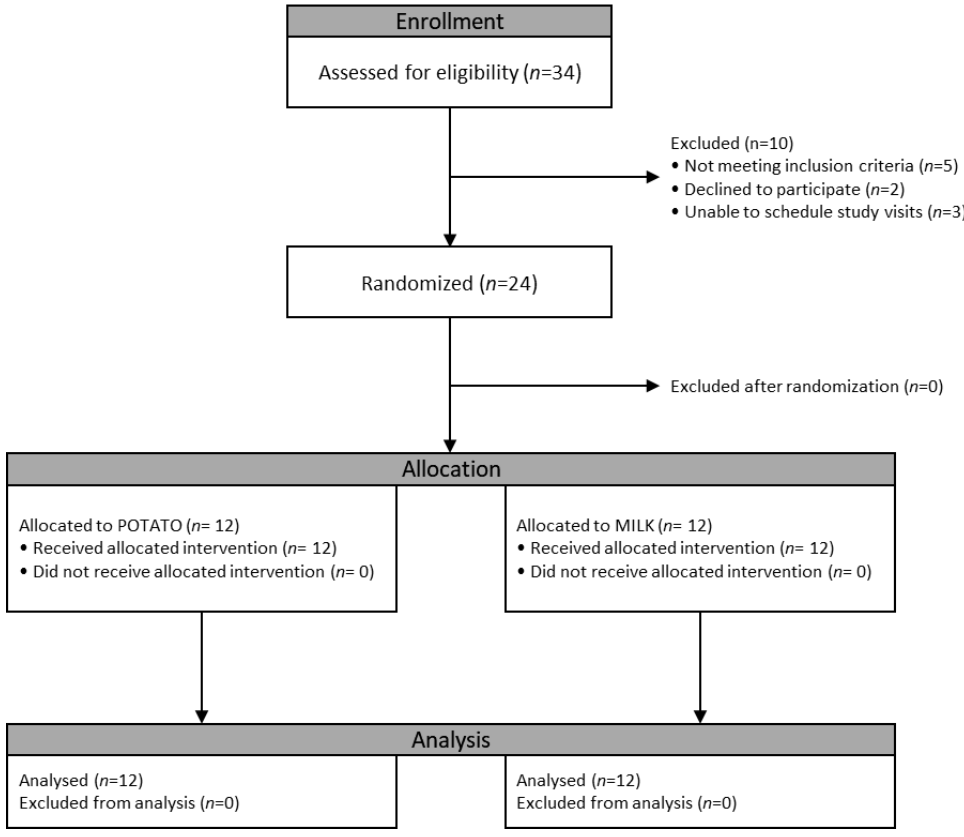
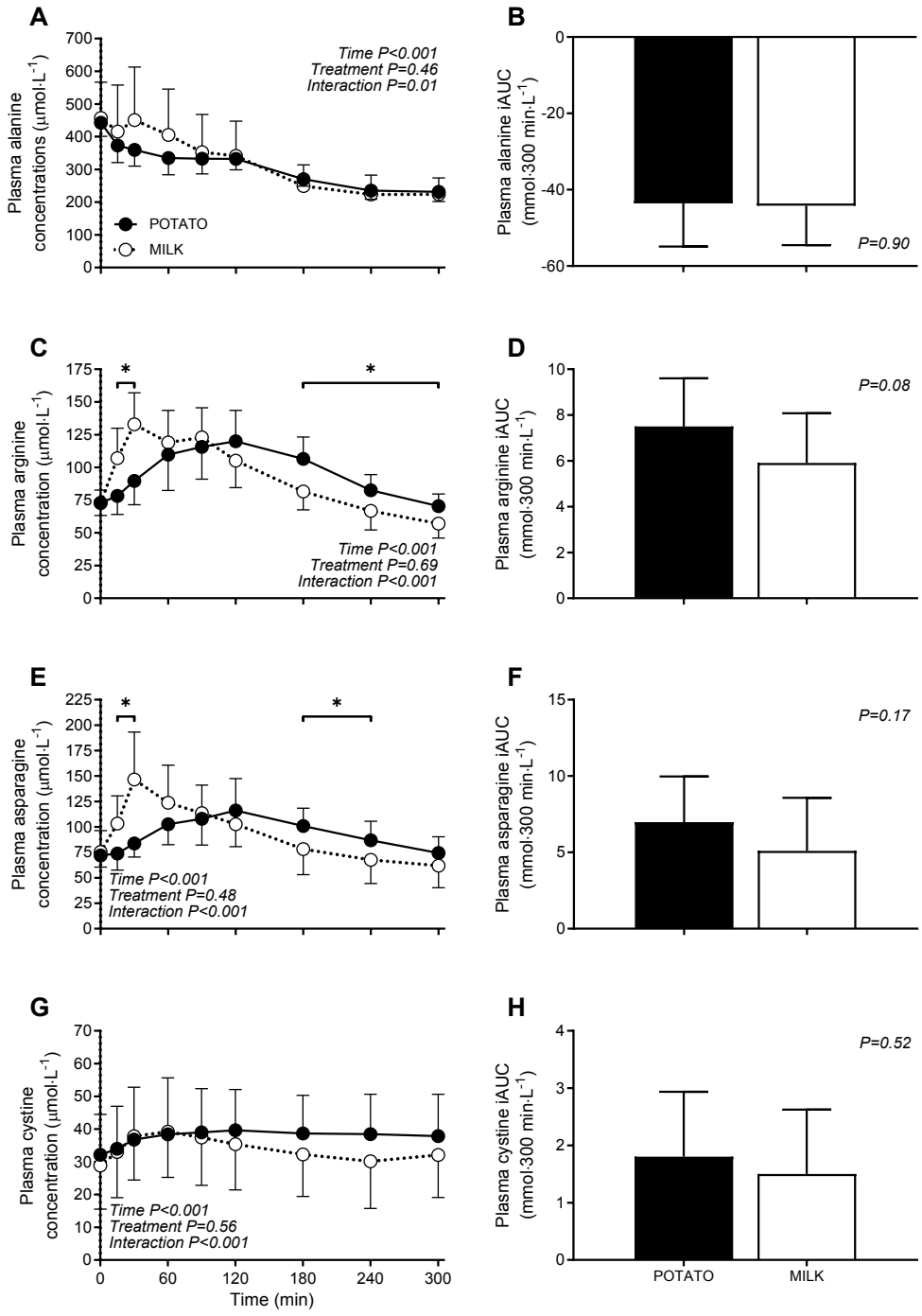
SUPPLEMENTAL MATERIAL



SUPPLEMENTAL FIGURE 7.1 CONSORT flow diagram. CONSORT, Consolidated Standards of Reporting Trials.



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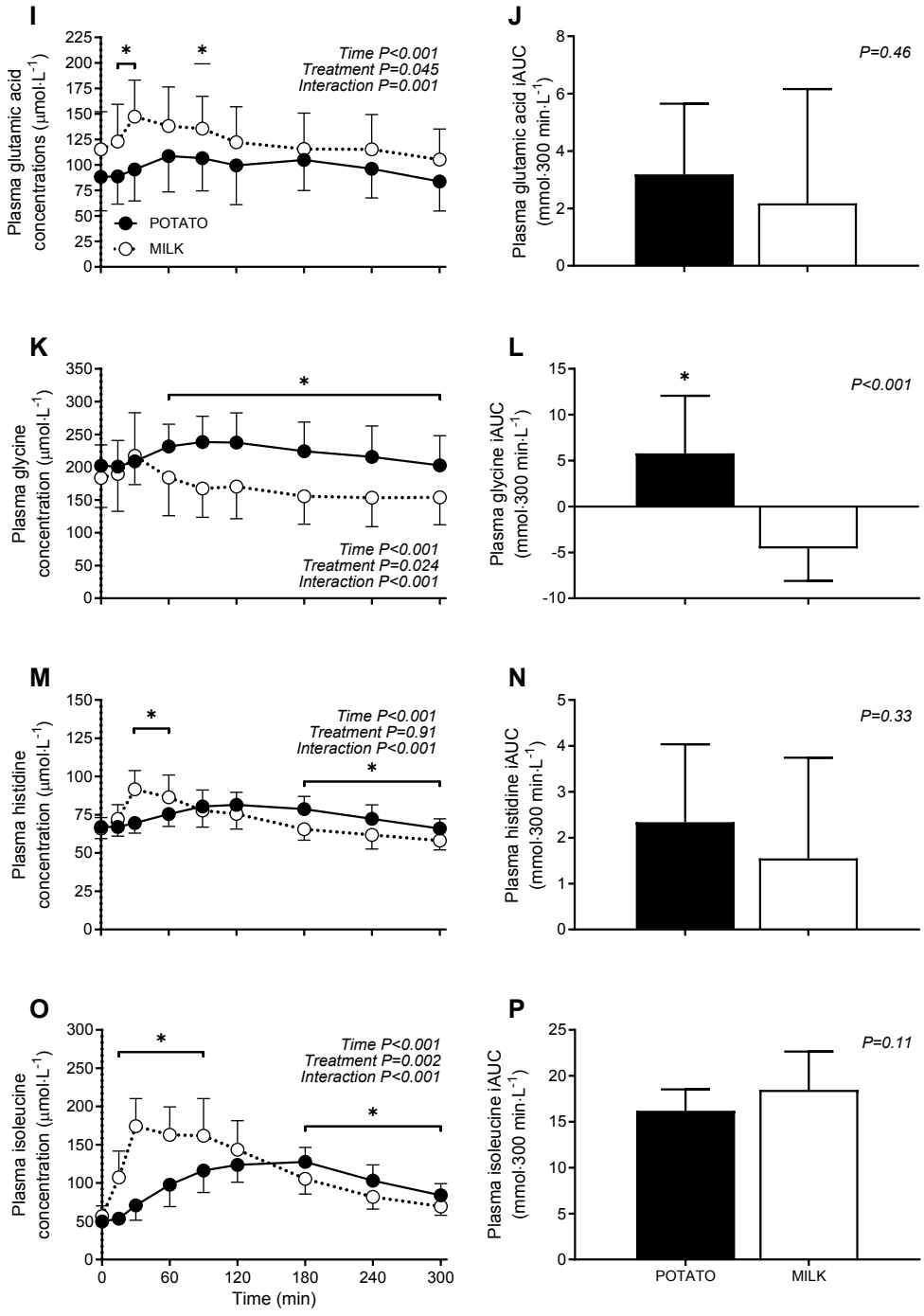

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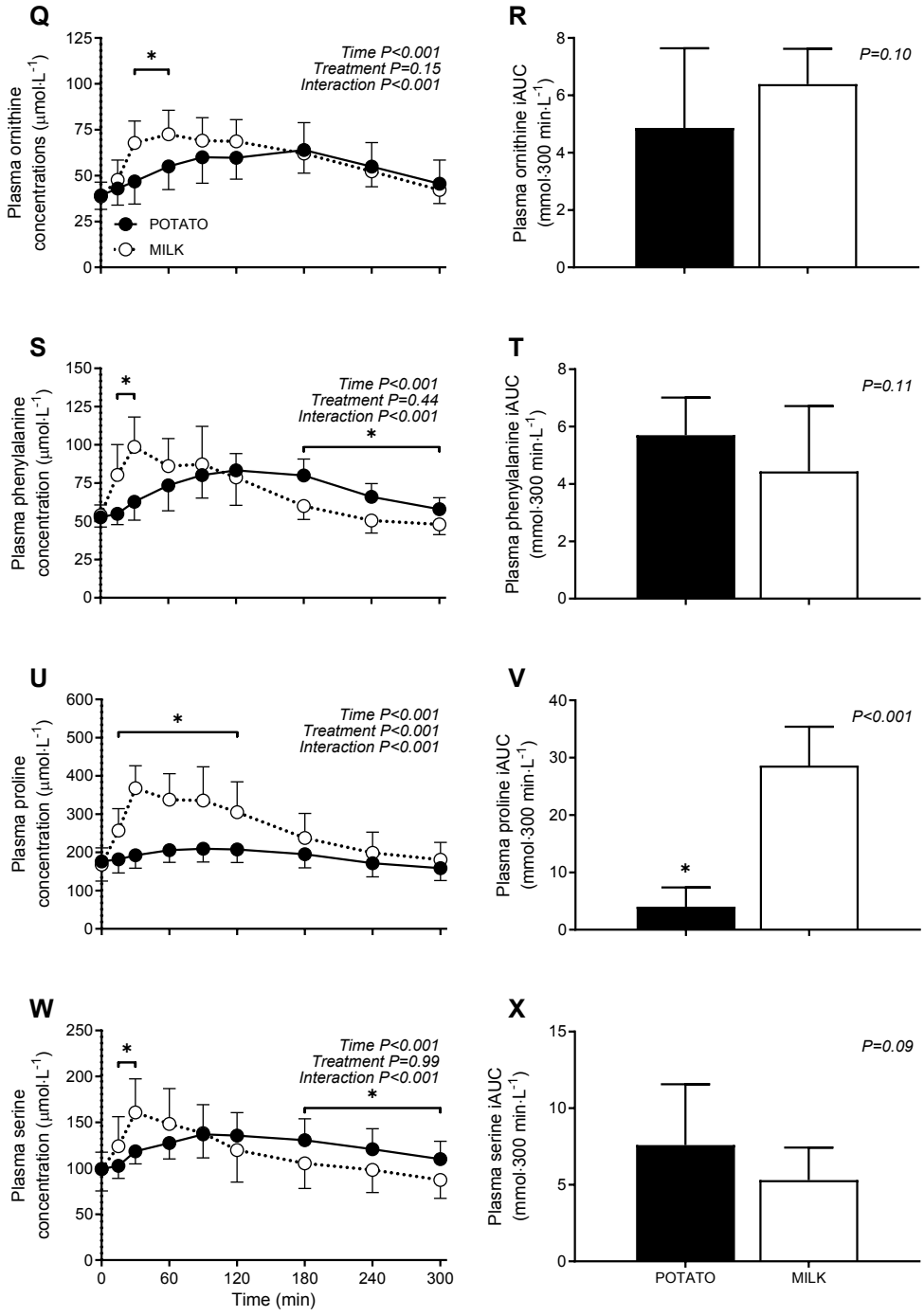

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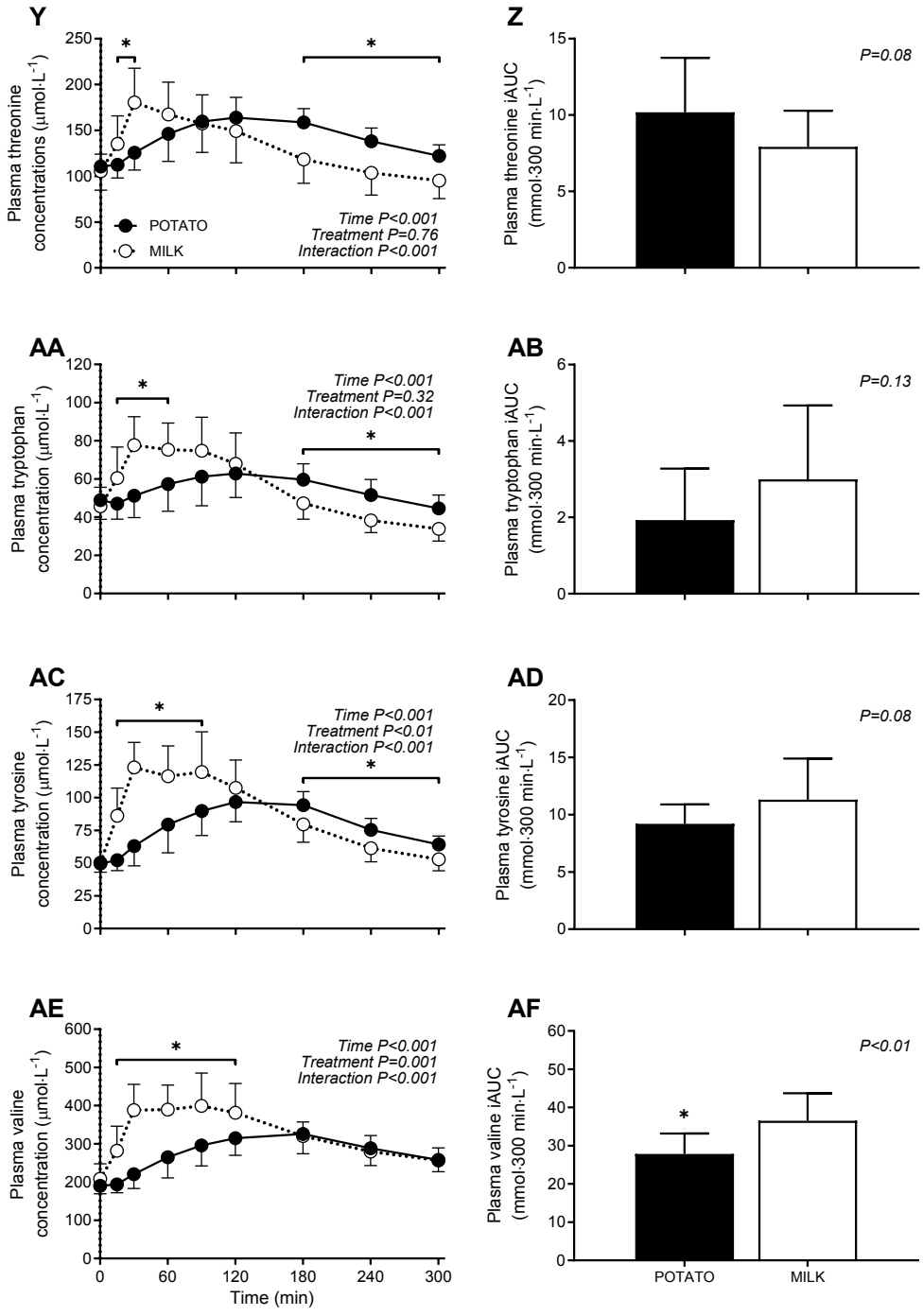

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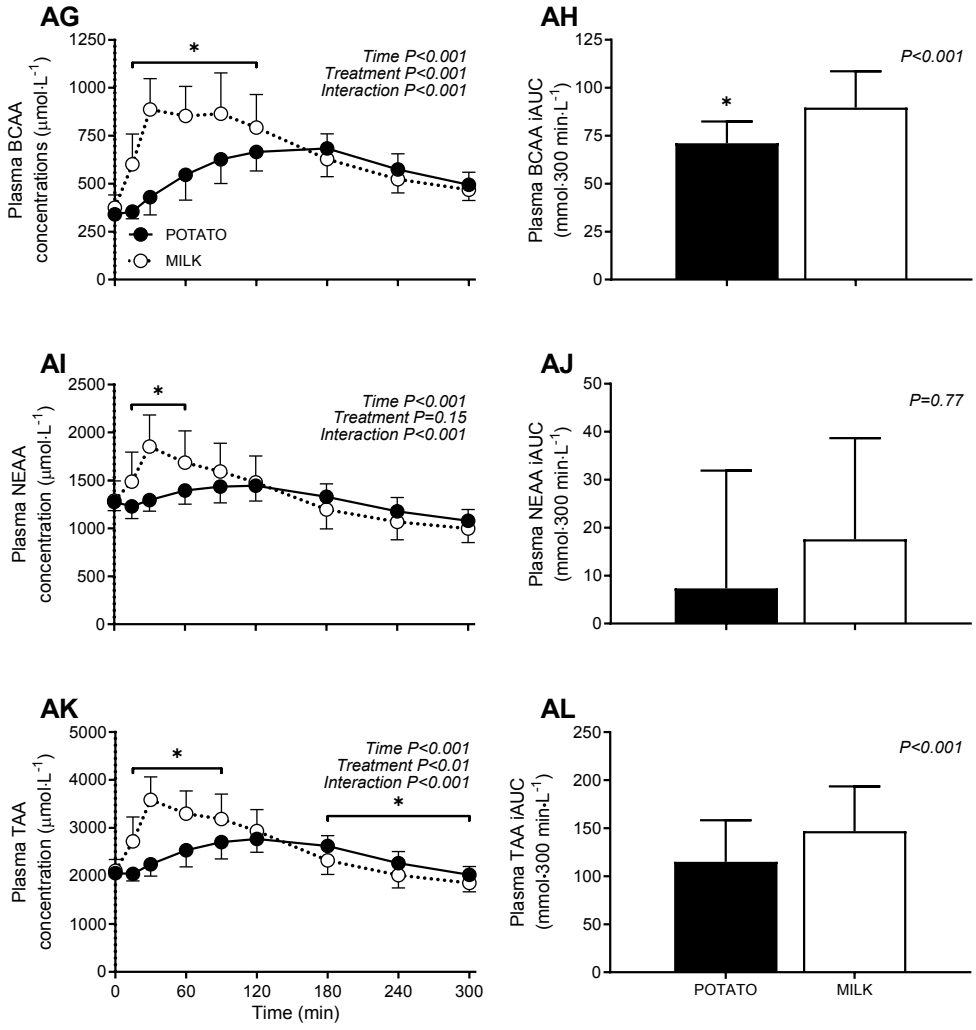
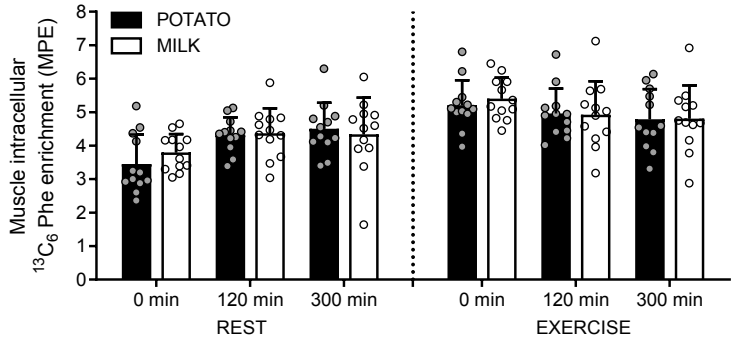
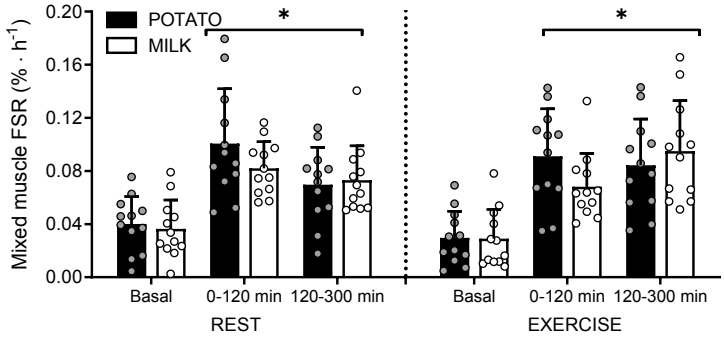


SUPPLEMENTAL FIGURE 7.2 Post-prandial plasma amino concentrations during the 300 min post-prandial period following the ingestion of POTATO *vs* MILK. Time 0 min represents time of beverage intake. Panels B, D, F, H, J, L, N, P, R, T, V, X, Z, AB, AD, AF, AH, AJ, AL represent the 0-5 h incremental area under curve (iAUC) following protein ingestion. POTATO (30 g potato derived protein), MILK (30 g milk protein). Values represent means ± standard deviation; * significantly different for POTATO *vs* MILK ($P<0.05$). Repeated measures ANOVA with time as within-subject variable and interventional drink (treatment) as between-subject variable, and independent samples t-test were used to determine differences between groups.



SUPPLEMENTAL FIGURE 7.3 Muscle intracellular L-[*ring*-$^{13}C_6$] Phenylalanine enrichments at different time points following ingestion of POTATO vs MILK during rest and during recovery following exercise in healthy, young males (*n*=12 per group). Phe: Phenylalanine, POTATO: 30 g potato protein, MILK: 30 g milk protein. Bars represent means ± standard deviation, dots represent individual values. Independent samples *t*-test: POTATO *vs* MILK: REST: *P*=0.26, *P*=0.86, and *P*=0.67 for basal, 0-120, and 0-300 min, respectively. EXERCISE: *P*=0.49, *P*=0.92, *P*=0.97 for basal, 0-120, and 0-300 min, respectively.



SUPPLEMENTAL FIGURE 7.4 Mixed muscle fractional synthetic rate (FSR) determined with intra-cellular enrichments as precursor pool at different time points following ingestion of POTATO *vs* MILK during rest and during recovery following exercise in healthy, young males (*n*=12 per group). POTATO: 30 g potato protein, MILK: 30 g milk protein. Bars represent means ± standard deviation, dots represent individual values. *significantly different from basal; *P*<0.05. Independent samples *t*-test: POTATO *vs* MILK: REST: *P*=0.67, *P*=0.17, and *P*=0.51 for basal, 0-120, and 0-300 min, respectively. EXERCISE: *P*=0.95, *P*=0.08, *P*=0.58 for basal, 0-120, and 0-300 min, respectively.

CHAPTER 8

INGESTION OF AN AMPLE AMOUNT OF MEAT SUBSTITUTE BASED UPON A LYSINE-ENRICHED, PLANT-BASED PROTEIN BLEND STIMULATES POSTPRANDIAL MUSCLE PROTEIN SYNTHESIS TO A SIMILAR EXTENT AS AN ISONITROGENOUS AMOUNT OF CHICKEN IN HEALTHY, YOUNG MEN

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ABSTRACT

Background

Plant-based proteins are considered to be less effective in their capacity to stimulate muscle protein synthesis when compared with animal-based protein sources, likely due to differences in amino acid contents.

Objective

We compared the postprandial muscle protein synthetic response following the ingestion of a lysine-enriched plant-based protein product with an isonitrogenous amount of chicken.

Methods

In a randomized, double blind, parallel-group design, 24 healthy young males (24±4y) received primed continuous L-[*ring*-$^{13}C_6$]-phenylalanine infusions while ingesting 30g potato derived protein or 30g milk protein following a single bout of unilateral resistance exercise. Blood and muscle biopsies were collected for 5 hours following protein ingestion to assess post-prandial plasma amino acid profiles and mixed muscle protein synthesis rates at rest and during recovery from exercise.

Results

Twenty-four men (age: 24±5 y; BMI: 22.9±2.6 kg·m^{-2}) participated in this parallel, double-blind, randomised controlled trial and consumed 40 g protein as a lysine-enriched wheat and chickpea protein product (Plant, n=12) or chicken breast fillet (Chicken, n=12). Primed, continuous intravenous L-[ring-$^{13}C_6$]-phenylalanine infusions were applied while repeated blood and muscle samples were collected over a 5h postprandial period to assess plasma amino acid responses, muscle protein synthesis rates, and muscle anabolic signalling responses. Postprandial plasma leucine and essential amino acid concentrations were higher following Chicken (P<0.001), while plasma lysine concentrations were higher throughout in Plant (P<0.001). Total plasma amino acid concentrations did not differ between interventions (P=0.181). Ingestion of both Plant and Chicken increased muscle protein synthesis rates from post-absorptive: 0.031±0.011 and 0.031±0.013 to postprandial: 0.046±0.010 and 0.055±0.015%·h^{-1}, respectively (P-time<0.001), with no differences between Plant and Chicken (*time x treatment* P=0.068).

Conclusion

Ingestion of 40 g protein in the form of a lysine-enriched plant-based protein product increases muscle protein synthesis rates to a similar extent as an isonitrogenous amount of chicken in healthy, young men. Plant-based protein products sold as meat replacers may be as effective as animal-based protein sources to stimulate postprandial muscle protein synthesis rates in healthy, young individuals.

INTRODUCTION

Skeletal muscle mass maintenance is regulated by the balance between muscle protein synthesis and breakdown rates. The stimulation of muscle protein synthesis by food intake (i.e. dietary protein ingestion and subsequent aminoacidemia) and physical activity (i.e. resistance type exercise) are key factors responsible for the maintenance of skeletal muscle mass. The amplitude and duration of the muscle protein synthetic response to protein ingestion is modulated by the amount [1-3], source [4-7], and type of protein [4, 8] that is consumed, as well as the matrix in which it is embedded [9-11]. In addition, the essential amino acid (EAA) composition of the protein that is consumed, and the leucine content in particular, plays a key role in the postprandial stimulation of muscle protein synthesis [12].

The consumption of plant-based protein sources and the use of plant-based protein isolates and concentrates in food formulations and products is increasing worldwide, which is mainly due to the increasing awareness regarding food sustainability and the lower production cost of plant-based proteins [13]. However, based upon their digestibility and/or amino acid composition, plant-based protein sources are generally considered of a lesser quality when compared to animal-based proteins [14-16]. In accordance, the postprandial muscle protein synthetic response to the ingestion of plant-based proteins has been shown to be lower when compared to the ingestion of an isonitrogenous amount of animal-based protein [6, 7, 17, 18]. The lesser anabolic properties of plant-based proteins have been attributed to the lower EAA content and the shortage of specific amino acids such as leucine, lysine, and/or methionine [14, 15, 19, 20]. Since all amino acids are required as precursors for *de novo* muscle protein synthesis, the lack of one or more amino acids may compromise the postprandial muscle protein synthetic response. Though there are only few studies that have assessed muscle protein synthesis rates following the ingestion of plant-based proteins, a lower muscle protein synthetic response to the ingestion of soy [7, 17, 18] and wheat [6] have been consistently reported when compared with the ingestion of animal-based protein sources such as milk or beef.

To compensate for the proposed lesser anabolic potential of plant-based proteins, more of the plant-based protein could be consumed to induce a similar postprandial increase in muscle protein synthesis rates when compared to a high quality animal-based protein [6]. Although effective, strongly increasing the dose of plant-based proteins to compensate for their lower anabolic properties may not always be practical or feasible. Other strategies to increase the anabolic potential of a plant-based protein source may be to fortify with specific amino acids or the use of specific blends of various plant-based proteins that have opposing differences in their specific shortages of one or more amino acids. Recent innovations in food processing and the selection of specific plant-based protein blends may optimize the quality of a plant-based protein meal and, as such, increase the postprandial muscle protein synthetic response [21-23]. As a result, there is an extensive range of plant-based protein products (as alternatives to meat consumption) available on the market; their capacity to stimulate muscle protein synthesis rates, however, has not yet been assessed. We aimed to

compare the muscle protein synthetic response following the ingestion of an ample amount of a plant-based, whole-food protein source (a meat alternative) with an equivalent amount of an animal-based protein source. We hypothesised that ingestion of a lysine-enriched plant-based protein product can increase muscle protein synthesis rates in healthy individuals. Furthermore, we hypothesised that the postprandial muscle protein synthetic response following the ingestion of an ample amount of such a plant-based meat alternative would not differ from the ingestion of an isonitrogenous amount of chicken. To test our hypothesis, we assessed post-absorptive and postprandial muscle protein synthesis rates using contemporary stable isotope methodology following ingestion of 40 g protein provided via a lysine-enriched wheat and chickpea protein-based product or an isonitrogenous amount of chicken in 24 healthy, young men.

MATERIALS AND METHODS

Participants

Twenty-four healthy, young, recreationally active men (age 18-35 y, BMI 18-27.5 $kg \cdot m^{-2}$) volunteered to participate in this parallel, double-blind, randomised controlled trial (recreationally active was defined as engaging in sports or structured exercise ≤3 d/week and not participating in any structured resistance exercise program). Participants' characteristics are presented in **Table 8.1**. The flowchart of participant enrolment is shown in **Supplemental Figure 8.1**. This study was registered at the Netherlands Trial Register (NTR6380) and was conducted between June 2017 and October 2017 at Maastricht University, Maastricht, the Netherlands. All participants were informed on the purpose of the study, the experimental procedures, and possible risks before providing informed written consent to participate. The procedures followed were in accordance with the ethical standards of the medical ethics committee of Maastricht University Medical Centre+ on human experimentation and in accordance with the Helsinki Declaration of 1975 as revised in October 2013. The study was independently monitored by the Clinical Trial Centre Maastricht.

Pretesting

Participants underwent an initial screening session to assess height, weight, blood pressure, and body composition (by dual-energy X-ray absorptiometry (DEXA); Hologic Inc., DXA; Discovery A, QDR series, Marlborough, USA). Whole-body and appendicular (sum of lean mass of both arms and legs) lean mass and body fat was determined using the software package Apex (en-CORE 2005, version 4.0.2. Hologic, Marlborough, USA) and reference values from the National Health and Nutrition Examination Survey (NHANES) population-based dataset [24]. Participants were deemed healthy based on their responses to a medical questionnaire and screening results. All participants were instructed to refrain from strenuous physical activity and alcohol consumption for 3 d before the experimental trial. On the evening before the experimental trial, all participants consumed a pre-packaged standardized meal (Aviko Maaltijdpannetje, Aviko, Steenderen, the Netherlands) containing

TABLE 8.1 Participants' characteristics

	PLANT	MILK
Age (y)	24 ± 4	24 ± 5
Body mass (kg)	77.6 ± 14.5	71.3 ± 7.4
BMI (kg·m^{-2})	23.5 ± 2.9	22.2 ± 2.1
Diastolic blood pressure (mmHg)	71 ± 14	67 ± 6
Systolic blood Pressure (mmHg)	128 ± 13	121 ± 10
Lean body mass (kg / %)	60.7 / *78.0* ± 12.2 / *2.9*	54.2 / *76.2* ± 5.4 / *5.3*
Fat mass (kg / %)	15.1 / *19.6* ± 3.3 / *3.3*	15.3 / *21.1* ± 4.9 / *5.6*
Appendicular lean mass (kg / %)[1]	28.3 / *36.2* ± 6.3 / *2.0*	25.1 / *35.3* ± 3.2 / *3.2*

Values are mean ± standard deviation. [1]Appendicular lean mass was calculated by the sum of lean mass of both arms and legs. Data were analysed by unpaired Student's t-test, $P<0.05$. No significant differences were observed between groups. BMI: body mass index. n=12 per group

55% energy as carbohydrate, 30% energy as fat, and 15% energy as protein before 08:00 PM, after which they remained fasted.

Dietary intervention

Participants were randomly assigned to consume a 40 g protein in the form of either 230 g of a baked lysine-enriched plant-based meat substitute (Plant; Tereos, Marckolsheim, France) or 175 g of baked chicken breast (Chicken). We selected an existing plant-based meat alternative typically available on the market. As plant-based meat substitutes usually provide more fat and/or carbohydrate relative to the amount of protein when compared with animal-based products, we compared products based on the same amount of protein provided. The lysine-enriched, plant-based protein product was composed of a blend of wheat and chickpea flour (60/40 ratio) and supplemented with 5% free lysine/100 g (L-Lysine monohydrochloride), up to ~200% of the recommended levels of the FAO/WHO, in order to fortify the lysine content in the product that was naturally lacking in lysine and below the recommended intake levels according to the FAO standards. The product was produced by extrusion of the protein blend into small shredded, diced pieces at temperatures <100°C followed by cooking at about 135°C. A staff member not involved in the study generated random assignment of the treatments and participant codes using a computerized list randomiser (www.random.org), participants were sequentially allocated to a treatment according to the random assignment list that was stored in a closed cabinet. Meals were prepared by a staff member not involved in the study and served on an identical white plate and provided with the randomization code, making them blinded to both participants and researchers. Both meals were presented in identical form and appearance (small, diced pieces) and baked for 9 min in 7 g of olive oil (15% extra virgin olive oil, Albert Heijn, the Netherlands) in a frying pan. No additional flavouring was added. Qualitative measurements on palatability were taken directly after consumption of the meals by providing participants with visual analogue scales (VAS), these are presented in the **Supplemental Results**. Macronutrient breakdown and amino acid composition are shown in **Table 8.2** and **8.3**, and the appearance of specific amino acids

following ingestion of Plant and Chicken and methods for determining amino acid content are provided in the **Supplemental Figures.** The amino acid content of both interventions was determined as previously described [15]. In short, approximately 5 mg of freeze-dried Plant or Chicken was hydrolysed in 3 mL 6 M HCl for 12 h at 110 °C. After hydrolysis, HCl was evaporated under nitrogen stream, while heated to 120 °C and the dried amino acids were reconstituted in 5 mL 0.1 M HCl. Amino acids were measured by using ultra-performance liquid chromatograph mass spectrometry (UPLC-MS; ACQUITY UPLC H-Class with QDa; Waters, Saint-Quentin, France).

TABLE 8.2 Macronutrient composition of protein meals

	Per 100 g		*Per serving size*	
	PLANT	CHICKEN	PLANT	CHICKEN
Serving size (g)			230	174
Energy content (kJ)	559	461	1286	802
Protein (g)	17.4	23.0	39.9	39.9
Carbohydrates (g)	11.1	0	18.2	0
Fat (g)	6.5	1.8	10.7	3.1

n=12 per group

Experimental protocol

At 07:30 AM, participants arrived at the laboratory after an overnight fast. A peripheral intravenous catheter was inserted into an antecubital vein for stable isotope amino acid infusion, and a second catheter was inserted into a dorsal hand vein on the contralateral arm for arterialised blood sampling (the hand was placed in a hot box (60°C) for 10 min before sample collection (24)). After taking a baseline blood sample (t = −180 min), the plasma phenylalanine pool was primed with a single dose of L-[ring-$^{13}C_6$]-phenylalanine (2.25 μmol·kg^{-1}) and subsequently a continuous intravenous infusion of L-[ring-$^{13}C_6$]-phenylalanine (0.05 μmol kg^{-1}·min^{-1}) was initiated (t = −180 min) with use of a calibrated IVAC 598 pump. While resting in a supine position, blood samples were taken at t = −90, −60, and −30 min relative to meal ingestion. At t = 0 min, a blood sample and a muscle biopsy sample from the *M. vastus lateralis* of a randomly selected leg were collected to assess post-absorptive muscle protein synthesis. Subsequently, participants received a protein meal corresponding to their randomly assigned treatment [Plant (n = 12) or Chicken (n = 12)]. All subjects ingested a water beverage (and were instructed to consume this consistently throughout their meal) with enriched ~4% L-[ring-$^{13}C_6$]-phenylalanine to minimise dilution of the steady-state plasma L-[ring-$^{13}C_6$]-phenylalanine precursor pool implemented by the constant infusion. Arterialised blood samples were then collected at t = 15, 30, 60, 90, 120, 150, 180, 240, and 300 min. A second and third muscle biopsy sample were collected at t = 120 and t = 300 min to determine postprandial muscle protein synthesis rates from t = 0–120, 120–300, and 0–300 min. Blood samples were collected into EDTA-containing tubes and centrifuged at 1000*g* for 15 min at 4°C. Aliquots of plasma were frozen in liquid nitrogen and stored at −80°C. Muscle

TABLE 8.3 Amino acid composition of raw product

	g per 100 g protein		*g per serving size*	
	PLANT	CHICKEN	PLANT	CHICKEN
Alanine	2.50	4.76	1.00	1.90
Arginine	3.19	4.19	1.27	1.68
Aspartic acid	3.07	7.02	1.23	2.81
Asparagine	ND[1]	ND[1]	ND[1]	ND[1]
Cysteine	0.58	0.14	0.23	0.06
Glutamic Acid	20.28	9.08	8.11	3.63
Glycine	2.80	3.25	1.12	1.30
Histidine	1.52	2.31	0.61	0.93
Isoleucine	2.10	2.33	0.84	0.93
Leucine	5.78	6.08	2.31	2.43
Lysine	8.68	6.54	3.47	2.61
Methionine	0.87	2.27	0.35	0.91
Ornithine	ND[1]	ND[1]	ND[1]	ND[1]
Phenylalanine	4.82	2.94	1.93	1.17
Proline	10.11	2.56	4.04	1.02
Serine	4.84	2.93	1.93	1.17
Threonine	2.27	3.22	0.91	1.29
Tyrosine	1.25	1.20	0.50	0.48
Valine	2.35	2.42	0.94	0.97
TAA	77.02	63.24	30.81	25.30
EAA	28.40	28.11	11.36	11.24
BCAA	10.23	10.82	4.09	4.33
NEAA	48.61	35.13	19.45	14.05

TAA, sum of total amino acids; EAA, essential amino acids; BCAA, branched-chain amino acids; NEAA, non-essential amino acids. [1]Not detectable. *n*=12 per group.

samples were collected with use of a 5 mm Bergström needle custom-adapted for manual suction [25]. Samples were obtained from separate incisions from the middle region of the *M. vastus lateralis*, ~15 cm above the patella and ~3 cm below entry through the fascia, under 1% xylocaine local anaesthesia with adrenaline (1:100.000). Muscle samples were freed from any visible non-muscle material, immediately frozen in liquid nitrogen, and stored at −80°C until further processing.

Plasma and muscle analysis

Details of analysis related to the determination of plasma (glucose, insulin, amino acids, plasma L-[ring-$^{13}C_6$]-phenylalanine, and mixed plasma proteins) as well as muscle data (mixed muscle protein-bound L-[ring-$^{13}C_6$]-phenylalanine enrichments and protein signalling) are presented in the **Supplemental Methods**.

Muscle analysis

A piece of wet muscle (~50-70 mg) was freeze dried for 48 h. Collagen, excessive blood and other non-muscle materials were subsequently removed from the muscle fibers under a light microscope. The isolated muscle fiber mass was weighed and 35 volumes (7x wet weight of isolated muscle fibers x wet-to-dry ratio 5:1) of ice-cold 2% perchloric acid (PCA) was added. Thereafter, the tissue was homogenized by sonification, and centrifuged to separate the supernatant from the protein pellet. The supernatants containing the muscle tissue free amino acids were purified, and derivatized before analysis by GC-MS, similarly as for the plasma L-[*ring* $^{13}C_6$]-phenylalanine enrichments. The protein pellet was washed 3 times with 1 mL 2% PCA. The amino acids were liberated from the mixed muscle enriched protein fraction by adding 2 mL of 6M HCl and heating to 110°C for 15.5 h. The hydrolysed mixed muscle protein fractions were dried under a nitrogen stream while heated to 110°C. The dried mixed muscle protein fraction was dissolved in a 50% acetic acid solution. The amino acids from the mixed muscle protein fraction were passed over a Dowex exchange resin (AG 50W-X8, 100-200 mesh hydrogen form; Bio-Rad, Hercules, CA, USA) using 2M NH4OH. Subsequently, the purified amino acid solution was dried under a nitrogen stream at room temperature, followed by derivatization to their N(O,S)-ethoxycarbonyl-ethylesters. The ratio of $^{13}C/^{12}C$ of mixed muscle protein-bound phenylalanine was determined using gas chromatography-combustion-isotope ratio mass spectrometry (GC-IRMS; Delta V, Thermo Scientific, Bremen, Germany) by monitoring ion masses 44, 45 and 46. Standard regression curves were applied from a series of known standard enrichment values against the measured values to assess the linearity of the mass spectrometer and to account for any isotope fractionation which may have occurred during the analysis.

Calculations

The present study involved the infusion of L-[ring-$^{13}C_6$]-phenylalanine combined with muscle biopsy and arterialised venous blood sampling to determine the fractional synthesis rates (FSR) of mixed muscle proteins in the basal and postprandial state and were calculated by using the standard precursor-product equation:

$$FSR = \left(\frac{(\Delta E_p)}{(E_{precursor} \cdot t)} \right) \cdot 100\%$$

where *ΔEp* is the increment in muscle protein-bound L-[ring-$^{13}C_6$]-phenylalanine enrichment after an incorporation period (in mole percent excess, MPE). $E_{precursor}$ is the weighted average plasma L-[ring-$^{13}C_6$]-phenylalanine enrichment during the tracer incorporation period (MPE) and *t* is the incorporation time (h). Weighted mean plasma enrichments were calculated by taking the measured enrichments between consecutive time points and correcting for the time between these sampling time points. For basal FSR, plasma protein samples at *t* = −180 min and muscle biopsy samples at *t* = 0 min were used; for postprandial FSRs muscle biopsy samples at *t* = 0, 120, and 300 min were used.

Statistical analysis

All data are expressed as mean ± SD. Subjects' characteristics and baseline data (including basal FSR and anabolic signalling) were analysed using unpaired, two-tailed Student's t-tests. The primary outcome of the study was mixed muscle FSR (change from basal to postprandial period), secondary outcomes included plasma glucose, insulin, amino acid concentrations (changes over time, total area under the curve; AUC, and time to peak), and anabolic signalling responses (changes over time). Two-factor repeated measures ANOVA with time as within-subject factor and intervention as between-treatment factor was used to compare differences over time in plasma glucose, insulin, amino acid concentrations and enrichments, anabolic signalling, and FSR (basal to the 0-120 min and 120-300 min postprandial period, and basal to the cumulative 0-300 min postprandial period). In case of significant time x treatment interactions, separate analyses were performed to determine time-effects for each treatment (one-factor repeated measures ANOVA with Bonferroni post-hoc tests to identify time differences) and between-treatment effects for each time-point (two-tailed Student's t-test). Peak values, time to peak, and AUC were calculated for plasma time curves and differences were determined using unpaired, two-tailed Student's t-tests. Based upon previous studies [6], a sample size of 12 subjects per intervention including a 10% dropout rate was calculated, using a 2-sided statistical test (P<0.05, 95% power, effect size 1.8), to detect differences in FSRs between treatments. For all analyses, statistical significance was set at P<0.05. All calculations were performed using SPSS (IBM Statistics, version 25.0, IBM Corp., Armonk, USA).

RESULTS

Plasma glucose and insulin concentrations

Plasma glucose and insulin concentrations are shown in **Figure 8.1.** Following the ingestion of the 40 g protein meal, plasma glucose concentrations (**Figure 8.1A**) increased to a greater extent in Plant when compared to Chicken (*time x treatment* P<0.001). Plasma glucose concentrations reached peak values at 30±0 min in Plant (6.1±0.2 mmol·L^{-1}) and were higher when compared with peak values in Chicken (5.4±0.4 mmol·L^{-1} at 140±59 min; P<0.001). Following protein ingestion, plasma insulin concentrations (**Figure 8.1B**) increased to a greater extent after Plant ingestion when compared with Chicken (*time x treatment* P<0.001). Plasma insulin concentrations in Plant peaked at 38±19 min, reaching concentrations of 205±73 pmol·L^{-1}, and were higher when compared with Chicken, reaching peak values of 111±39 pmol·L^{-1} at 78±37 min (P<0.05). Plasma insulin responses (AUC) were higher at 0-2 h (P=0.003) and 0-5 h (P=0.031) in Plant when compared with Chicken.

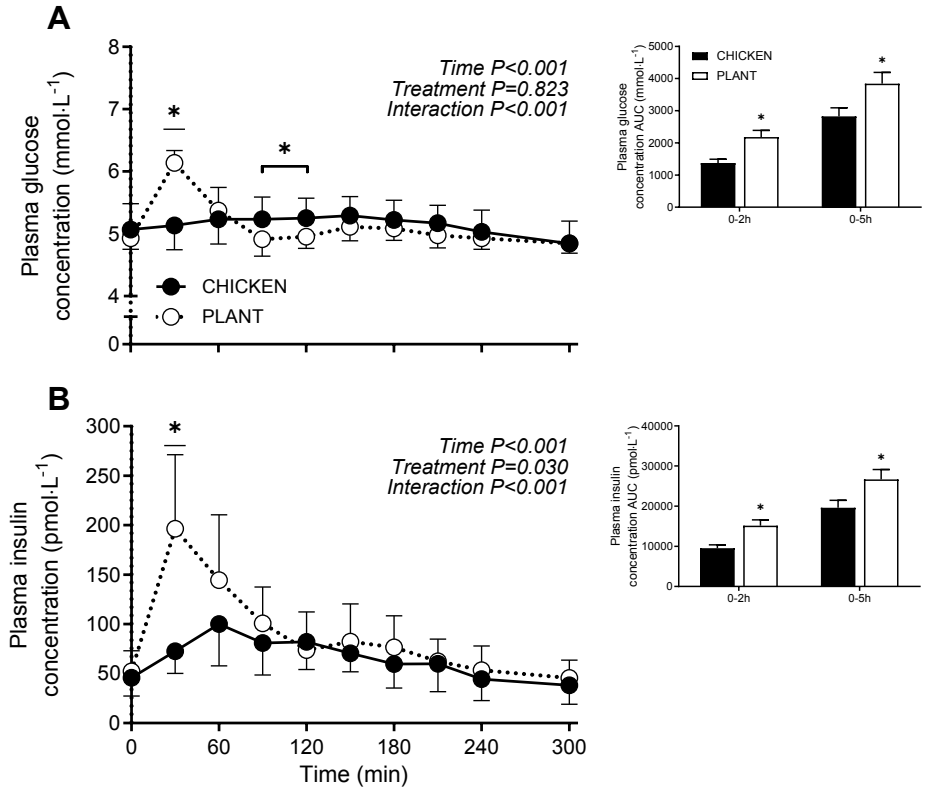

FIGURE 8.1 Plasma glucose (A) and insulin (B) concentrations (mmol·L^{-1} and pmol·L^{-1}, respectively) in 24 healthy, young individuals following the ingestion of 40 g protein of either a lysine-enriched wheat and chickpea protein product (Plant; *n*=12) or chicken breast fillet (Chicken; *n*=12). Values represent means ± standard deviation. Insets represent AUC. Data were analysed by repeated measures (time x treatment) ANOVA. Bonferroni post-hoc test was used to locate differences over time. *Indicates a significant difference between treatments, *P*<0.05.

Plasma AA concentrations

Plasma amino acid concentrations are shown in **Figure 8.2** and **8.3**. Plasma leucine concentrations (**Figure 8.2A**) increased following meal ingestion, but to a greater extent in Chicken when compared with Plant (*time x treatment P*<0.001). Plasma leucine concentrations were higher in Chicken (peak values: 290±28 μmol·L^{-1}) when compared with Plant (peak values: 200±34 μmol·L^{-1}) from *t* = 60-240 min (*P*<0.05). The AUC of plasma leucine concentrations was higher in Chicken in the 0-2 h and 0-5 h postprandial period when compared with Plant (both, *P*<0.001). Plasma lysine concentrations (**Figure 8.2B**) rapidly increased following Plant ingestion (*time x treatment P*<0.001), reaching peak concentrations of 517±77 μmol·L^{-1} and were higher when compared to Chicken (peak values: 324±28 μmol·L^{-1}) throughout the postprandial period from *t* = 15-90 min (*P*<0.001). Plasma methionine concentrations (**Figure 8.2C**) increased to a greater extent following ingestion of Chicken when compared to Plant (*time x treatment P*<0.001) and remained elevated in Chicken from 30–300 min following protein ingestion (*P*<0.05).

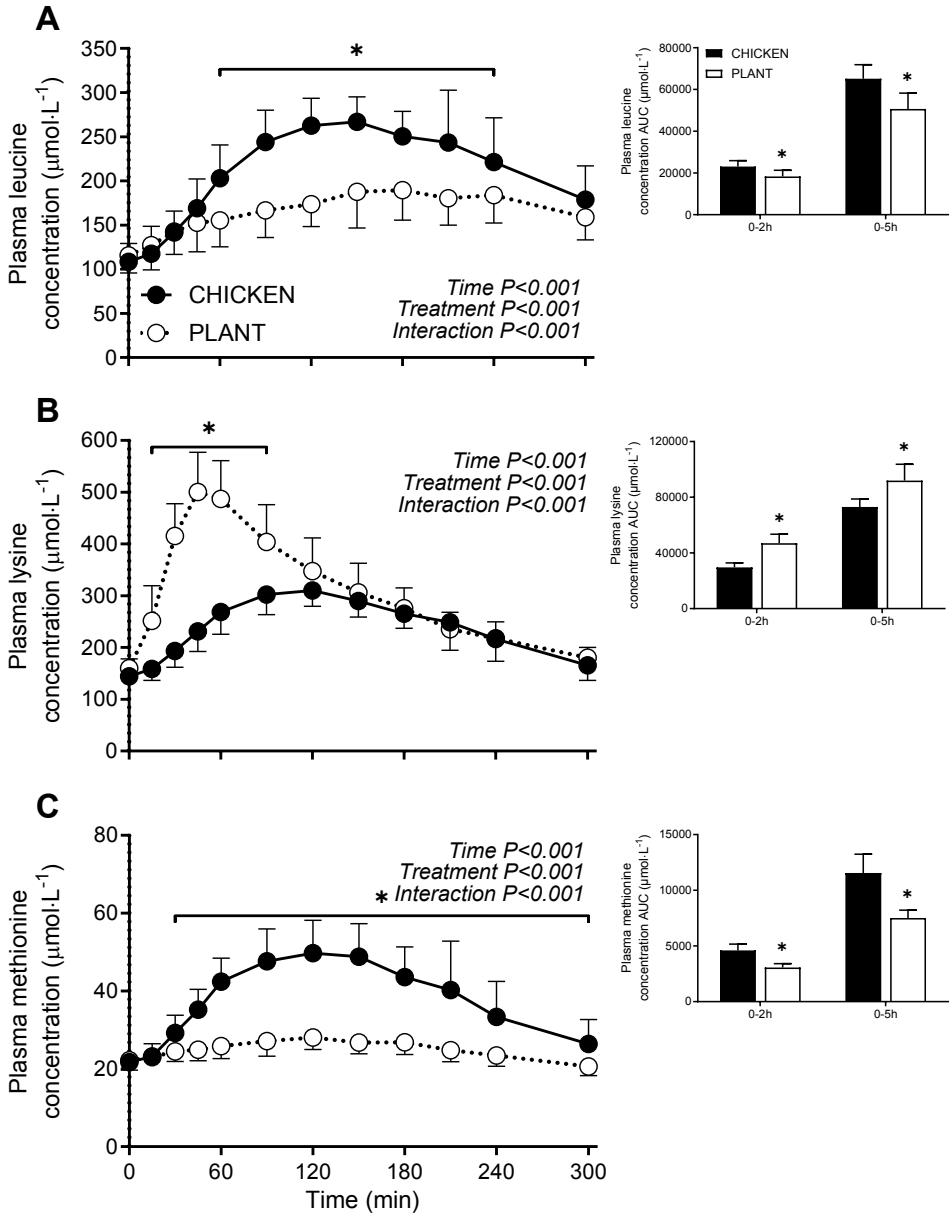

FIGURE 8.2 Plasma leucine (A), lysine (B), and methionine (C) concentrations in 24 healthy, young individuals following the ingestion of 40 g protein of either a lysine-enriched wheat and chickpea protein product (Plant; *n*=12) or chicken breast fillet (Chicken; *n*=12). Values represent means ± standard deviation. Insets represent AUC. Data were analysed by repeated measures (time x treatment) ANOVA. Bonferroni post-hoc test was used to locate differences over time. *Indicates a significant difference between treatments, *P*<0.05.

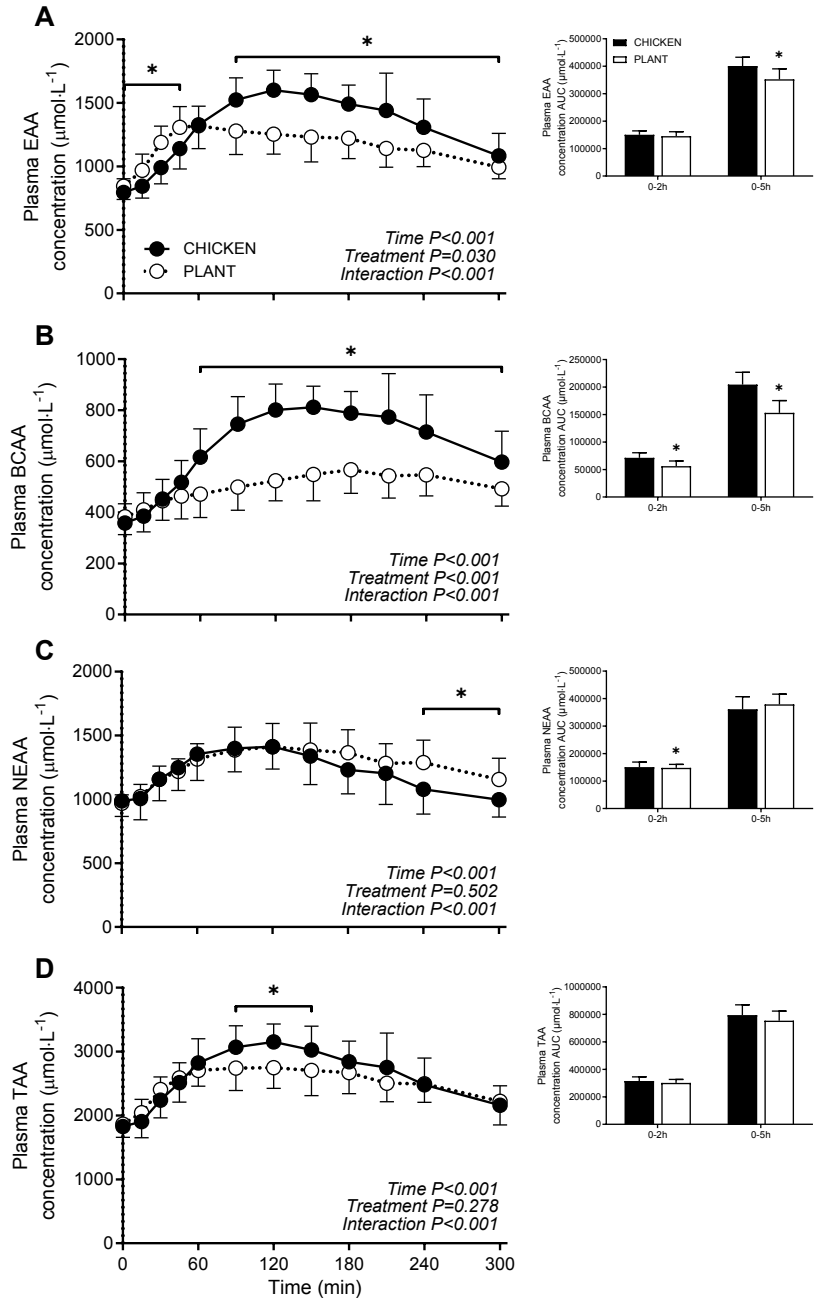

FIGURE 8.3 Sum of plasma essential amino acids (A), branched-chain amino acids (B), non-essential amino acids (C), and the sum of all amino acids (D) in 24 healthy, young individuals following the ingestion of 40 g protein of either a lysine-enriched wheat and chickpea protein product (Plant; *n*=12) or chicken breast fillet (Chicken; *n*=12). Values represent means ± standard deviation. Insets represent AUC. Data were analysed by repeated measures (time x treatment) ANOVA. Bonferroni post-hoc test was used to locate differences over time. *Indicates a significant difference between treatments, *P*<0.05. EAA: essential amino acids, BCAA: branched-chain amino acids, NEAA: non-essential amino acids, TAA: sum of all amino acids.

The sum of specific subgroups of plasma amino acids are shown in **Figure 8.3**. Plasma EAA (**Figure 8.3A**) increased following protein ingestion in both interventions (*time x treatment* $P<0.001$; time effect, both $P<0.001$), and were higher following Plant when compared with Chicken from t = 0-45 min though conversely from t = 90-240 min ($P<0.05$). The AUC of EAAs did not differ during the 0-2 h postprandial period ($P=0.438$) but was lower in Plant when compared with Chicken over the 5 h postprandial period ($P<0.01$). Plasma branched chain amino acids (BCAA; **Figure 8.3B**) were higher in Chicken when compared with Plant from 60 min after protein meal ingestion and throughout the remainder of the postprandial period (*time x treatment* $P<0.001$, post-hoc all, $P<0.05$). The AUC of the BCAAs was higher in Chicken when compared with Plant in both the 0-2 h and 0-5 h phase ($P<0.05$). Plasma non-essential amino acids (NEAA; **Figure 8.3C**) differed between treatments (*time x treatment* $P<0.05$) and were higher in Plant when compared with Chicken from t = 240-300 min following meal ingestion (all, $P<0.05$). The sum of all amino acids (TAA; **Figure 8.3D**) differed between interventions (*time x treatment* $P<0.05$) and were higher in Chicken when compared with Plant from t = 90-150 min following meal ingestion (all, $P<0.05$). The AUC of NEAA and TAA did not differ between interventions (both, $P>0.05$). Individual plasma amino acid concentrations of alanine, arginine, asparagine, aspartic acid, beta-alanine, cystine, glutamic acid, glycine, histidine, isoleucine, ornithine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine are presented in **Supplemental Figure 8.2** and **8.3**.

Muscle protein synthesis rates

Prior to ingestion of the meal, plasma L-[ring-$^{13}C_6$]-phenylalanine weighted mean enrichments averaged 6.6±0.6 MPE in Chicken and 6.7±0.4 MPE in Plant with no differences between interventions ($P=0.940$; **Supplemental Figure 8.4**). Plasma L-[ring-$^{13}C_6$]-phenylalanine enrichments increased directly following the ingestion of the protein meal (main time effect, $P<0.001$), but returned rapidly to baseline steady state levels. Postprandial plasma L-[ring-$^{13}C_6$]-phenylalanine weighted means averaged 6.5±0.7 MPE in Chicken and 6.6±0.4 in Plant, with no differences between interventions (*time x treatment* $P=0.323$).

Mixed muscle protein synthesis rates are shown in **Figure 8.4**. Basal muscle protein synthesis rates did not differ between interventions (Chicken: 0.031±0.013 %·h^{-1} and Plant: 0.031±0.011 %·h^{-1}, $P=0.884$). Muscle protein synthesis rates increased from the basal to the 0-5 h postprandial period (0.056±0.015 %·h^{-1} in Chicken and 0.046±0.010 %·h^{-1} in Plant; main time effect, $P<0.001$; **Figure 8.4A**) but did not differ between treatments (*time x treatment* $P=0.068$, main treatment effect, $P=0.369$). Similarly, postprandial FSR in the early, 0-2 h (0.057±0.021 %·h^{-1} in Chicken and 0.048±0.016 %·h^{-1} in Plant) and late, 2-5 h postprandial period (0.052±0.023 %·h^{-1} in Chicken and 0.044±0.027 %·h^{-1} in Plant) increased when compared with basal rates (main time effect, $P<0.001$), with no differences between treatments (*time x treatment* $P=0.562$, main treatment effect $P=0.261$; **Figure 8.4B**).

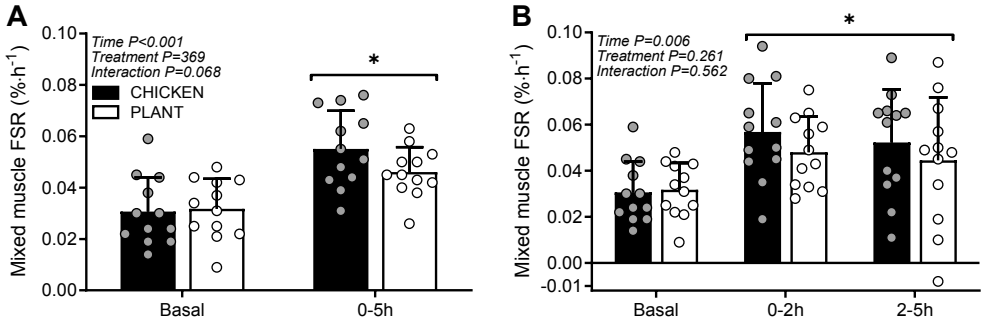

FIGURE 8.4 Mixed muscle fractional synthetic rates (%·h^{-1}) during the basal and 0-5h postprandial period (A) and the early (0-2h) and late (2-5h) postprandial response (B), using intravenous L-[ring-$^{13}C_6$]-phenylalanine infusions in 24 healthy, young males following the ingestion of 40 g protein of either a lysine-enriched wheat and chickpea protein product (Plant; *n*=12) or chicken breast fillet (Chicken; *n*=12). Bars are means and dots represent individual values. Data were analysed with unpaired Student's t-test (between treatments) and repeated measures (time x treatment) ANOVA. FSR, fractional synthesis rates.

Muslce protein signaling

Key anabolic muscle signalling proteins are shown in Figure 8.5. No differences over time or between groups were observed in phosphorylation status of mammalian target of rapamycin (mTOR^{Ser2448}), p70 ribosomal protein S6 kinase (p70S6k^{Thr389}), ribosomal protein S6 (rS6$^{Ser235/236}$), and eukaryotic initiation factor 4E binding protein-1 (4E-BP1$^{Thr37/46}$) at 2 and 5h after protein ingestion (*P*>0.05).

DISCUSSION

In the present study, we compared the muscle protein synthetic response following the ingestion of 40 g protein in the form of a lysine-enriched wheat and chickpea protein-based product with the ingestion of an isonitrogenous amount of chicken in healthy, young men. The ingestion of both an ample amount of the plant-based protein product as well as an isonitrogenous amount of chicken strongly increased postprandial muscle protein synthesis rates when compared to post-absorptive muscle protein synthesis rates, with no differences observed between the protein sources.

The interest in plant-based products as alternative protein sources is increasing worldwide due to their proposed contribution to better health and greater sustainability [13]. However, it is generally reported that plant-based proteins have lesser anabolic properties when compared to animal-based proteins. This has been attributed to the lower EAA contents (leucine in particular) and deficiencies in specific amino acids (lysine and methionine) in various plant-based proteins. The combination of different plant-based protein sources and the fortification with deficient free amino acids have been suggested as effective strategies to increase the anabolic properties of plant-based protein sources. Such plant-based protein food products, aiming to replace meat or poultry, are becoming increasingly

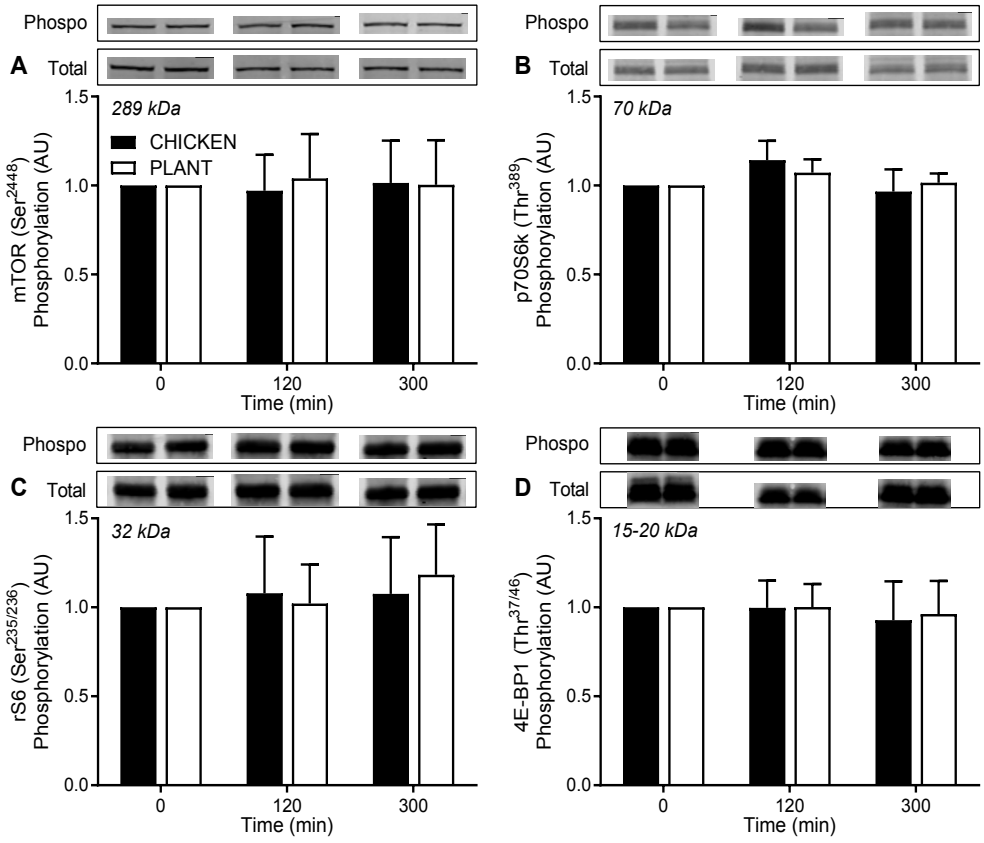

FIGURE 8.5 Muscle protein expression (ratio between phosphorylated/total protein content) of mammalian target of rapamycin (mTORSer2448; **A**), phosphorylation of p70 ribosomal protein S6 kinase ($p70S6k^{Thr389}$; **B**), ribosomal protein S6 ($rS6^{Ser235/236}$; **C**), and eukaryotic initiation factor 4E binding protein-1 ($4E\text{-}BP1^{Thr37/46}$; **D**) in 24 healthy, young individuals in the post-absorptive state and following the ingestion of 40 g protein of either a lysine-enriched wheat and chickpea protein product (Plant; *n*=12) or chicken breast fillet (Chicken; *n*=12) with representative blots for phosphorylated and total protein expression of each protein (inset). Values represent means ± standard deviation. Data were analysed with unpaired Student's t-test (between treatments) and repeated measures (time x treatment) ANOVA. No significant main effects were detected. AU, arbitrary units.

popular. However, their capacity to stimulate postprandial muscle protein synthesis has never been investigated. Therefore, in the present study, we assessed postprandial muscle protein synthesis rates following ingestion of an ample, 40 g amount of protein, provided in the form of 230 g baked meat substitute (composed of a lysine-enriched blend of wheat and chickpea protein) or 175 g of baked chicken breast fillet. The products were matched for the amount of protein ingested and, as such, differed in carbohydrate, fat, and total energy content (Plant: 559 kJ and Chicken: 461 kJ per serving). To our knowledge, this is the first study to compare postprandial protein handling following ingestion of a whole-food plant-based protein product with an equivalent amount of protein derived from animal-based origin.

Following protein ingestion, greater increases in plasma glucose and insulin concentrations were observed following consumption of the plant-based product when compared to an isonitrogenous amount of chicken (**Figure 8.1**). Plasma EAA and BCAA concentrations increased to a greater extent following ingestion of the 40 g protein as chicken (**Figure 8.3**), with higher postprandial plasma leucine and methionine concentrations when compared with the ingestion of the plant-based protein source (**Figure 8.2**). To compensate for any potential limiting effect of the low lysine and EAA content of wheat and chickpea protein [15], the plant-based protein product was fortified with free lysine at ~200% of the recommended levels of the FAO/WHO ((18); **Table 8.3**) and as such suitable for consumers as a meat substitute. As a result, postprandial plasma lysine concentrations further increased following the ingestion of Plant when compared with Chicken. No differences between interventions were observed in postprandial NEAA concentrations or the sum of all amino acids when assessed over the entire 5-h postprandial period (**Figure 8.3**). As such, despite that the products were protein-matched and had similar EAA contents (~28 g/100 g, **Table 8.2**), the higher energy content and a greater amount of fat and carbohydrate of the plant-based protein product likely attenuated protein digestion and amino acid absorption and, as such, contributed to the attenuated postprandial rise in plasma amino acid availability when compared with the ingestion of the isonitrogenous amount of baked chicken [10, 26-29].

The few studies that assessed the muscle protein synthetic response following plant-based protein ingestion have shown lower postprandial muscle protein synthesis rates when compared to animal-based proteins [6, 7, 17, 18]. The lesser anabolic properties of plant-based protein isolates (such as wheat) compared with high-quality animal-based protein sources (such as milk) may be compensated for by ingesting more protein [6]. However, strongly increasing the protein dose may not always be a feasible and practical strategy to increase the anabolic properties of a plant-based protein meal, since this would further increase both the volume as well as the energy content of a meal. In the present study, ingestion of 40 g protein in the form of the plant-based protein product increased muscle protein synthesis rates by ~68% when compared with post-absorptive muscle protein synthesis rates (**Figure 8.4A**; $P<0.001$). Clearly, a measurable increase in muscle protein synthesis rates can be observed following the consumption of an ample amount of a plant protein-based meat substitute. The selected plant-based meat alternative was produced using a blend of wheat and chickpea protein isolates and further fortified with lysine to achieve levels recommended by the FAO/WHO ((18); **Table 8.3**). Whether the lysine fortification was required to support the postprandial increase in muscle protein synthesis cannot be derived from this study design. More work will be needed to assess whether plant-derived protein blends with or without (free) amino acid fortification are required to allow significant increases in muscle protein synthesis rates following ingestion of more moderate amounts and different compositions of plant-based protein products as well as more complete, mixed meals.

To allow a comparison of the postprandial muscle protein synthetic response to the ingestion of a plant-based meat substitute with a high-quality animal-based protein source, we included a control trial in which we provided young individuals with an isonitrogenous amount of

chicken. Despite the greater postprandial rise in plasma EAA, and leucine in particular, following the ingestion of chicken, we observed no significant differences in postprandial muscle protein synthesis rates assessed over the 5 h period between the interventions (**Figure 8.4A**; *time x treatment P*=0.068, main treatment effect *P*=0.369). Though we did observe a trend of a greater overall response in muscle protein synthesis rates in the Chicken treatment, this trend was no longer present when we assessed the postprandial muscle protein synthetic response in the early (0-2 h) and late (2-5 h) postprandial phase (**Figure 8.4B**; *time x treatment P*=0.237 and 0.394, respectively). In line, we did not detect any substantial differences in myocellular anabolic signalling following ingestion of the plant-based protein product and chicken (*P*>0.05, **Figure 8.5**). No detectable rise in phosphorylation status of mTOR, p70S6k, rS6, and 4E-BP1 were observed 2 and 5 h after protein ingestion in either treatment. These findings may seem inconsistent to the observed substantial postprandial rise in muscle protein synthesis rates. However, it should be noted that signalling responses merely provide snapshot measurements in time and do not necessarily serve as a proxy for the rise in muscle protein synthesis rates. It is likely that (transient) differences in anabolic signalling occurred prior to the biopsy collection at 2 h following protein ingestion. Nevertheless, these data clearly show that the ingestion of an ample amount of plant-based meat substitute has the capacity to stimulate muscle protein synthesis to an extent similar to the ingestion of an equivalent amount of animal-based protein source. It is evident that more work will be required to define the factors, such as the amount of protein consumed, that may contribute to the presence or absence of differences in the postprandial muscle protein synthetic response to the ingestion of plant versus animal-based protein foods. Relevant factors will likely include the dose [1, 3, 6, 30], protein source [2, 5-7, 18], matrix of the food [9-11], food processing [31, 32], and preparation of the foods [33-35], as well as the population consuming these products [36].

There is a growing popularity and accessibility of plant-based protein sources and the consumption of plant-based proteins has increased with campaigns, such as 'Meatless Mondays' and 'flexitarianism' that are advocating a more plant-based diet [37-39]. As a response, the industry has been investing in the development and production of a growing range of plant-based protein food products [13, 40]. Previous studies that assessed muscle protein synthesis rates following the ingestion of plant-based protein sources have generally been limited to the ingestion of protein isolates derived from soy [5, 7, 17, 18], wheat [6], or blends of casein, whey, and soy [21-23] in the form of a liquid protein drink. Moreover, commercially available plant-based protein products are naturally higher in carbohydrate and fat (and consequently energy content) when compared to animal-based protein sources and, therefore, might be less effective in their capacity to stimulate muscle protein synthesis. To date, studies assessing postprandial muscle protein synthesis rates following protein containing whole-food products or meals are lacking. We [41, 42] and others [43, 44] have defined several dietary factors that can modulate protein digestion and amino acid absorption and the subsequent muscle protein synthetic response to protein ingestion. Such factors include the amount and type of protein, macro- and micronutrient composition of the meal, food density and meal composition, food texture, food matrix, food processing, food preparation and temperature (i.e. heating or cooling), and mastication. While most of these

modifications seem to affect protein digestion and amino acid absorption kinetics [10, 28, 33], their impact on postprandial muscle protein synthesis rates remain to be resolved [10, 11, 28, 33]. We matched the meals for protein content and provided an ample amount that would be typically ingested during dinner. Here we show that when an isonitrogenous amount of plant-based protein is consumed and the deficit of one specific amino acid is replaced, a plant-based meat replacement can be as effective as an animal-based protein source to stimulate muscle protein synthesis in healthy, young adults. Though the long-term effect of plant-based protein consumption on protein metabolism needs to be further explored, plant-based meat substitutes may be applied in a regular diet without compromising the capacity to support muscle mass maintenance in young individuals. Whether the fortification of plant-based meat substitutes with other specific amino acids is required to induce a proper anabolic response and how different plant-based protein blends can be combined to improve the amino acid profile and maximize the anabolic properties of plant-based meat replacers remains questions to be addressed in further studies.

In conclusion, the ingestion of an ample amount of lysine-enriched plant-based protein product increases muscle protein synthesis rates in healthy, young men. The muscle protein synthetic response to the ingestion of an ample amount of protein (i.e. 40 g) of such a lysine-enriched plant-derived protein blend does not differ from the ingestion of an isonitrogenous amount of chicken. Plant-based protein products sold as meat replacers may be as effective as animal-based protein sources to stimulate postprandial muscle protein synthesis rates in healthy, young individuals.

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Conflicts of Interest

LJCvL has received research grants, consulting fees, speaking honoraria, or a combination of these, from Friesland Campina, Tereos, and Nutricia Research. IWKK and LBV have received speaking honoraria from Nutricia Research. None of the other authors have disclosed any conflicts of interest.

Authorship

IWKK, CLB, LCPGMdG, LBV, and LJCvL formulated the research question and designed the study. IWKK, PJMP, and TS conducted the experimental trials. JvK and AHZ performed the blood and muscle analyses. IWKK performed the (statistical) data analysis, data interpretation, and wrote the manuscript together with LBV and LJCvL. IWKK and LJCvL had primary responsibility for final content. All authors read and approved the final content of the manuscript.

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SUPPLEMENTAL METHODS

Dietary intervention

Meals were prepared by a staff member that was not involved in the study and were served on a white plate provided with the randomization code, making them blinded to both participants and researchers. The appearance of the 2 products was similar and presented in an identical form (small, diced pieces), see pictures below. In addition, we instructed the participants not to comment on the smell and taste and not to make any speculations of what they were consuming.

PLANT

230 g meat substitute
providing 40 g protein

CHICKEN

174 g chicken breast filet
providing 40 g protein

The amino acid content of the raw products were determined by the Dumas combustion method was used to determine nitrogen content using the Vario MAX cube CN (Elementar Analysensysteme, Germany). Protein content was calculated by multiplying the determined nitrogen content by 6.25 as the standard nitrogen-to-protein conversion factor. Amino acid profile of the products was determined by acid hydrolysis in triplicate of the raw products (~500 mg wet weight). Specifically, the amino acids were liberated by adding 2 mL of 6M HCl and heating to 110°C for 12 h. The hydrolysed free amino acids were subsequently dried under a nitrogen stream while heated to 120°C. Before analysis by ultraperformance liquid chromatography-mass spectrometry (UPLC-MS; ACQUITY UPLC H-Class with QDa; Waters, Saint-Quentin, France), the hydrolysate was dissolved in 5 mL of 0.1 M HCl and 20 µL of AccQ/Tag derivatizing reagent solution (Waters, Saint-Quentin, France) was a for the plasma amino acid concentration analysis.

Plasma analysis

Plasma glucose and insulin concentrations were analysed using commercially available kits (ref. no. A11A01667; glucose HK CP, ABX Diagnostics, Montpellier, France, and Human Insulin specific RIA, ref. no. HI-14K, Millipore, Billerica, USA, respectively).

Plasma amino acid concentrations were measured by using ultra-performance liquid chromatograph mass spectrometry (UPLC-MS, ACQUITY UPLC H-Class with QDa; Waters, Saint-Quentin, France). 50 µL of blood plasma was deproteinized using 100 µL of 10 % SSA with 50 µM of MSK-A2 internal standard (Cambridge Isotope Laboratories, Massachusetts, USA). Subsequently, 50 µL of ultra-pure demineralized water was added and samples were centrifuged (15 min at 14000 rpm). After centrifugation, 10 µL of supernatant was added to 70 µL of Borate reaction buffer (Waters, Saint-Quentin, France). In addition, 20 µL of AccQ-Tag derivatizing reagent solution (Waters, Saint-Quentin, France) was added after which the solution was heated to 55 °C for 10 min. An aliquot of 1 µL was injected and measured using UPLC-MS. The amino acids analysed were alanine, arginine, asparagine, aspartic acid, beta-alanine, cystine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, ornithine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine.

For plasma L-[ring-$^{13}C_6$]-phenylalanine enrichment measurements, plasma phenylalanine was derivatized to the tert-butyldimethylsilyl (TBDMS) derivative with N-tert-butyldimethylsilyl-N-methyltrifluoroacetamide (MTBSTFA), and the ^{13}C enrichments were determined by electron ionization gas chromatography-mass spectrometry (GC-MS; Agilent 7890A GC/5975C MSD; Agilent Technologies) using selected ion monitoring of masses 336 and 342 for unlabelled and labelled (ring-$^{13}C_6$) phenylalanine, respectively. Standard regression curves were applied from a series of known standard enrichment values against the measured values to assess the linearity of the mass spectrometer and to account for any isotope fraction which may have occurred during the analysis. Phenylalanine enrichments were corrected for the natural level of ^{13}C isotopes.

Mixed plasma proteins from blood samples at *t*=-180 min were used to determine baseline L-[ring-$^{13}C_6$]-phenylalanine enrichments to allow calculation of basal muscle protein synthesis rates using the single biopsy approach [1]. Mixed plasma proteins were isolated from blood by using perchloric acid (PCA) to a final concentration of 2 %. Samples were centrifuged at 1000 *g* at 4 °C for 10 min, and the supernatants were removed. The mixed plasma protein pellet was washed 3 times with 2 % PCA and dried. Amino acids were liberated by adding 6 M HCl and were heated at 120 °C for 15–18 h. Thereafter, the enrichments in hydrolysed mixed plasma protein samples were assessed using the same procedures as the muscle protein-bound samples.

Muscle analysis

Mixed muscle protein L-[ring-$^{13}C_6$]-phenylalanine enrichments were extracted from ~50 mg of wet muscle tissue. After the muscle was freeze-dried, collagen, blood, and other non-muscle fibres were removed from the muscle fibres under a light microscope. The isolated muscle fibre mass was weighed and 35 volumes (7x wet weight of isolated muscle fibres x wet-to-dry ratio 5:1) of ice-cold 2% PCA was added and the sample was homogenized and centrifuged. The tissue protein pellet was washed three times with 1.5 mL of ice-cold 2% PCA and hydrolysed in 3 mL of 6 M HCl overnight at 120°C. The free amino acids were then dissolved in 50% acetic acid solution and passed over cation exchange AG 50W-X8 resin

[mesh size: 100–200, ionic form: hydrogen (Bio-Rad Laboratories)] columns. The amino acids were eluted with 2 M NH_4OH for measurement of L-[ring-$^{13}C_6$]-phenylalanine enrichment in tissue protein. To determine the L-[ring-$^{13}C_6$]-phenylalanine enrichment of tissue protein, the purified amino acids were derivatized into their *N*(O,S)-ethoxycarbonyl ethyl ester derivatives with ethyl chloroformate (ECF). The derivatives were then measured by GC-C-IRMS (Thermo Fisher Scientific Delta V, Bremen, Germany) using a DB-17MS-column (30 m x 0.25 mm x 0.5 µm; Agilent J+W scientific GC column, Santa Clara, CA, USA) and monitoring of ion masses 44, 45, and 46. Standard regression curves were applied to assess the linearity of the mass spectrum and to account for isotopic fractionation.

Western blot analysis was performed on muscle tissue samples at t = 0, 120, and 300 min. A portion (~20 mg) of each muscle sample frozen for biochemical analyses was homogenized in 7 volumes Tris buffer (20 mM Tris-HCL, 5 mM EDTA, 10 mM Na-pyrosphospate, 100 mM NaF, 2 mM Na3VO4, 1% Nonident P-40; pH 7.4) supplemented with protease and phosphatase inhibitors: aprotinin 10 µg·mL^{-1}, leupeptin 10 µg·mL^{-1}, benzamidin 3 mM and PMSF 1 mM. After homogenization, each muscle extract was centrifuged for 10 min at 10,000 g (4°C) and sample buffer (final concentration: 60 mM Tris, 5% glycerol, 20 mg·mL^{-1} SDS, 0.1mM DTT, 20 µg·mL^{-1} bromophenol blue) was added to the supernatant. The supernatant was extracted and boiled for 5 min at 100 °C and put on ice after sample buffer was added to the sample. Immediately before analyses, the muscle extraction sample was warmed to 50 °C and centrifuged for 1 min at 1,000 g (room temperature, RT). The total amount of sample loaded on the gel was based on a Bradford protein assay performed and checked on gel after a Coomassie staining. With the exception of mTOR, protein samples were run on a Criterion gel 4-20% (Biorad Order No. 567-1094) for 90 min at 150 V (constant voltage) and transferred onto a Trans-blot Turbo 0.2 µm nitrocellulose membrane (Biorad Order No. 170-4159) in 7 min at 2.5 A and 25 V. mTOR samples were run on a Criterion Tris acetate gel (Biorad Order No. 345-0130) and transferred onto a Trans-blot Turbo 0.2 µm nitrocellulose membrane (Biorad Order No. 170-4159) in 10 min at 2.5 A and 25 V. Specific proteins were detected by overnight incubation at 4°C on a shaker with specific antibodies in 50% Odyssey blocking buffer (Part No. 927-40000, Li-Cor Biosciences, Lincoln, USA) in PBS, after blocking for 60 min at RT in 50% Odyssey blocking buffer in PBS. Polyclonal primary phospho-specific antibodies were purchased from Cell Signaling Technology (Danvers, USA) and included anti-total mammalian target of rapamycin (mTOR; no. 2972S), anti-phosphorylated mTOR Ser2448 (no. 2971L), anti-p70 ribosomal protein S6 kinase (p70S6K; no. 9202L) and anti-phosphorylated p70S6K Thr^{389} (no. 9205L), anti-total S6 ribosomal (no. 2217L), anti-phosphorylated S6 ribosomal Ser$^{235/236}$ (no. 4856S), anti-total 4E-BP1 (no. 9452L) and anti-phosphorylated 4E-BP1 Thr$^{37/46}$ (no. 9459L). Following incubation, membranes were washed 3 times 10 min in 0.1% PBS Tween 20 and once for 10 min in PBS. Next, samples were incubated (1 h at RT) with infrared secondary antibodies, donkey anti-rabbit IRDYE 680 (Li-Cor, Cat. No. 926-32223, dilution 1:50000) and donkey anti-mouse IRDYE 800CW (Li-Cor, Cat. No. 926-32212, dilution 1:10000) dissolved in 50% PBS Odyssey blocking buffer. After a final wash step (3 x 10 min) in 0.1% Tween 20-PBS and once 10 min in PBS, protein quantification was performed by scanning on an Odyssey Infrared Imaging System (LI-COR Biotechnology, Lincoln, NE).

Ponceau S staining after blotting was used to standardize for the amount of protein loaded. Phosphorylation of mTOR, p70S6K, ribosomal S6, and 4E-BP1 were expressed relative to the total amount of each protein (arbitrary units; AU).

SUPPLEMENTAL RESULTS

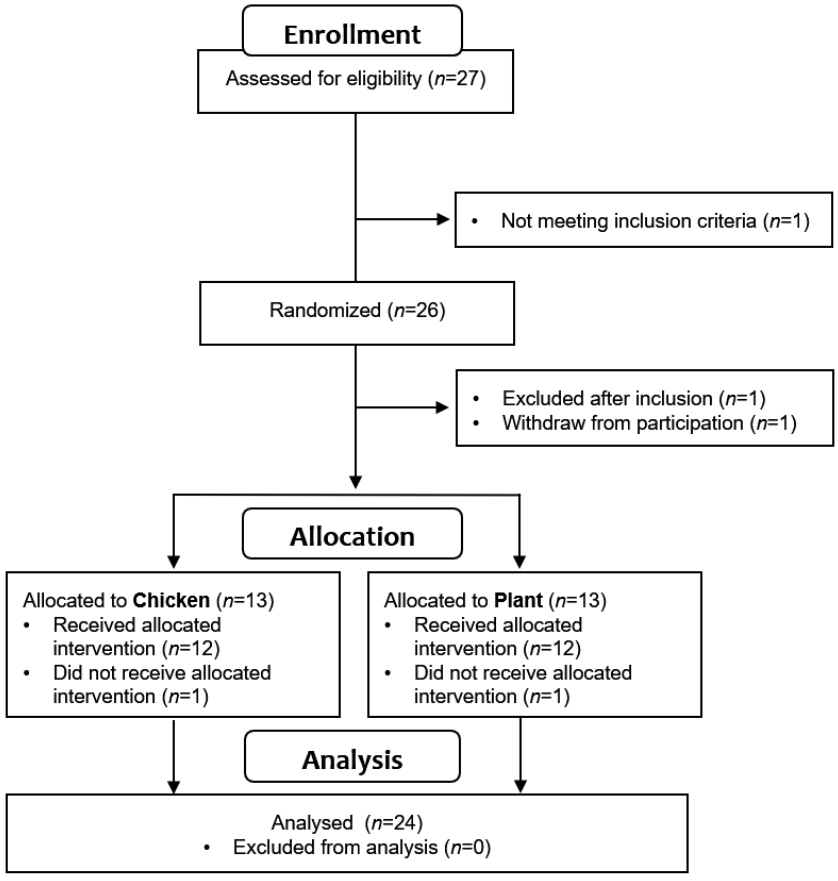
Qualitative measurements on palatability were taken directly after consumption of the meals by providing participants with visual analogue scales (VAS), VAS scores are presented in **Supplemental Table 8.1**. Questions on 'general taste experience', 'taste sensation in the mouth', 'taste addiction', and 'texture experience' did not differ between treatment groups ($P>0.05$), while questions as 'I have eaten food that has a similar taste', 'I have eaten foods like this before', 'I am satisfied with the taste', and 'Would you use the product more regular?' were lower in Plant when compared with Chicken ($P<0.05$).

SUPPLEMENTAL TABLE 8.1 VAS scores on palatability questions

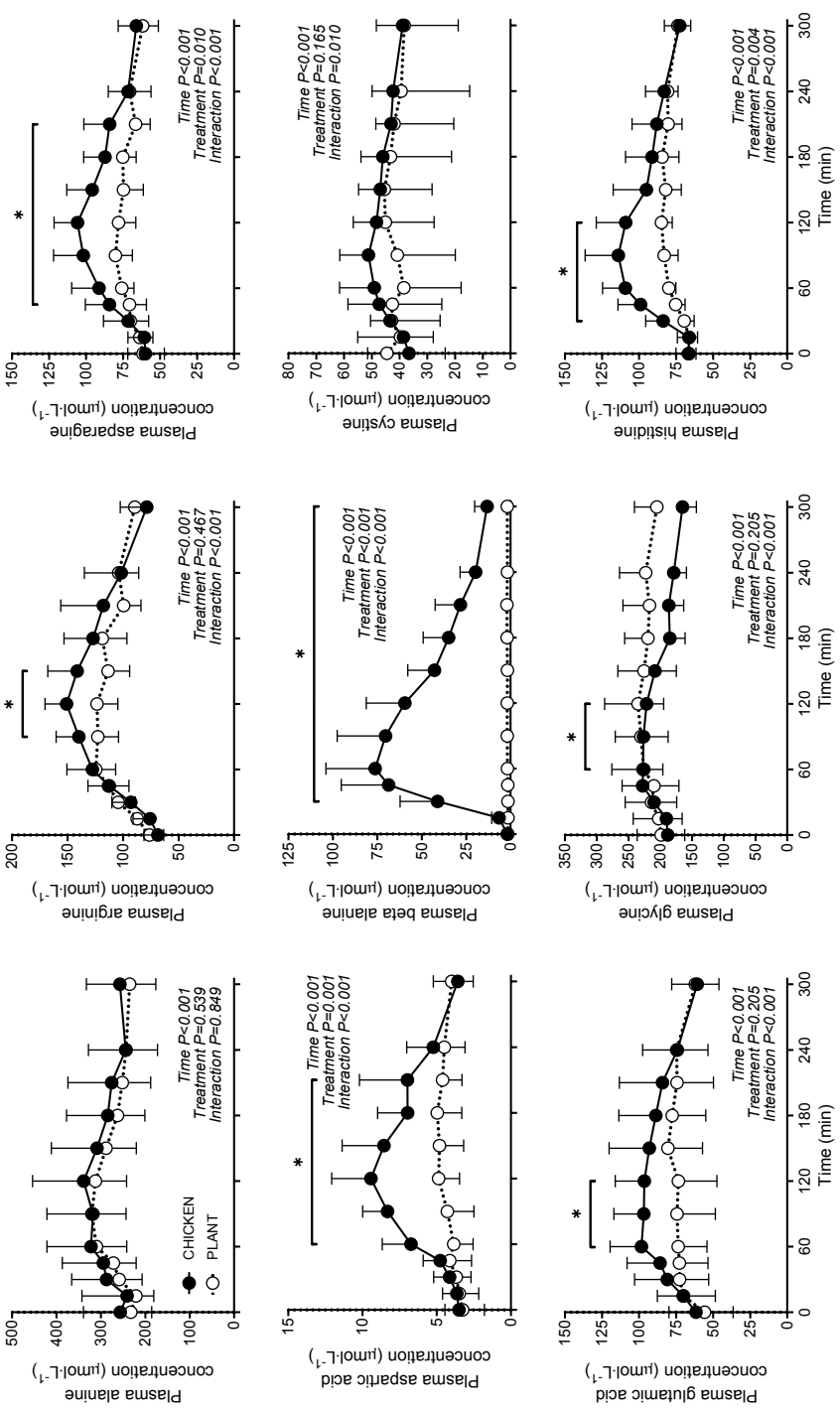
	PLANT	CHICKEN
General taste experience	43.3 ± 21.0	58.8 ± 19.0
General texture experience	48.7 ± 16.7	60.6 ± 18.3
Is the taste addictive?	21.3 ± 15.9	30.3 ± 18.8
Would you have another bite of this meal?	41.0 ± 22.7	50.1 ± 23.0
Are you satisfied with the taste?	46.4 ± 20.1	67.2 ± 23.3*
How would you rate the taste in your mouth?	47.3 ± 15.1	49.7 ± 15.4
Have you ever eaten foods with a similar taste?	38.0 ± 20.7	63.2 ± 20.1*
Have you ever eaten foods like this?	37.9 ± 28.5	64.8 ± 23.5*
How likely would your family or friends like this meal?	37.9 ± 19.5	60.3 ± 25.2*
Would you use this product more often?	39.1 ± 23.5	58.1 ± 19.9*
How likely would you buy this product?	37.1 ± 24.7	55.0 ± 19.9

VAS: visual analogue scale. Values are mean ± SD. Data were analysed by unpaired Student's t-test. *Indicates a significant difference between treatments, $P<0.05$. $n=12$ per group

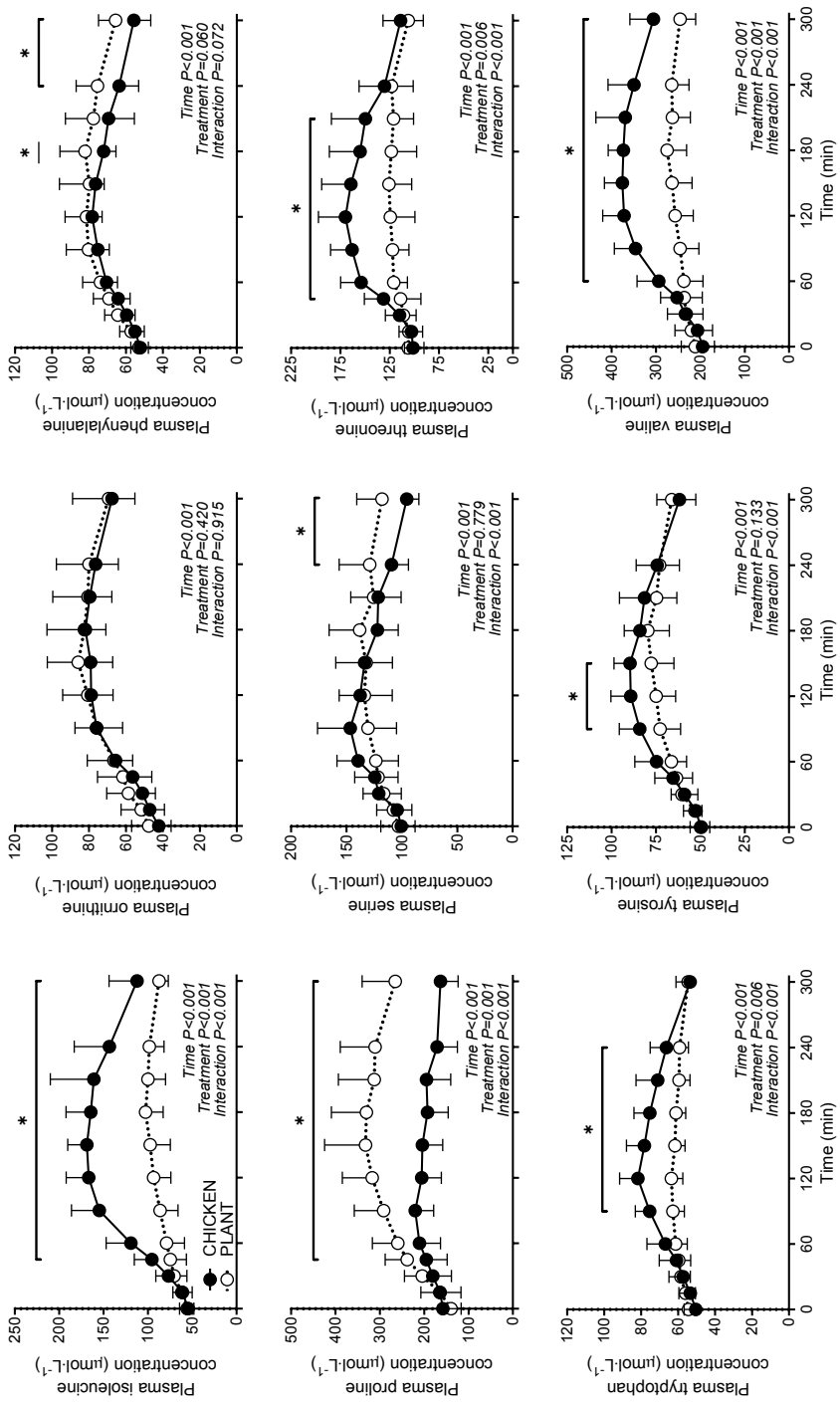
SUPPLEMENTAL FIGURES



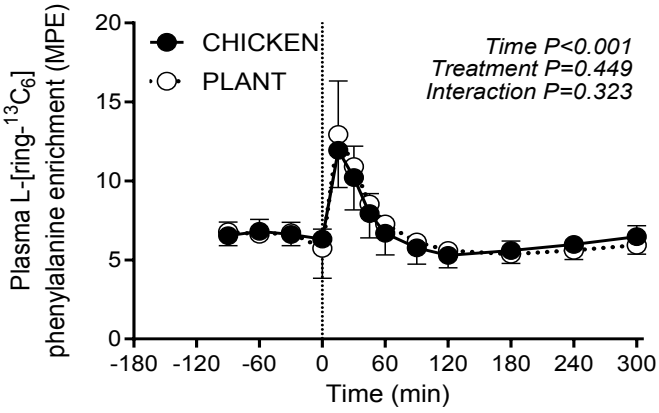
SUPPLEMENTAL FIGURE 8.1 Flow diagram of participants' enrollment procedure according to the CONSORT (Consolidated Standards of Reporting Trials). Plant: 40 g protein in the form of a baked lysine-enriched wheat and chickpea protein product, Chicken: 40 g protein in the form of baked chicken breast filet.



SUPPLEMENTAL FIGURE 8.2 Mean (±SD) plasma alanine, arginine, asparagine, aspartic acid, beta-alanine, cystine, glutamic acid, glycine and histidine, concentrations in 24 healthy, young males following the ingestion of 40 g protein of either a lysine-enriched wheat and chickpea protein product (Plant; *n*=12) or chicken breast filet (Chicken; *n*=12). Data were analysed by repeated measures (time x treatment) ANOVA. Bonferroni post-hoc test was used to locate differences over time. *Indicates a significant difference between treatments, *P*<0.05.



SUPPLE SUPPLEMENTAL FIGURE 8.3 Mean (±SD) plasma isoleucine, ornithine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine concentrations in 24 healthy, young males following the ingestion of 40 g protein of either a lysine-enriched wheat and chickpea protein product (Plant; *n*=12) or chicken breast filet (Chicken; *n*=12). Data were analysed by repeated measures (time x treatment) ANOVA. Bonferroni post-hoc test was used to locate differences over time. *Indicates a significant difference between treatments, *P*<0.05.



SUPPLEMENTAL FIGURE 8.4 Mean (±SD) plasma L-[ring-$^{13}C_6$]-phenylalanine enrichments in 24 healthy, young males following the ingestion of 40 g protein of either a lysine-enriched wheat and chickpea protein product (Plant; *n*=12) or chicken breast filet (Chicken; *n*=12). Data were analysed by repeated measures (time x treatment) ANOVA. Bonferroni post-hoc test was used to locate differences over time. Basal period: time x treatment interaction, *P*=0.309, main time effect, *P*=0.01, main group effect, *P*=0.700, Postprandial period: time x treatment interaction, *P*=0.322, main time effect, *P*<0.001, main group effect, *P*=0.449. No significant differences were observed between treatment groups.

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CHAPTER 9

GENERAL DISCUSSION

Protein is a key dietary macronutrient to support growth, repair, and maintenance of skeletal muscle tissue, as it stimulates muscle protein synthesis [1, 2]. Hence, sufficient protein ingestion is of key importance in the day-to-day diet. Although protein is present in many different food items, not all dietary protein sources are equally effective in stimulating (muscle) protein synthesis. Plant-derived proteins (such as soy protein isolate) are historically observed to elicit an inferior muscle anabolic response following ingestion compared to animal derived protein[3-5]. The lesser anabolic response of plant-derived protein isolates is thought to be related to a suboptimal amino acids composition. However, there are many different plants from which protein can be isolated, all of which hold a unique amino acids composition [6]. In this dissertation we assessed the anabolic properties of pea (**Chapter 3**), wheat (**Chapter 4**), corn (**Chapter 5**), and potato (**Chapter 7**) protein, which were compared with a reference animal-based protein (i.e. milk protein isolate). The amino acid composition of these plant-derived protein isolates differ substantially, with an insufficient amount of lysine and/or methionine content in several plant-based proteins, which are suggested to dictate their anabolic profile [6, 7]. Hence, we aimed to compensate for the poor amino acids composition of these plant-based proteins by blending (50-50) them with milk protein, to observe whether this would augment the post-prandial rise in muscle protein synthesis rates (**Chapters 4** & **5**). In a similar fashion, we evaluated the response following ingestion of a blend of complementary plant-derived proteins (wheat + corn + pea), providing the same amount of leucine as milk protein (**Chapter 6**).

In general, plasma amino acid availability following protein ingestion was lower for plant *vs* animal derived protein isolates or concentrates (**Chapters 3-8**). These differences were reduced, but still substantial following ingestion of the protein blends (**Chapters 4-6**). We observed a substantial rise in post-prandial muscle protein synthesis rates following the ingestion of all included plant-based proteins. More importantly, this increase in post-prandial muscle protein synthesis rates following ingestion of plant-based proteins was not different compared with the consumption of the animal-based reference protein (i.e. milk protein) (**Chapters 3-8**). However, it is important to consider to which extent the results observed following ingestion of protein isolates can be translated to the whole foods they are derived from. Whole foods contain many other factors apart from protein amino acid composition, that may influence protein digestion, amino acid absorption, and anabolic signaling. The subsequent muscle protein synthetic response following whole foods ingestion is, therefore, not solely dependent on the protein quality itself, but on the characteristics of the entire food product (**Figure 9.1**).

WHOLE FOODS

Protein is just one of the many components of the whole food matrix. The food matrix characteristics that determine the anabolic response following food ingestion can be divided in 3 main characteristics. These involve: protein density, macronutrient composition, and the presence of other nutritional factors (**Figure 9.1**).

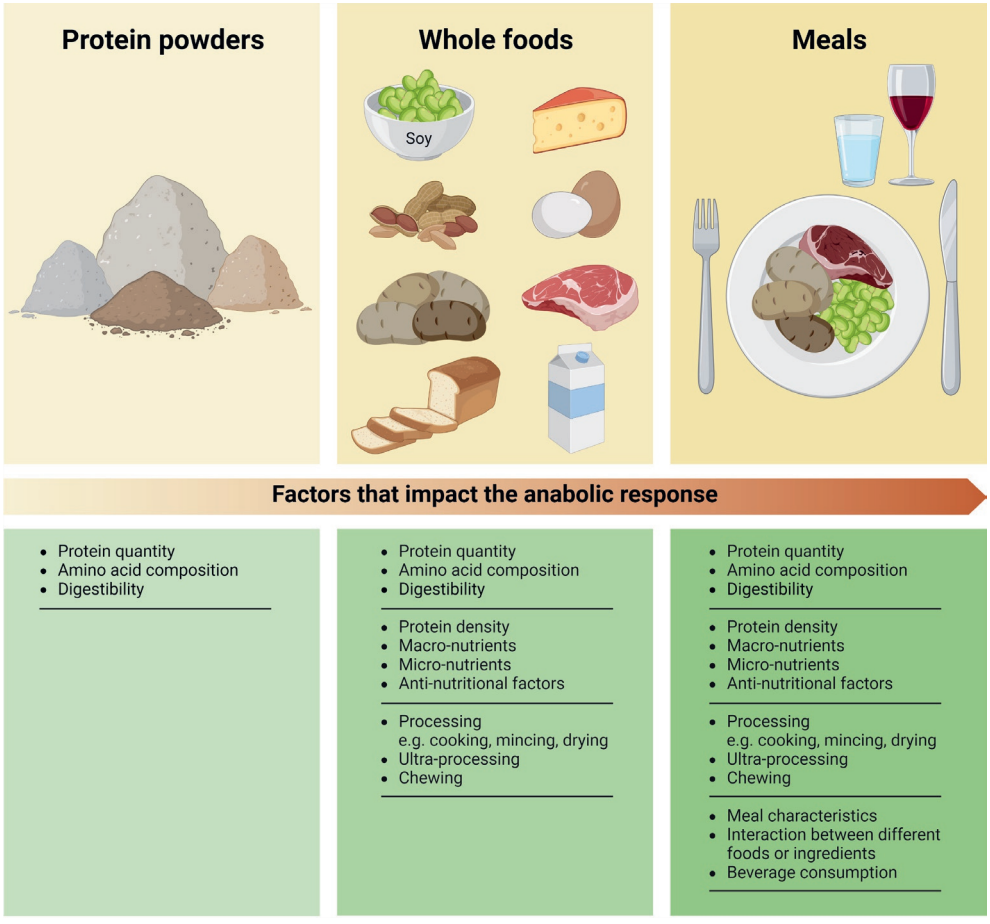

FIGURE 9.1 Overview of the various factors that impact the muscle protein synthetic response following food intake. When moving from protein concentrates/isolates to whole foods and complex meals, more and more factors will impact the anabolic response to food ingestion, factors beyond those of isolated proteins only. *Figure created with BioRender.com.*

Protein density

Protein concentrates and isolates provide 60-90% protein [6], which can easily be dissolved in water and subsequently ingested as a single beverage. However, the protein content of whole foods, and especially of plant origin, is lower. Therefore, it can become challenging to consume a sufficient amount of protein when consuming plant based whole foods only. **Chapter 7** shows that potato protein has a favorable amino acid composition, e.g. does not show any essential amino acid deficiencies, and is, therefore, considered to be a high quality protein. In addition, this dissertation shows that when a potato protein isolate is ingested, it is rapidly digested and absorbed and effectively stimulates muscle protein synthesis at rest and during recovery from a single bout of exercise (**Chapter 7**). However, when considering potato as a whole-food, 100 g of potato only contains ~2 g of protein [8]. It would require an unrealistic ~1.5 kg of potatoes to consume an "optimal" amount of 20-30 g protein needed

to maximize muscle protein synthesis rates after a single meal in young individuals [9-11]. Thus, although potatoes are considered to be a high quality protein source, due to the low amount of protein, their contribution to the daily habitual dietary protein intake may be negligible. Many other vegetables share this problem, and contain a low amount of protein (e.g. brown rice, corn, quinoa, oats, Chapter 2). Therefore, when considering which whole foods to consume, it is not only the protein quality that is important, but also the amount of protein the food product contains. As a result, not every food product is equally suitable to fulfill the daily protein needs. Instead, there needs to be a balanced consideration for protein quality and quantity.

Macronutrient composition

The use of protein isolates and concentrates in research has the advantage to assess the anabolic properties of protein in isolation, without other confounding factors. In whole foods, however, protein is combined with other macro-nutrients that could impact protein digestion and amino acids absorption and, as such, post-prandial protein synthesis rates. In line, co-ingestion of carbohydrate and/or fat results in delayed protein digestion and amino acid absorption. Previous work in our group [12-15] as well as others [16, 17] have shown that post-prandial plasma amino acid availability is strongly impacted by the co-ingestion of fat and or carbohydrates with protein. Despite these differences in post-prandial plasma availability, there was little effect on the post-prandial rise in muscle protein synthesis rates [12]. This does not imply that macronutrient composition is not an important factor modulating the post-prandial muscle protein synthetic response to protein ingestion, but simply shows that macronutrient composition forms merely one of many factors that may modulate the capacity to stimulate post-prandial muscle protein synthesis. Recent work investigating differences in the anabolic response to the ingestion of protein isolates versus the equivalent amount of protein provided as a whole food product has shown that other factors besides macronutrient composition are likely to modulate protein digestion and amino acid absorption, and the subsequent capacity to stimulate muscle protein synthesis [18]. It is important to consider that protein quality and quantity of whole foods can be negatively affected by other macronutrients limiting protein accessibility, and thereby reducing post-prandial amino acid availability of whole foods when compared to protein isolates.

Other nutritional factors

Besides macronutrients, whole foods also contain various other nutritional factors which may affect protein digestion and amino acid absorption in the gut. Due to the presence of anti-nutritional factors (i.e. compounds in the food that interfere with digestion and absorption of the available protein), plant-based protein sources like maize, oat, pea and potato, tend to exhibit lower digestibility compared with animal-based protein sources [19-21]. A reduced or slower release of protein derived amino acids in the circulation can attenuate the post-prandial rise in muscle protein synthesis rates [22]. Besides an often incomplete essential amino acid profile, the presence of anti-nutritional factors in plant based protein sources, is suggested to be the most limiting factor for the anabolic response following food ingestion.

In contrast, whole foods may also contain specific (micro)nutrients that support anabolic signaling and, therefore, contribute towards promoting muscle protein synthesis [23]. Vitamins (e.g. vitamin D [24]), minerals (e.g. magnesium [25], zinc [26]), unsaturated fats [27] have been shown to augment mTOR signaling. Egg yolk, for example, contains a high concentration of various minerals, vitamins, and lipids. Interestingly, ingestion of a whole egg (i.e. egg white + yolk) results in a significant greater rise in post-prandial muscle protein synthesis rates compared with ingestion of an isonitrogenous amount of egg white only [28]. This indicates that other factors in whole foods, apart from protein itself, may play an (in) direct role in stimulating post-prandial muscle protein synthesis.

Collectively, the composition and characteristics of a whole food can (in)directly influence protein digestion and amino acid availability, and can subsequently affect the anabolic response and/or signaling following food ingestion. The integration of all these factors in whole foods is complex, with a further level of complexity added when the food products are being processed prior to consumption.

FOOD PROCESSING

Though some whole foods can be consumed raw, such as fruits and vegetables, most foods need some degree of processing to ensure palatability, safety, and digestibility [29]. Processing of food has been around since the prehistoric times. For at least 250.000 years humans have been using fire for cooking as a form of food processing [30]. During the ancient and medieval times more complex forms of food processing were introduced, like sun-drying, smoking, baking bread, cheese-making, steaming, and others (**Figure 9.1**). These basic food processing methods improved food preservation, taste and reduced microbial activity. In the late 20th and early 21st century the main driver for food processing was to allow mass production, processed foods became fast and affordable, and sometimes even more affordable than the whole foods from which they were derived from. But, concerns began to rise on the nutritional values of processed foods. Many preservation methods can affect whole food micro- and macronutrient composition. Added sugar, fat, and oils increase the calorie content without adding nutritional value, which may have long-term health consequences, which is still a hot topic of ongoing debate [31-35].

When specifically considering protein rich whole foods, food processing can have an equally detrimental as well as positive impact on the bioavailability and/or biofunctionality. Meat, for example, is considered to be a high quality protein source and has been observed to be a potent stimulator of muscle protein synthesis [36, 37]. Whereas meat can be consumed as steak, it can also be ground down to minced beef. Ingestion of minced beef, results in a more rapid digestion and absorption when compared to the ingestion of beef steak [38]. Thus, even though the protein and macronutrient composition of beef remains exactly the same, processing (i.e. mincing) can change the food matrix, thereby modulating the capacity to

rapidly digest the protein and absorb the protein derived amino acids, and in turn affecting the anabolic potential of the ingested food.

Alternatively, food products can be processed to exhibit a different macronutrient composition. When food processing is aimed at increasing protein availability, this often, but not always, results in dehydration, and partial removal of anti-nutritional factors and macronutrients. The protein digestion and amino acid absorption of these products are substantially improved when compared to their original whole food products [29]. Such fabricated food products will also make it easier to ingest substantial amounts of protein from sources otherwise relatively low in protein. Cheese, tofu and seitan are, for example, popular protein rich products which have a higher protein content when compared to the original whole foods they originate from (milk, soy bean, and wheat, respectively). Recent developments in the food industry, also show a higher grade of food processing used to produce high protein meat substitutes from plant-derived ingredients (e.g. a vegan burger). These meat substitutes are often a combination of whole food grains and pulses as main ingredients (e.g. wheat, pea, soy bean, chick pea) [39]. Protein isolates are added to these products to provide a protein content similar to meat. Although processing food can potentially improve protein digestion and amino acid availability, the intrinsic properties of the proteins remain intact. This means, if a protein source has deficiencies in certain amino acids, those deficiencies will remain following processing, unless added as a free amino acid (**Chapter 1**). In **Chapter 8** of this thesis, we showed that adding lysine to a plant-based meat substitute indeed increases plasma lysine availability. However, the availability of all other essential amino acids was still substantially lower when compared to chicken breast filet (**Chapter 8**). Therefore, in order to provide a sufficient amount of essential amino acids and attain a muscle protein synthetic response similar to an animal protein source, also for meat substitutes it would be recommended to provide an ample amount of protein (i.e. ~30 g; **Chapter 1**).

During food preparation

Nowadays, the majority of food is processed before consumption, this often involves heating or cooking of food. Heating can result in denaturation of protein structures which improves protein digestibility, and thereby increases plasma amino acid availability [1, 40-42]. For example, protein derived plasma amino acid availability is higher following ingestion of fully cooked meat when compared to rare meat [43]. In line, ingestion of cooked eggs results in a greater post-prandial plasma amino acid availability when compared to the ingestion of raw eggs [44, 45]. Besides an effect on protein structures, cooking can also improve protein digestibility indirectly. Trypsin inhibitors in soy beans are inactivated by cooking, thereby indirectly improving protein digestion and subsequent amino acid availability [21, 46].

Heating can, however, also have a negative impact on protein digestion and/or amino acid availability following ingestion of whole foods. Prolonged heating at high temperatures can, in the presence of other macronutrients (i.e. sugar and fat), result in protein glycation and cross-linking via the Maillard reaction. As a result proteins can aggregate, and amino acids can become modified, thereby reducing protein digestibility (e.g. via modification of trypsin

cleavage sites) and amino acid availability [47-49]. Apart from changes in protein structures, there are numerous other effects of heating, such as oxidation of amino acids, which reduces amino acid availability. Therefore, preparing food, especially by heating, can substantially influence not only the availability, but also the quality of the ingested protein. Common food processing practices like heating are not commonly taken into consideration when studying the anabolic response to food ingestion. Furthermore, not only during cooking, but also during eating itself there are several factors affecting how food is broken down into its macro- and micronutrients, such as chewing efficiency and dental health [50], and body position during and immediately following food consumption (i.e. sitting upright vs laying down; [51]) have been shown to impact food/protein digestion and amino acid absorption kinetics.

Collectively, food processing can result in the same food product to have a different availability/anabolic response. This happens during food manufacturing, during preparation of the food by the consumers, but also during food consumption itself. Therefore, not only the food of choice, protein quantity and quality are important, but also the way the food is processed. Finally, when foods have been prepared, they will not be consumed in isolation, but will likely be part of a more complex, composite meal.

MEAL COMPOSITION

Whole food products and the processing of those products can influence the anabolic response following ingestion of protein rich foods. However, in our diet we generally do not eat single foods, but rather consume a combination of various food items as part of a complete meal, which will influence the anabolic response following food ingestion. The factors affecting this response can be divided in 2 categories: The characteristics of the meal itself, and the interaction between different food items within the entire meal (**Figure 9.1**).

Meal characteristics

A meal is composed of different food products, the characteristics of each food item may contribute towards the post-prandial response of the entire meal. According to Dutch food guidelines [52] a dinner meal is considered to be healthy, when it contains vegetables, oils and fats (during preparation), fish, pulses, meat, egg or nuts, whole grain pasta or couscous, brown rice, or potatoes, and water. Cheese, yoghurt, and fruit are considered optional for a dinner meal and are typically served as a dessert. Historically, a Dutch dinner meal consists of meat or fish, vegetables (e.g. string beans, cauliflower, broccoli) and potatoes. In such a meal, the majority of the protein ingested (~80%) originates from consuming meat or fish (100-120 g per serving, providing ~30 g protein), only a small amount (~20%) of the total protein composition is generally attributed to plant-derived foods. In the past years, however, there has been a trend towards the consumption of more plant- instead of animal derived foods. This transition is considered to be a positive development for general health, as more vegetables are included in the meal, and for a more sustainable food production [53]. However, replacing meat or fish with vegetables and/or pulses, has consequences for

the meal's protein content. A vegetarian or vegan meal contains more vegetables which lowers the overall protein content when compared with a meal containing meat or fish. Though there are a few exceptions (e.g. soy beans), the amount and volume of food that needs to be ingested in order to meet the recommended protein intake for a single meal (20-30 g) from consuming exclusively plant-based sources, is at least 2-3 times more, when compared to a single serving including meat or fish (**Chapter 1**). Therefore, total meal volume and caloric intake will increase when only consuming whole food plant based products in a single meal. For example, a single portion of cooked lean beef (~100 g, ~150 kCal) already provides ~30 g protein, adding potatoes (~200 g) and vegetables (e.g. ~150 g string bean) to provide a complete meal would result in a meal of ~450 g, ~350 kCal, and ~35 g protein. When composing a meal of similar protein content (~35 g) from a variety of popular plant-based foods, such as soy beans (~120 g), chick peas (~120 g) and quinoa (~200 g), the total caloric intake is ~60 % higher (~560 kCal, 440 g) when compared to the alternative meal containing meat. Given that gastric emptying and subsequent availability of protein derived amino acids will be slower with increasing meal caloric intake [17], the protein content of the individual products needs to be taken into account when composing a meal. This is of particular relevance in individuals with increased protein needs, or for those who have an overall lower food consumption, such as elderly, or hospitalized individuals [54, 55]. Careful selection of plant-based foods with the highest protein contents, such as soy beans and chick peas, could, in part, prevent an excessive meal volume. Another clear example of this is the use of plant-based meat alternatives that compensate for the, in general, low protein content of plant-based food, such as described in **Chapter 8**. The possibilities have rapidly expanded over the last years, providing plant-based alternatives for burgers, sausages, meatballs, and others, not only for individuals adhering to a vegan/vegetarian diet, but also for individuals who just want to eat less animal-based foods. The protein /amino acid bioavailability and functionality of these plant-based meat alternatives *in vivo* in humans is largely unknown. However, given their increased popularity and generally higher protein contents, more research involving meat alternatives and how they compare to the meat they substitute is warranted, in order to provide proper recommendations for the use of these products.

Interaction of food products within a meal

Upon meal consumption different food items are ingested, and subsequently, digested simultaneously within the gut. The fact that various foods come together in the stomach results in an interaction between these different foods items. This interaction plays a major role in protein digestion and amino acid absorption, and may attenuate the subsequent anabolic response. Eating beef steak, for example, with a lot of high fiber and anti-nutritional factors containing vegetables likely decreases the anabolic response of the ingested protein due to a substantial delay in digestion of the beef and thereby the amino acid absorption [56].

In general a meal is combined with the consumption of a beverage. Such a beverage can simply be a glass of water, but also a soft drink containing a lot of refined sugars or an alcoholic drink which can also add a lot of carbohydrates to the meal. Currently, very little is known

regarding the effects of co-ingesting non-caloric liquid (like water, or non-caloric soft drink) during a meal on protein digestion and amino acid absorption, or muscle protein synthesis rates. Given that carbohydrate co-ingestion with protein slows down protein digestion [12], it would not be surprising that consuming an energy rich soft drink (containing a lot of refined sugars) will delay gastric emptying and protein digestion when compared to consuming water. Since the beginning of time, alcohol containing beverages, like beer or wine, have played key roles in the context of meal consumption in the evening, and represents a large social aspect that makes individuals enjoy eating. However, alcohol consumption *per se* is known to reduce the post-prandial muscle protein synthetic response, possibly related to an attenuated phosphorylation of mTOR and p70S6K [57], and slower gastric emptying [58]. However, it remains to be determined to which extent alcohol consumption (2-3 servings) would affect the muscle protein synthetic response following meal ingestion.

Collectively, not only the entire food matrix and preparation of whole foods determines protein digestion, amino acid absorption and the subsequent muscle protein synthetic response, it is also important to consider the whole meal characteristics, as well as the interaction of all the individual processed or unprocessed food products that are consumed within or with this meal.

WAY FORWARD

In the past decade research has mainly focused on investigating the various individual aspects that may influence the anabolic response following protein ingestion (i.e. by using protein isolates with or without the addition of isolated nutrients). These studies have been essential in increasing our understanding of the various factors (like protein amount, protein source, timing of protein ingestion, and the addition of other macronutrients, but also age, sex and exercise) that can affect the post-prandial anabolic response. However, it also made the scientific field realize that relative small variations in whole food characteristics and/or meal compositions may be of importance, and may significantly affect the post-prandial muscle protein synthetic response. Therefore, in a first step, future research should focus on transitioning towards a more integrative and translational approach, in which the anabolic response is investigated in a more practical setting such as following whole meal consumption. Additionally, it is of interest to evaluate the anabolic response when adhering to a specific diet for a prolonged period of time. In line with this thesis, and given the current transition towards a more plant-based diet, it is of particular interest to compare the anabolic response following omnivorous vs vegan whole food meals and the impact of prolonged changes in habitual diet.

In a second step, future studies are required to establish the general impact of transitioning towards a more plant based diet on muscle health. Currently, most research studies assess the impact of adhering to a plant-based diet on general measures like body mass, blood pressure, lipid profiles, risk of developing cardiovascular diseases, and type II diabetes mellitus

in humans [59-62]. However, transitioning towards a more plant-based diet can also have substantial impact on muscle health [63], which in turn is related to the ability of performing physical activity, and the development of the previously mentioned metabolic diseases [64]. Therefore, measures of muscle protein synthesis and muscle mass should be incorporated in such studies, to obtain a broader insight into the potential positive and negative effects of transitioning towards a plant-based diet.

Thirdly, it has been well-established that protein digestion and amino acid absorption are affected by aging [65]. As such, the recommended amount of protein ingested per meal may differ between young and older adults [66]. When studying the impact of plant-based proteins, in isolation or when combined within a meal or larger diet, it should be assessed both in the young and older population. This will further aid consumers, health professionals, and policy makers to make an informed decision on how much and which proteins to ingest. To establish this, future nutritional guidelines should define the protein needs in various young and older populations.

Finally, the sex bias in translational research involving human volunteers is a well-documented and a concerning issue [67]. Historically, clinical research has often focused on male subjects, and as a result, there has been a significant underrepresentation of females in many studies. This gender disparity can have serious implications for the understanding of health and disease, as well as the development of medical treatments and interventions. This imbalance has been suggested to be emerged due to concerns with the heterogeneity of the studied population, convention, convenience, and/or limited financial resources [67]. In the field of muscle physiology and protein metabolism research, female underrepresentation is equally present and needs continuous consideration in future research. Although no differences in basal protein synthesis rates have been observed, post-prandial muscle protein synthesis rates seem to be higher in females compared with males, when the same absolute amount of protein is consumed [68]. This discrepancy may, in part, be explained by the difference in skeletal muscle mass between males and females. As the anabolic response to the ingestion of the same absolute amount of protein may differ between sexes, it is key that studies are performed in both males and females

CONCLUSIONS

The current dissertation is the first to evaluate the anabolic properties of various plant-derived protein isolates. Plant-derived proteins contain lower amounts of essential amino acids when compared to animal-derived proteins (**Chapters 2-5**). This discrepancy is also reflected in a substantially lower post-prandial plasma essential amino acid profile following protein ingestion (**Chapters 3-6**). However, the muscle protein synthetic response following ingestion of 30 g of wheat, corn, pea, and potato protein do not differ when compared to ingesting 30 g milk protein in young men (**Chapters 3-5** and **7**). During recovery from exercise, potato protein ingestion stimulates muscle protein synthesis to the same extent

as milk protein (**Chapter 7**). Similarly, ingestion of a lysine enriched meat substitute (40 g protein) stimulates muscle protein synthesis to an extent not different from ingesting the same amount from chicken breast filet, showing that meat substitutes can have a similar anabolic response to meat consumption (**Chapter 8**).

In general, we do not eat or drink protein isolates, we consume single whole foods or combine different foods within composite meals. Current scientific evidence would suggest that plant-based foods would result in a lower anabolic response when compared to animal-based foods, as result of the combination of a lower protein content, lower essential amino acid content, and higher amount of anti-nutritional factors. The scientific field would benefit from using a more inclusive approach when evaluating the anabolic properties of protein. Moving away from evaluating the post-prandial response to ingesting protein isolates and concentrates, and focus on the consumption of whole foods as well as different foods items combined within a meal or diet, will proof to be critical to take the next step towards evaluating the true anabolic potential of our foods.

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ADDENDUM

SUMMARY

Skeletal muscle health is essential for well-being and functional capacity in humans. More specifically, skeletal muscle plays an important role in metabolism and allows us to perform physical activities (such as walking, cycling, and exercising) and activities of daily living that are required to maintain our physical independence (such as getting up from a chair). Muscle is comprised of many different proteins that work together to facilitate muscle contraction. These muscle tissue proteins are composed of smaller building blocks, also known as amino acids. There are 20 different amino acids which can be used to build proteins. As a result, each protein in our body is built from a unique combination of these amino acids. In order to keep our muscles healthy and functional, new muscle proteins are continuously synthesized, while old proteins are broken down. Dietary protein plays an essential role as it provides the prerequisite amino acids to synthesize new protein. Besides being a building block, amino acids also directly stimulate the production of new muscle proteins. To synthesize new muscle protein, not only the quantity but also the specific type of dietary protein ingested plays an important role. The type of protein consumed can be divided in two main categories, animal- (meat, fish, milk, egg) and plant-derived (beans, wheat, pea) proteins. This dissertation investigated whether plant-derived protein ingestion can stimulate muscle protein synthesis rates, and whether this would differ from ingesting an animal-derived protein in healthy, young males.

In recent years, plant-derived proteins received considerable attention as they are considered to be a sustainable alternative when compared to animal-derived protein production, requiring less water, land, and energy resources. Furthermore, there is an increased interest in reducing the *per capita* meat consumption, which corresponds with the increasing popularity of vegetarian and vegan diets. Since protein is crucial for the maintenance, growth, and recovery of skeletal muscle tissue, it is important to investigate to which extent this can be established by consuming plant-derived proteins. In **chapter 2**, we discuss the differences between plant- and animal-derived proteins, and elaborate on how these differences can affect muscle protein synthesis rates. Plant-based protein sources contain less protein, when compared to animal-based protein sources, and do not have all essential amino acids available in sufficient amounts. A lower amount of essential amino acids in the diet is thought to result in reduced muscle protein synthesis rates. Plant protein sources also contain more fibers and anti-nutritional factors, delaying protein digestion and amino acid absorption, which may result in an attenuated muscle protein synthetic response. For these reasons, plant-derived protein sources are regarded as lower quality protein sources compared to animal-derived protein sources. However, one can (in part) compensate for this reduced quality, by: 1) consuming protein concentrates/isolates (**Chapters 3, 4, 5, 7**); 2) enriching a plant-derived protein with the limiting essential amino acid(s) (**Chapter 8**); 3) Combining plant-derived proteins with animal-derived proteins, or combining multiple plant-derived proteins which complement each other's amino acid composition (**Chapters 3, 4, 6**); 4) ingesting more of a lower quality protein (**Chapters 3, 4, 5, 6, 7, 8**).

In **chapters 3, 4, and 5** the intake of 30 grams plant protein concentrate derived from pea, wheat, and corn have been investigated for their capacity to stimulate muscle protein

synthesis rates in healthy, young males. Furthermore, we assessed whether this stimulation differed from that observed following ingestion of 30 grams animal-derived dairy protein. The increase in the amount of circulating essential amino acids following ingestion of the plant-derived proteins was only half of the increase following ingestion of the same amount of milk-derived protein. Especially the amount of lysine (wheat and corn) and methionine (wheat and pea) in the circulation were low following the ingestion of plant-derived proteins. Despite the low plasma availability of these essential amino acids, a substantial increase in muscle protein synthesis rates was observed following ingestion of plant-derived proteins. Contrary to our hypothesis, this stimulation of muscle protein synthesis did not differ from the stimulation following ingestion of the animal-derived milk protein. We hypothesized that the muscle protein synthesis rate following ingestion of the plant-derived protein would be lower, when compared to the ingestion of the same amount of milk-derived protein. For this reason, an additional comparison was made in **chapters 4 and 5**, between the ingestion of 30 grams milk-derived protein, and either a blend of 15 grams milk protein plus 15 grams wheat protein (**Chapter 4**), or a blend combining 15 grams milk protein plus 15 grams corn protein (**Chapter 5**). By blending a plant-derived protein with a milk-derived protein, the amino acid composition of the protein drink improved. Subsequently, we assessed whether the protein blend stimulated the muscle protein synthetic response to same extent compared to the ingestion of milk-derived protein only. Despite the observation that less essential amino acids were present in the circulation following ingestion of the protein blends, no differences were observed in the muscle protein synthetic response between the ingestion of the protein blends (milk + corn or milk + wheat) when compared with the ingestion of milk-derived protein only.

Chapter 6, is the first to show that an exlcusive plant-derived protein blend (15 grams wheat + 7.5 grams corn + 7.5 grams pea) can effectively stimulate muscle protein synthesis rates in healthy, young males. Subsequently, the muscle protein synthetic response following the plant-derived protein blend was compared with the ingestion of an equivalent amount of milk-derived protein. In line with **chapters 3, 4, and 5**, the amount of essential amino acids (mainly methionine and lysine) in the circulation following ingestion of the plant-derived protein mixture, was only half of the amount present following ingestion of milk-derived protein. Also the amount of leucine available in the circulation following ingestion of the plant-derived protein mix was lower (16%) when compared to the ingestion of milk-derived protein. Ingestion of the plant-derived protein blend resulted in a substantial increase in muscle protein synthesis rates, which did not differ from the rates observed following ingestion of milk-derived protein.

Besides protein ingestion, physical activity is known to be a potent stimulus to increase muscle protein synthesis rates and support overall muscle tissue health. In **chapter 7**, we investigated the capacity of a plant-derived protein to stimulate muscle protein synthesis at rest, as well as during recovery from a single bout of resistance exercise in healthy, young males. In general, plant-derived proteins contain a lower amount of leucine, lysine, and/or methionine. However, potato-derived protein distinguishes itself from other plant-derived

proteins by containing sufficient amounts of all essential amino acids. For this reason, we assessed whether ingestion of potato-derived protein can stimulate muscle protein synthesis rates to the same extent when compared with the ingestion of milk protein. The amount of essential amino acids present in the circulation was lower following ingestion of potato-derived protein, when compared to milk-derived protein. This was particularly evident throughout the first 1.5 hours following protein ingestion. Potato-derived protein ingestion resulted in an effective stimulation of muscle protein synthesis rates at rest and during recovery from a single bout of resistance exercise in healthy, young males. This stimulation of muscle protein synthesis rates was not different when compared to the ingestion of milk-derived protein.

The increasing interest in plant-derived protein rich foods has led to an increase in the development and availability of plant-derived meat substitutes. In chapter 8, the capacity of a protein rich meat substitute to stimulate muscle protein synthesis rates was investigated. Here we determined whether the muscle protein synthetic response to the ingestion of the plant-based meat substitute differed from the ingestion of an equivalent amount of meat. In this research study, the ingestion of a lysine-enriched meat substitute, composed out of wheat- (60%) and chick pea- (40%) derived protein, was compared with the ingestion of an equivalent amount of chicken breast filet. The post-prandial essential amino acid concentrations were lower following ingestion of the plant-derived meat substitute, compared to ingestion of the chicken breast filet. In contrast, the lysine availability in the blood circulation was strongly elevated following ingestion of the meat substitute when compared to the chicken breast filet. Ingestion of the meat substitute strongly increased the muscle protein synthesis rates and did not differ from the synthesis rates observed following ingestion of chicken breast filet in healthy, young males.

In summary, from this dissertation we can conclude that, when a sufficient amount of plant-derived protein (30 grams) is consumed in healthy, young males, muscle protein synthesis rates are effectively stimulated. Furthermore, the stimulation of muscle protein synthesis is not different from the response following ingestion of the same amount of milk-derived protein. Also, the ingestion of a blend of plant- and animal-derived protein, and a blend of different plant-derived proteins, result in a strong increase in muscle protein synthesis rates, a stimulation which is not different from ingesting an equal amount of milk-derived protein. We can further conclude that when a sufficient amount of protein (30 grams) is ingested, the low availability of one or several essential amino acids in plant-derived proteins, does not represent a limiting factor for the acute increase in muscle protein synthetic rates in healthy, young males. It is important, however, to consider that our nutrition does not consist out of protein isolates/concentrates, but rather represent whole-food products and complex meals. To further investigate the anabolic properties of plant-derived proteins, more research needs to focus on what we actually eat: whole-foods and complex meals (Chapter 9).

SAMENVATTING

Gezonde skeletspieren zijn van essentieel belang voor het lichaam om goed te kunnen functioneren. Skeletspieren zijn belangrijk voor een goede stofwisseling, het uitvoeren van fysieke activiteit (zoals lopen, fietsen, en sporten), en voor het onafhankelijk kunnen uitvoeren van dagelijkse activiteiten (zoals het opstaan vanuit een stoel). Spieren zijn opgebouwd uit verschillende soorten eiwitten die samen werken om spiercontracties, en daarmee beweging van gewrichten, mogelijk te maken. Eiwitten zijn op hun beurt opgebouwd uit kleinere bouwstenen die aminozuren worden genoemd. Er zijn in totaal 20 verschillende aminozuren die voor de opbouw van eiwitten worden gebruikt. Ieder eiwit in het lichaam bestaat uit een unieke verhouding tussen deze aminozuren. Om de spieren gezond en functioneel te houden vindt continue opbouw van nieuwe en afbraak van oude spiereiwitten plaats in ons lichaam. Eiwitten in de voeding spelen een essentiële rol bij het aanleveren van aminozuren voor de opbouw van nieuwe spiereiwitten, en stimuleren daarnaast ook direct de aanmaak van spiereiwitten. Hierbij is niet enkel de inname van een voldoende hoeveelheid eiwitten van belang, maar ook het soort eiwit dat wordt ingenomen. Er wordt veelal een onderscheid gemaakt tussen dierlijke (vlees, vis, melk, ei) en plantaardige (bonen, tarwe, erwten) eiwitten. In dit proefschrift is onderzocht of de inname van een plantaardig eiwit de spiereiwitopbouw kan stimuleren en of dit verschilt van de inname van een dierlijk eiwit bij gezonde jonge mannen.

Eiwitten van plantaardige afkomst hebben de afgelopen jaren veel aandacht gekregen vanwege duurzaamheidsvoordelen ten opzichte van eiwitten met een dierlijke afkomst. Plantaardige eiwitten worden gezien als duurzaam omdat deze geproduceerd worden met minder gebruik van water, land, en energie. Daarnaast is er een toenemende interesse in minder vleesconsumptie en neemt de populariteit van een vegetarisch of veganistisch voedingspatroon toe. Gezien het feit dat eiwit van cruciaal belang is voor het onderhoud, de groei en het herstel van skeletspieren, is het belangrijk om te onderzoeken in hoeverre dit ook bewerkstelligd kan worden met de inname van plantaardige eiwitten. In **hoofdstuk 2** bespreken we de verschillen tussen plantaardige en dierlijke eiwitten en hoe dit de spiereiwitopbouw kan beïnvloeden. In vergelijking met dierlijke eiwitten bevatten plantaardige eiwitbronnen minder eiwit, en zijn niet alle essentiële aminozuren in voldoende mate aanwezig. De algemene gedachte is dat een lagere hoeveelheid essentiële aminozuren in de voeding kan resulteren in een verminderde spiereiwitopbouw. Plantaardige eiwitbronnen bevatten ook meer vezels en anti-nutriënten die de vertering van eiwit en de opname van aminozuren kunnen vertragen, wat ook kan resulteren in een verminderde stimulatie van de spiereiwitopbouw. Om deze redenen worden plantaardige eiwitten als lagere kwaliteit eiwitten gezien dan dierlijke eiwitten. Dit kan echter (deels) gecompenseerd worden door: **1)** gebruik te maken van eiwit concentraten/isolaten (**Hoofdstuk 3, 4, 5, 7**); **2)** een plantaardig eiwit te verrijken met het limiterende essentiële aminozuur (**Hoofdstuk 8**); **3)** plantaardige eiwitten te combineren met dierlijke eiwitten of een combinatie te gebruiken van meerdere plantaardige eiwitten welke elkaars aminozuursamenstelling aanvullen (**Hoofdstuk 3, 4, 6**); **4)** meer van een lagere kwaliteit eiwit in te nemen (**Hoofstuk 3, 4, 5, 6, 7, 8**).

In **hoofdstuk 3, 4, en 5** is onderzocht of de inname van 30 gram plantaardig eiwitconcentraat afkomstig van erwt, tarwe, en mais in staat is om de spiereiwitopbouwsnelheid na inname te stimuleren in gezonde jonge mannen. Daarnaast is bekeken hoe deze stimulatie zich verhoudt tot de inname van 30 gram (dierlijk) melkeiwit. De resultaten laten zien dat de toename in de hoeveelheid essentiële aminozuren aanwezig in de bloedsomloop na inname van de plantaardige eiwitten slechts de helft is van de hoeveelheid aanwezig na inname van een zelfde hoeveelheid ingenomen melkeiwit. Voornamelijk de hoeveelheid lysine (tarwe en mais) en methionine (tarwe en erwt) is laag in de bloedsomloop na inname van de plantaardige eiwitten. Ondanks de lage beschikbaarheid van deze essentiële aminozuren in het bloed, is een substantiële toename in de spiereiwitsopbouwsnelheid te zien na inname van de plantaardige eiwitten. Tegen onze verwachting in is deze stimulatie van de spiereiwitsopbouw dus niet verschillend ten opzichte van de inname van het dierlijke melkeiwit. Onze hypothese was dat de spiereiwitopbouwsnelheid na inname van het plantaardige eiwit lager zou zijn dan na inname van eenzelfde hoeveelheid melkeiwit. Om deze reden is in **hoofdstuk 4 en 5** een extra vergelijking gemaakt tussen de inname van een mengsel van 15 gram melkeiwit en 15 gram tarwe eiwit (**hoofdstuk 4**), of een mengsel van 15 gram melk eiwit en 15 gram mais eiwit (**hoofdstuk 5),** met de inname van 30 gram melkeiwit. Door een plantaardig eiwit met melkeiwit te mengen, wordt de aminozuur samenstelling van de eiwitdrank verbeterd. Vervolgens is bekeken in welke mate dit eiwit mengsel de spiereiwitopbouwsnelheid stimuleert in vergelijking met de inname van alleen het dierlijke melkeiwit. Er waren minder essentiële aminozuren aanwezig in het bloed na inname van het eiwit mengsel, dan na inname van enkel het melkeiwit. De resultaten lieten echter zien dat de spiereiwitopbouwsnelheid even sterk gestimuleerd werd na inname van het eiwitmengsel (melk + mais of melk + tarwe eiwit) als na de inname van melkeiwit alleen.

In **hoofdstuk 6** is voor het eerst onderzocht of een volledig plantaardig eiwitmengsel (15 gram tarwe + 7.5 gram mais + 7.5 gram erwt) in staat is om de spieropbouwsnelheid te stimuleren bij gezonde jonge mannen. Vervolgens werd wederom deze opbouwsnelheid vergeleken met de inname van eenzelfde hoeveelheid dierlijk melkeiwit. De hoeveelheid leucine in het plantaardig eiwitmengsel was gelijk aan die van de melkeiwit drank. In lijn met **hoofdstuk 3, 4 en 5**, was de toename in de hoeveelheid essentiële aminozuren (met name methionine en lysine) aanwezig in de bloedsomloop na inname van het plantaardige eiwitmengsel slechts de helft van de hoeveelheid na inname van het melkeiwit. Er was ook minder leucine beschikbaar in de bloedsomloop na inname van het eiwitmengsel dan na inname van het melkeiwit, maar dit verschil was gering (16%). Inname van de plantaardige eiwit mengsel resulteerde in een substantiële stimulatie van de spiereiwitopbouwsnelheid die niet verschillend was van de inname van het melkeiwit.

Behalve eiwitinname is ook fysieke activiteit belangrijk om spieren gezond te houden en de aanmaak van nieuwe spiereiwitten te stimuleren. In **hoofdstuk 7** is onderzocht in hoeverre een plantaardig eiwit de spiereiwitopbouw kan stimuleren in rust als ook tijdens het herstel van een enkele krachttrainingssessie bij gezonde jonge mannen. Over het algemeen bevatten plantaardige eiwitten een lagere hoeveelheid leucine, lysine en/of methionine.

Aardappeleiwit onderscheid zich echter van andere plantaardige eiwitten door te beschikken over een voldoende hoeveelheid van alle essentiële aminozuren. Om deze reden is het plantaardige aardappeleiwit concentraat vergeleken met het dierlijke melkeiwit als referentie. De hoeveelheid essentiële aminozuren aanwezig in het bloed was lager na inname van aardappeleiwit dan na inname van het melkeiwit. Dit was met name zichtbaar in de eerste 1.5 uur na inname van de eiwitten. Inname van het plantaardige aardappel eiwit resulteerde in een effectieve stimulatie van de spiereiwitopbouwsnelheid, zowel in rust als tijdens herstel van de krachttraining, in gezonde jonge mannen. Deze stimulatie van spiereiwitopbouw was niet verschillend in vergelijking met de inname van het melkeiwit.

De toenemende interesse in plantaardige eiwitrijke voeding leidt tot een toename in de ontwikkeling en beschikbaarheid van plantaardige vleesvervangers. In **hoofdstuk 8** is onderzocht in welke mate een eiwitrijke vleesvanger in staat is om de spiereiwitopbouwsnelheid te stimuleren in gezonde jonge mannen. Vervolgens werd bepaald of deze spiereiwitopbouwsnelheid stimulatie verschilt met vergelijkbaar 'traditioneel' vlees. In dit onderzoek is gekeken na de inname van een lysine verrijkte vleesvervanger, op basis van tarwe (60%) en kikkererwten (40%) eiwit, en vergeleken met de inname van eenzelfde hoeveelheid eiwit (40 gram) aan kipfilet bij gezonde jonge mannen. De essentiële aminozuren concentratie in de bloedsomloop was lager na inname van de plantaardige vleesvervanger dan na inname van de kipfilet. Daarentegen was de hoeveelheid lysine in de bloedsomloop na inname van de vleesvervanger sterk verhoogd. Inname van de vleesvanger verhoogde de spiereiwitopbouwsnelheid sterk, maar deze was niet verschillend van de opbouwsnelheid na inname van kipfilet bij gezonde jonge mannen.

Kort samengevat kunnen we uit dit proefschrift concluderen, dat wanneer een voldoende hoeveelheid plantaardig eiwit (30 gram) wordt geconsumeerd, een effectieve stimulatie plaatsvindt van de spiereiwitopbouwsnelheid bij gezonde jonge mannen. Daarnaast zien we dat deze stimulatie van de spiereiwitopbouw niet verschilt met die na inname van eenzelfde hoeveelheid melk eiwit. Ook de inname een mengsel van plantaardige en dierlijke eiwitten, en een mengsel van verschillende plantaardige eiwitten resulteert in een sterke stimulatie van de spiereiwitopbouwsnelheid, welke niet verschilt van die na inname van een equivalente hoeveelheid melk eiwit. Hieruit kunnen we verder concluderen dat wanneer een voldoende hoeveelheid eiwit (30 gram) wordt ingenomen, de lage beschikbaarheid van één of enkele essentiële aminozuren in plantaardige eiwitten geen beperkende factor vormt voor de acute stimulatie van de spiereiwitopbouwsnelheid bij gezonde jonge mannen. Het is echter belangrijk om in acht te nemen dat onze voeding niet bestaat uit eiwit isolaten/concentraten, maar uit volledige voedingsproducten en maaltijden. Om de anabole eigenschappen van plantaardige eiwitten verder te onderzoeken, is het belangrijk dat meer onderzoek zich richt op wat daadwerkelijk gegeten wordt: volledige voedingsproducten en complete maaltijden (**Hoofdstuk 9**).

IMPACT

The main aim of this dissertation was to investigate the anabolic properties of plant-derived proteins, and to evaluate how they compare with animal-derived proteins in their capacity to stimulate muscle protein synthesis. This thesis extends on previous work reporting on the inferior anabolic properties of soy protein isolate ingestion when compared with animal-derived protein in humans [1-3]. This dissertation evaluated the plasma amino acid profiles and muscle protein synthesis rates following ingestion of wheat, corn, pea, and potato derived protein in comparison to animal-derived milk protein in healthy, young men. The amino acid profiles following ingestion of 30 g of the selected plant-derived proteins showed lower plasma essential amino acid availability, when compared to the ingestion of the same amount of milk-derived protein, especially for leucine, lysine, and methionine. Despite a lower essential amino acid provision, muscle protein synthesis rates were strongly increased following ingestion of the selected plant-derived protein sources. Importantly, this muscle protein synthetic response did not differ when compared to the ingestion of the same amount of milk protein. Similar results were observed when potato protein isolate was ingested during recovery from exercise. Furthermore, we presented data on the anabolic properties of protein blends, in which different plant-derived proteins are combined, with or without animal-derived protein. We showed that despite lower plasma essential amino acid availability, the ingestion of the selected protein blends increased muscle protein synthesis rates, which was not different from the increase observed following ingestion of milk protein. Lastly, we reported that ingestion of a lysine enriched plant-based meat substitute, consisting of wheat- and chickpea-derived protein, increases muscle protein synthesis rates to an extent not different from chicken breast filet, when both provide 40 g protein. Collectively, we can conclude, that ingestion of an ample amount of plant-derived protein (i.e. ≥30 g) stimulates muscle protein synthesis to an extent not different from ingesting the same amount of animal-derived protein in healthy, young men.

Stimulating muscle protein synthesis is fundamental for maintaining muscle health and support the exercise induced muscle reconditioning response [4]. When protein is ingested in close proximity to physical activity or exercise, the increased muscle protein synthetic response is greater and prolonged, when compared with the stimulation observed following exercise or protein ingestion only [5-8]. However, it is important to note that not all proteins are the same and do not have the same potential to stimulate muscle protein synthesis rates [9, 10]. Therefore, this thesis provides valuable information outlining the anabolic properties of various plant-derived proteins, their protein blends, and a plant-based meat substitute. The impact of this dissertation, as well as the implications for translation into practice will be discussed below.

Scientific relevance

This thesis broadens our understanding of the anabolic properties of plant-derived proteins. We have made substantial contributions to the scientific field, to better understand the important factors impacting the anabolic response following protein ingestion. Early studies on protein quality identified three main factors that would determine protein quality, and the subsequent anabolic response: 1) essential amino acid composition, 2) leucine content,

and 3) protein digestion and amino acid absorption of the specific protein or protein source [10]. Given that on all these factors plant-derived proteins are subpar when compared to animal-derived proteins, plant-derived proteins have traditionally been considered to exhibit a lower capacity to increase muscle protein synthesis rates [11]. This assumption was primarily based on earlier studies evaluating the anabolic properties following ingestion of soy protein isolates [1-3, 12]. Several, of these studies showed that soy protein ingestion stimulates muscle protein synthesis to a lower extent when compared to the ingestion of dairy-derived proteins, both at rest and during post-exercise recovery [1-3]. There are, however, many different plant protein sources with each holding their own unique properties [9, 10]. This thesis expands our knowledge by investigating the anabolic properties of wheat, corn, pea, and potato protein, protein blends, and a plant-based meat substitute *in vivo* in humans. The current work clearly shows that low essential amino acids contents of plant-derived proteins do not necessarily compromise the acute anabolic response when a sufficient amount of protein (i.e. ≥30 g) is ingested in healthy, young men. Therefore, neither protein characteristics, such as essential amino acid composition, nor the availability of essential amino acids in the circulation following ingestion, predict the muscle protein synthetic response following protein ingestion. Hence, statements on the anabolic properties of a specific protein can only be sustained when muscle protein synthesis rates have been assessed directly following the ingestion of that specific protein *in vivo* in humans.

With the work presented in this thesis we have substantially contributed to the current knowledge on the anabolic properties of plant-derived proteins and have provided many new directions for future research. It would be of interest to evaluate the muscle protein synthetic response of the selected proteins in older individuals. Given that older individuals suffer from anabolic resistance [13], it is of substantial interest to determine how plant-derived proteins compare with animal-derived proteins in stimulating muscle protein synthesis rates in this population. Secondly, the amount of protein ingested is of key importance to consider when evaluating plant-derived protein sources, particularly in older adults [14, 15]. A key question that should be addressed is whether older individuals need to ingest more plant-derived protein to elicit a similar anabolic response when compared with the ingestion of animal-derived protein. Furthermore, as discussed in chapter 9, there is a need to transition towards assessing the anabolic response following ingestion of whole foods and more complex meals, to take into account all interactions between foods and nutrients on protein digestion, amino acid absorption, and anabolic signaling originating from all other foods components besides protein.

Target groups and societal relevance

There is a global transition towards the consumption of more plant-derived proteins, which will become more important to attain future global protein needs [16-18]. Plant-derived proteins are considered to be a more sustainable protein source as production requires less water, land, and energy resources when compared to animal-derivedproteins [19]. Given the essential role of dietary protein in the maintenance of muscle health, it is key to understand the anabolic properties of plant-derived proteins.

The work in this thesis shows that wheat, corn, pea, and potato-derived protein isolates may potentially be used as effective alternative protein sources when transitioning towards more plant-based protein consumption in young, healthy individuals. In addition, we show that plant-derived protein blends, and plant-based meat substitutes, may also be considered as alternative protein sources when transitioning towards the consumption of more plant-derived proteins. This provides direction and opportunities for industry for the development of plant-derived protein rich products and meat substitutes, i.e. the type and amount of protein to be used to ensure an anabolic response similar to the ingestion of an equivalent amount of animal-derived products. We identified potato protein concentrate as an effective protein source to stimulate muscle protein synthesis rates at rest and during recovery from exercise. This is of special interest, since potato protein concentrate is produced from the waste product of potato starch extraction. Hence, this not only shows that plant-derived proteins can support the transition towards a more plant-based diet, but also supports the current drive towards a more circular economy. The studies presented in this thesis have been performed within a public-private partnership, in which a collaboration is found between the Dutch government, university and industry to perform pre-competitive research. Beside the financial support to perform independent scientific research studies, such a public-private partnership facilitates the translation of scientific discoveries into practical applications and products. Private (industry) partners generally have expertise in commercialization and manufacturing, and distribution which is key to bridge the gap between research findings and real-world implementation. This dissertation capitalizes on this collaboration by direct utilization of the results and products used by the private partners in the development of their consumer products.

In the athletic community there is an increasing interest towards specific plant-derived sports nutrition products to support athletes adhering to a vegan and vegetarian diet [20]. More than 80% of the sports nutrition market sales originates from protein products, including powders, bars, and drinks [21]. Given the combined interest towards plant-derived proteins and the continuously growing sports nutrition market, the application of plant-derived proteins in sports nutrition will substantially increase in the years to come. This dissertation, provides key knowledge in this transition by showing that potato-derived protein can effectively support post-exercise muscle protein synthesis rates, and thereby support muscle recovery and remodeling following exercise. Like in athletes, skeletal muscle recovery and/or reconditioning is of equal importance in many clinical situations. The loss of skeletal muscle mass and function is a well-known phenomenon during hospitalization [22, 23]. Apart from the negative effects of physical inactivity, the loss of muscle mass observed during hospitalization may be attributed to an insufficient protein intake as a direct consequence of the lower energy intake [24, 25]. Within the field of clinical nutrition there is equal interest in the use of more sustainable protein sources [26]. In addition, there will be a need to accommodate future patient choices to consume a more plant-based diet. Therefore, it is key to evaluate the anabolic responses of plant-derived protein administration in frail and older individuals, to support optimal recovery with the use of both animal as well as plant-derived protein.

Translation into practice

In today's society, there is a lot of information available and accessible to every individual, and people share their thoughts and opinions with the world by just a few clicks on social media. On the flipside, there is a large spread of misinformation, hype, and very narrow perspectives without scientific rigor. Especially when considering food supplements and diets excluding certain foods or food groups, it is important to elucidate the potential health consequences in an evidence-based manner. Throughout the completion of this dissertation we have not only provided scientific reports and presentations at scientific conferences, but also actively incorporated results in lectures in nutrition and exercise physiology educational courses. Outside the academic world we have, and still see an increasing interest in the transition towards the consumption of more plant-derived proteins, in which we are being approached to translate our knowledge to be applied by e.g. sports nutrition companies and the military.

The transition towards incorporation of more plant-derived protein in our diet, seems inevitable from both a consumers' choice as well as a sustainability perspective, to meet future global protein demands. Therefore, it is important to not only address the potential shortcomings in the anabolic response of plant-derived proteins, but to also give directions towards potential solutions. The scientific field should not be blinded by the initial studies showing a lower anabolic response following soy *vs* dairy protein ingestion [2, 3], but should rather support this transition by providing directions towards potential solutions. In chapter 2 we have given potential solutions to the lower quality of plant-derived proteins, i.e. 1) providing protein isolates, 2) fortifying the protein source with its limiting amino acids, 3) providing protein blends, and 4) consuming more of a lower quality protein. Future research should focus on translating these findings even further, by investigating the clinical relevance of the potential differential anabolic response between plant- and animal-derived proteins. Lastly, the knowledge obtained from plant-derived protein isolates and meat substitutes, requires further translation towards whole meals in the everyday kitchen.

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DANKWOORD

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lame jokes and remarks, and have been a major contributor to making the office not only productive, but also gezellig. Ieder team heeft echter ook afleiding nodig, iets waar Wesley graag voor zorgt. Desondanks kan ik jouw input en kritische blik waarderen, wanneer ik iets aan jouw vraag of wanneer we wat bespreken, dan doe je je ook echt de moeite om samen tot een antwoord of oplossing te komen.

De team manager

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De team artsen

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De echte coureurs van M3 cycling

Over de verschillende seizoenen hebben we verschillende samenstellingen gekend, met enkele eendagsvliegen, maar ook vaste waarden die altijd wel in waren voor een avondrit. Joan, Antoine, Freek, Kristin, Cindy, Imre, Andy, Evelien, Stefan, Joey, Lisanne, Lisa, Heather, Floris, Glenn, Birgitta, … en op de valreep heeft ook Noortje aan het einde van vorig seizoen haar bootje nog aangehaakt. Wat heb ik genoten van de nationale en internationale etappes die we samen hebben gereden, zelfs tot in de Ardennen (Stefan, Joey, Joan) en Luxemburg (Heather, Lisa, Floris). Het was een plezier om te zien dat jullie je goed amuseerden, op elke foto was wel iemand aan het lachen. Daarnaast was het mooi om te zien dat sommige van jullie snel beter en handiger werden op de fiets, met ieder zijn eigen kwaliteiten. Zo hebben we alle ingrediënten voor een succesvolle ploeg voorbij zien komen, klimgeiten, valken (afdalers), brommers en ook een dliesel.

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CURRICULUM VITAE

About the author

Philippe Pinckaers was born on September 10, 1991 in Eijsden, the Netherlands. He completed his secondary school in 2010 at Provinciale Secundaire School Voeren, Belgium. He obtained his Bachelor's degree in Biomedical Sciences at Maastricht University in 2013, during which he was selected for the Maastricht Research Based Learning program (MaRBLe excellence program). After obtaining his BSc degree, he started his MSc in Human Movement Sciences at Maastricht University. During his MSc he conducted an internship within the Muscle Metabolism Maastricht (M3) group of Prof. Dr. Luc van Loon at the department of Human Biology at Maastricht University. During this internship Philippe was involved in the doctorate studies from Dr. Jean Nyakayiru, Dr. Kristin Jonvik, and Dr. Jorn Trommelen, investigating the ergogenic potential of dietary nitrate supplementation. He completed his MSc degree (cum laude) with this thesis entitled "acute and chronic effects of nitrate supplementation on cycling time trial performance in well trained cyclists".

In May 2015, Philippe started as a research assistant within the M3 research group at Maastricht University. Together with Dr. Tyler Churchward-Venne, he performed studies focused on optimizing post-exercise skeletal muscle recovery by ingestion of different types and amounts of protein. Together they also investigated the effectiveness of leucine supplementation to attenuate skeletal muscle loss during short term immobilization, in healthy young and old males and females.

In April 2017, Philippe started his PhD at the department of Human Biology, within the School of Nutrition and Translational Research in Metabolism (NUTRIM) at Maastricht University, under the supervision of Prof. Dr. Luc van Loon, Dr. Tim Snijders, and Dr. Lex Verdijk. His studies focused on the anabolic properties of plant-derived proteins. In the years that followed, he performed several human intervention studies investigating the muscle protein synthetic response and amino acid bio-availability following ingestion of animal- compared with plant-derived proteins.

During his PhD, Philippe was awarded with a Poster Award during the 2020 NUTRIM symposium (runner-up), a Young Investigator Award during the 2020 European College of Sport Science conference (5th place), and a Gatorade Sport Science Institute Nutrition Award during the 2021 European College of Sport Science conference (3rd place). In addition, he was awarded the Naomi Cermak Memorial Graduate Travel Award in 2021. This award allowed him to travel to McMaster University, Hamilton, Canada, to collaborate on a study investigating the effect of exogenous ketone ester supplementation on time trial performance in trained cyclists, within the lab of Prof. Dr. Martin Gibala.

After defending his thesis, Philippe will continue at Maastricht University as a post-doctoral researcher.

Scientific publications

1. **Pinckaers PJM**, Weijzen MEG, Houben LHP, Zorenc AH, Kouw IWK, de Groot LCPGM, Verdijk LB, Snijders T, and van Loon LJC. The muscle protein synthetic response following corn protein ingestion does not differ from milk protein in healthy, young adults. *Amino Acids.* 2024 Feb 5; 56(8). https://doi.org/10.1007/s00726-023-03377-z.

2. **Pinckaers PJM**, Smeets JSJ, Kouw IWK, Goessens JPB, Gijsen APB, de Groot LCPGM, Verdijk LB, van Loon LJC, and Snijders T. Post-prandial muscle protein synthesis rates following the ingestion of pea-derived protein do not differ from ingesting an equivalent amount of milk-derived protein in healthy, young males. *European Journal of Nutrition.* 2024 Jan 16. https://doi.org/10.1007/s00394-023-03295-6.

3. **Pinckaers PJM**, Domić J, Petrick HL, Holwerda AM, Trommelen J, Hendriks FK, Houben LH, Goessens JP, van Kranenburg JM, Senden JM, de Groot LC, Verdijk LB, Snijders T, van Loon LJ. Higher Muscle Protein Synthesis Rates Following Ingestion of an Omnivorous Meal Compared with an Isocaloric and Isonitrogenous Vegan Meal in Healthy, Older Adults. *Journal of Nutrition.* 2023 Nov 15; S0022-3166(23)72723-5. https://doi.org/10.1016/j.tjnut.2023.11.004.

4. McCarthy DG, Bone J, Fong M, **Pinckaers PJM**, Bostad W, Richards DL, van Loon LJC, Gibala MJ. Acute Ketone Monoester Supplementation Impairs 20-min Time-Trial Performance in Trained Cyclists: A Randomized, Crossover Trial. *International Journal of Sport Nutrition and Exercise Metabolism.* 2023 Apr 25; 33(4):181-188. https://doi.org/10.1123/ijsnem.2022-0255.

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7. **Pinckaers PJM**, Kouw IWK, Gorissen SHM, Houben LHP, Senden JM, Wodzig WKHW, de Groot LCPGM, Verdijk LB, Snijders T, van Loon LJC. The Muscle Protein Synthetic Response to the Ingestion of a Plant-Derived Protein Blend Does Not Differ from an Equivalent Amount of Milk Protein in Healthy Young Males. *Journal of Nutrition.* 2023 Jan 14; 152(12):2734-2743. https://doi.org/10.1093/jn/nxac222.

8. **Pinckaers PJM**, Hendriks FK, Hermans WJH, Goessens JPB, Senden JM, van Kranenburg JMX, Wodzig WKHW, Snijders T, van Loon LJC. Potato protein ingestion increases

muscle protein synthesis rates at rest and during recovery from exercise in humans. *Medicine and Science in Sports and Exercise.* 2022 Sep 1; 54(9):1572-1581. https://doi.org/10.1249/mss.0000000000002937.

9. Kouw IWK, **Pinckaers PJM**, Le Bourgot C, van Kranenburg JMX, Zorenc AH, de Groot LCPGM, Verdijk L, Snijders T, van Loon LJC. Ingestion of an ample amount of meat substitute based on a lysine-enriched, plant-based protein blend stimulates postprandial muscle protein synthesis to a similar extent as an isonitrogenous amount of chicken in healthy, young men. *British Journal of Nutrition.* 2021 Dec 9; 1-11. https://doi.org/10.1017/s0007114521004906.

10. **Pinckaers PJM**, Trommelen J, Snijders T, van loon LJC. The Anabolic Response to Plant-Based Protein Ingestion. *Sports Medicine.* 2021 Sep; 51(Suppl 1):59-74. https://doi.org/10.1007/s40279-021-01540-8.

11. **Pinckaers PJM**, Kouw IWK, Hendriks FK, Van Kranenburg JMX, De Groot LCPGM, Verdijk LB, Snijders T, Van Loon LJC. No differences in muscle protein synthesis rates following ingestion of wheat protein, milk protein, and their protein blend in healthy, young males. *British Journal of Nutrition.* 2021 Dec 28; 126(12):1832-1842. https://doi.org/10.1017/s0007114521000635.

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Oral presentations

2023 NUTRIM symposium, Oral presentation: *"Higher muscle protein synthesis rates following ingestion of an omnivorous compared with an isonitrogenous vegan meal"*, November 22, Maastricht, The Netherlands.

Nutrition symposium Dutch Ministry of Defence, Invited lecture: *"Plant-based nutrition and muscle accretion"*. February 15, Utrecht, The Netherlands.

2021 European College of Sport Science congress, Online pre-recorded: *"Potato protein ingestion strongly increases muscle protein synthesis rates at rest and during recovery from exercise in vivo in humans"*. September 8-10, Online.

HAN University of Applied Sciences, Masterclass Strength training and plant-based protein, Invited online lecture: *"Anabolic properties of protein ingestion: Are plant proteins as good as animal proteins?"*. January 12, Online.

2020 European College of Sport Science congress, Online presentation: *"Dose response of dietary protein on muscle protein synthesis during recovery from endurance exercise in young men"*. October 28-30, Online.

2018 Food Valley Summit: Sports & Nutrition, Invited lecture: *"Anabolic properties of plant based proteins"*. October 11, Ede, The Netherlands.

European College of Sport Science congress, Oral presentation: *"The effects of ingesting different dairy proteins on skeletal muscle protein synthesis rates during recovery from concurrent exercise"*. July 4-7, Dublin, Ireland.

Poster presentations

2023 European College of Sport Science congress, Poster pitch: "*Higher muscle protein synthesis rates following ingestion of a beef-containing omnivorous meal compared with an isonitrogenous vegan meal"*. July 2023, Paris, France.

NUTRIM symposium, Poster pitch: "*Higher muscle protein synthesis rates following ingestion of an omnivorous compared with an isonitrogenous vegan meal"*. November 22, Maastricht, The Netherlands.

2022 International Biochemistry of Exercise Conference, Poster: "*Potato protein ingestion increases muscle protein synthesis rates at rest and during recovery from exercise"*. May 2022, Toronto, Ontario, Canada,

European College of Sport Science congress, Poster pitch: "*Leucine supplementation does not attenuate the decline in muscle protein synthesis rates or preserve leg muscle mass during short-term immobilization in young or older adults"*. September 2022, Seville, Spain.

NUTRIM symposium, Poster pitch: "Leucine does not attenuate the decline in muscle protein synthesis rates or muscle mass during immobilization". November 2022, Maastricht, The Netherlands.

2021 NUTRIM symposium, Poster pitch: "Potato protein ingestion strongly increases muscle protein synthesis rates at rest and during recovery from exercise". November 2021, Maastricht, The Netherlands.

2020 NUTRIM symposium, Online Poster pitch: "*The muscle protein synthetic response after ingestion of corn protein, milk protein and their protein blend in young males*". November 2020, Online.

2019 NUTRIM symposium, Poster pitch: "*Effects of the Dual-energy X-ray absorptiometry calibration factor on body composition assessment in men*". December 2019, Maastricht, The Netherlands.

2017 NUTRIM symposium, Poster pitch: "*Skeletal muscle protein synthesis rates following concurrent exercise: Effects of milk protein, whey, or casein ingestion*". December 2017, Maastricht, The Netherlands.

2016 NUTRIM symposium, Poster pitch: *Ketone Bodies and Exercise Performance: "The Next Magic Bullet or Merely Hype?"*. December 2016, Maastricht, The Netherlands.

Honours and Awards

2021 Naomi Cermak Memorial Graduate Travel Award

Gatorade Sport Science Institute Nutrition Award, at the European College of Sport Science, Online. 3rd place.

2020 Young Investigator Award at the European College of Sport Science, Online oral presentation. 5th place.

Poster presentation prize at the School for Nutrition and Translational Research in Metabolism (NUTRIM) symposium, Online. 2nd place.

2014 MSc graduated Cum Laude, Maastricht University

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