University of Nottingham
UK | CHINA | MALAYSIA

Investigating the Digestibility and Bioavailability of Selected Protein Sources in Older Adults

Jake Cox

14320667

Supervised by Matthew Brook, Philip Atherton, and Bethan Phillips

School of Medicine

University of Nottingham

MRes Thesis

29/09/2022

Table of Contents

Chapter 1- Protein Digestibility Review

1.1- Introduction

Current population trends demonstrate a globally ageing population, particularly in Europe where the ageing rate is highest (J. Li et al., 2019). An ageing population presents numerous health, social and economic challenges, including sarcopenia, which has been defined as the progressive loss of muscle mass and strength that occurs with ageing (Morley et al., 2001). The prevalence of sarcopenia has been estimated at 13-24% in individuals under the age of 70, and over 50% in individuals greater than 80 years old (Baumgartner et al., 1998). Sarcopenia has been shown to be associated with numerous health conditions and functional outcomes that impede quality of life, including type II diabetes mellitus (Mesinovic et al., 2019), Parkinson's disease (Vetrano et al., 2018), depression (Kilavuz et al., 2018), functional disability (Kilavuz et al., 2018), increased risk of falls (Landi et al., 2012; Yeung et al., 2019), and mortality (Arango-Lopera et al., 2013; Bunout et al., 2011). It has been estimated that the direct healthcare cost of sarcopenia in the United States in the year 2000 was approximately $18.5 billion (Janssen et al., 2004). Consequently, strategies to reduce the prevalence of sarcopenia and limit the social and economic burdens associated with this condition have been a central focus of research.

Nutritional interventions focusing on the role of protein intake for the maintenance of muscle mass have been widely researched (Robinson et al., 2018). Muscle protein turnover is regulated by a dynamic balance between muscle protein synthesis (MPS) and breakdown (MPB), with MPS being more important for regulation of muscle mass than MPB in normal conditions (Millward et al., 1976). Many factors contribute to determining the effectiveness of a protein source at increasing MPS. It has been well established that the provision of amino acids through dietary protein intake,

particularly essential amino acids (EAAs), is a potent stimulant to MPS (Volpi et al., 2003). EAAs are the amino acids which cannot be synthesised by the body and are therefore only obtainable through the diet. Of the nine EAAs, three of these, namely leucine, valine and isoleucine, have a branched side chain, and are considered to be the most potent for the stimulation of MPS (Plotkin et al., 2021). Among these three branched chain amino acids (BCAAs) leucine has received the greatest amount of attention from research as it produces the greatest increases in MPS and is able to stimulate MPS even without conditions of hyperaminoacidemia (Rieu et al., 2006). This is particularly important in older populations where there is a blunted anabolic response to protein feeding, meaning that MPS rates do not increase as much in response to protein ingestion as in younger individuals (Fujita & Volpi, 2006; Wall et al., 2015). However, enhanced leucine content of amino acid feeds results in a significant increase in MPS response in older individuals, where without this elevated leucine content, the MPS increase has been found to be negligible (Katsanos et al., 2006).

Other factors beyond amino acid composition are also important to consider when determining the effectiveness of a protein source at increasing MPS. Protein digestibility plays an important role in the efficacy of protein sources, as this dictates the rate and extent to which amino acids, particularly EAAs, are made available through the circulation to the muscle. There are many types of protein source with varying digestibility scores, calculated originally using the Protein Digestibility-Corrected Amino Acid Score (PDCAAS), and more recently, the Digestible Indispensable Amino Acid Score (DIAAS) (Herreman et al., 2020; Rutherfurd et al., 2015). Despite this ever-growing database of protein quality scores, there are still some notable limitations in the assessment of protein quality. It has been established that there is a reduction in the digestive capacity of the elderly due to reduced gastrointestinal tract (GIT) health, masticatory ability and appetite (Lee et al., 2021). Therefore, it is paramount to consider the effects this has on the true quality of a protein source in this population and how this may impact the subsequent bioavailability of amino acids and MPS increases. Additionally, there is

currently very little understanding of how mixing of protein sources impacts amino acid availability and MPS following consumption. Given that, within a real-world context, there are typically numerous protein sources consumed within a meal, a greater understanding of the effects of this would be highly beneficial.

This review will focus on the importance of protein digestibility and how the quantification of this has changed over time. There will then be a consideration for the different factors that determine how well an individual is able to digest a protein source, followed by a discussion of different potential mechanisms by which protein digestibility can be improved and how this may benefit populations such as the sarcopenic elderly.

1.2- Determining Protein Digestibility

The determination of protein quality depends on three phases between the consumption of the protein and the creation of new muscle mass. The first of these phases is the digestion of the protein source into its amino acid constituents. This is followed by the absorption of amino acids through the small intestine into the circulation, where they can be transported and made available to the muscle. The final phase is the utilization of amino acids to promote MPS once they reach the muscle. Whilst much research has focused on the utilisation of amino acids at the muscle level, to better determine the efficacy of protein sources for MPS enhancement, there should be consideration of the holistic process, with examination of protein digestibility, amino acid bioavailability, and utilisation at the muscle.

1.2.1- Oro-Faecal Balance

The most basic method to quantify the digestibility of a protein source is to use the oro-faecal balance method, where the change in amino acid quantity between absorption and excretion is calculated, providing an estimate for amino acid digestion and uptake (Trommelen et al., 2021). This technique has been widely criticised for inaccuracies related to the contribution of microbial metabolism in the large intestine which affects amino acid balance but does not contribute to absorption. The extent of this effect has been examined previously through comparisons between amino acid digestibility using faecal sampling with sampling from the terminal ileum in pigs and humans respectively (Holmes et al., 1974; Rowan et al., 1994). The comparison between ileal and faecal sampling in humans can be seen in table 1.1, where there were significant differences in amino acid digestibility values between sampling techniques for nine of the 18 amino acids analysed (Rowan et al., 1994). Notably, the authors reported that the oro-faecal balance method tends to overestimate the digestion of most amino acids, with notable exceptions being methionine and lysine, which are typically underestimated.

Table 1.1. Amino acid digestibility scores for adult humans fed a diet consisting of meat, vegetable, cereal and dairy products. * Significant difference between faecal and ileal amino acid digestibility scores ($P < 0.05$), ** ($P < 0.01$), *** ($P < 0.001$). (Rowan et al., 1994).

Amino Acid	**Ileal**	**Faecal**
Essential		
Histidine	90.2	92.2*
Isoleucine	90.9	90.6
Leucine	91.9	92.8
Lysine	93.6	93.2
Tryptophan	76.7	82.6*
Methionine	93.1	83.3***

Phenylalanine	89.6	91.3***
Threonine	84.7	88.8**
Valine	89.7	90.9
Non-Essential		
Alanine	88.1	87.9
Aspartate	87.3	89.7*
Cysteine	85.5	90.7
Glutamate	93.6	94.6
Proline	89.9	94.6**
Glycine	71.5	86.5***
Serine	86.5	91.9***
Tyrosine	88.7	90.1
Arginine	90.2	92.6

1.2.2- Ileal Sampling

To circumvent the inaccuracies caused by microbial activity, the oro-ileal balance method has been used where samples are collected from the terminal ileum using ileostomies or a nasal-ileal tube (Deglaire et al., 2009). It has been well established that the oro-ileal technique is more accurate than fecal approaches for determination of the digestibility of protein sources (Maughan & Smith, 2012). Despite this, sampling from the terminal ileum is an invasive protocol and has therefore been used sparingly in humans. Instead, animal models have been proposed for examination of protein digestibility using ileal sampling. There has been much research involving the use of rats and pigs for determination of protein digestibility, with both models producing relatively similar results for most amino acids, particularly essential amino acids (Moughan et al., 2012). However, there is a consensus that the pig digestive system more closely resembles the human digestive system physiologically, and previous research has indicated that this is a suitable model for assessing differences in protein digestibility in healthy adult humans (Deglaire et al., 2009). Consequently, ileal sampling of pigs for protein digestibility

values has some utility, but there is still a need for a method which facilitates the assessment of digestibility of protein sources in target populations such as the elderly or clinical populations.

1.2.3- Dual Stable Isotope Tracer

More recently, the dual stable isotope tracer technique has been utilised for the assessment of protein digestibility. This is a new, non-invasive technique, which is beneficial as it can be applied to humans, allowing the assessment of protein digestibility in different target populations (Moughan & Wolfe, 2019). The dual stable isotope tracer technique involves feeding of a stable isotopically labelled test protein alongside a reference protein which is labelled with a different stable isotope that has a known digestibility value. The known digestibility value of the reference protein is calculated by comparing the reference protein with a differently labelled free amino acid mix representing the amount of amino acids that would be available if complete digestion of the protein source took place. The use of a reference protein rather than a direct comparison of the test protein to an isotopically labelled free amino acid mix improves the validity of this measure as there are differences in the rates of absorption, metabolism and utilisation between peptides derived from protein digestion and free amino acids (Bandyopadhyay et al., 2021). Following the feeding of the test protein, reference protein and free amino acids, the appearance of plasma amino acids is monitored by regular blood sampling. The appearance of amino acids with the test label can be compared to the appearance of those with the reference label to provide a measure of ileal digestibility of the test protein based on the known digestibility of the reference protein. U-^{13}C-labelled spirulina protein has been validated as a useful reference protein, as it is a commercially available, high-quality protein that has been found to have a high digestibility score of 85.2% (Devi et al., 2018).

As previously mentioned, one of the most notable strengths of the dual stable isotope tracer technique is its non-invasiveness, allowing the application of this method to a variety of populations. Numerous studies have been carried out assessing the digestibility of various protein sources in healthy Indian adults, as it has been suggested that the high prevalence of environmental enteric dysfunction in this population may influence digestive capacity for protein sources (Kashyap et al., 2018). This series of studies has assessed the digestibility of various protein sources, including high quality protein sources such as hen's egg and meat (Kashyap et al., 2018), widely available protein sources in lower and middle income countries such as goat's milk (Kashyap et al., 2021) and common legume proteins in vegetarian diets including chickpea, yellow pea and mung bean (Kashyap et al., 2019). These studies have produced some interesting findings, including the inverse relationship between reduced intestinal function within a normal range and protein digestibility, though this needs further investigation in individuals who have impaired intestinal function (Kashyap et al., 2021). Significant improvements in protein digestibility have also been reported through treatment of protein sources, such as the dehulling of mung beans (Kashyap et al., 2019), highlighting that the dual stable isotope tracer technique is a robust technique for measuring protein digestibility.

Additionally, some studies have applied the dual stable isotope tracer technique to measure the digestibility of protein sources in Indian infants (< 2 years) and primary school age children (Devi et al., 2020; Shivakumar et al., 2019). The study be Devi et al (2020) focused specifically on moderately stunted children and demonstrated high digestibility values for both extruded chickpea (89.0%) and yellow pea (88.0%). There was some suggestion that the extrusion preparation process may aid digestibility as significant differences were observed between extruded chickpea and yellow pea for lysine (79.2 ± 4.2% vs 75.0 ± 4.2%, $P < 0.05$) and proline (76.5 ± 4.4% vs 72.0 ± 3.8%, $P < 0.05$). However, it should be noted that the extrusion process in this study was only applied to chickpea, with a comparison being made to yellow pea. This may not give an accurate representation of the effects of extrusion as chickpea and yellow pea have been shown to have different digestibility values for many amino acids in

adults using the dual stable isotope tracer technique (Kashyap et al., 2019). To assess the role of extrusion using the dual stable isotope tracer technique more accurately, the same protein source should be used with or without extrusion processing. Despite this, studies involving dual stable isotope tracers in children and infants do demonstrate the potential applicability of this technique across a variety of populations. Future studies could aim to target a range of populations, with the elderly and sarcopenic being potential targets given the importance of improving protein digestibility to increase muscle mass for improved function in these individuals.

Dual stable isotope tracer techniques have excellent potential for the assessment of digestibility of different protein sources. However, it is important to note that this technique does rely on some assumptions which still need to be addressed in future research. One such assumption is that there is a limited effect of stable isotopic labelling and the position of this isotopic label on metabolism pathways of the amino acids. There is some evidence to support this assumption when administering stable isotope tracers intravenously, with there being no reported differences in phenylalanine flux using ^{13}C, ^{15}N or ^{2}H phenylalanine stable isotope tracers in the fasted state (Krempf et al., 1990; Marchini et al., 1993). However, it should be noted that the study by Marchini et al (1993) did identify a significant difference in phenylalanine flux between $^{2}H_2$-phenylalanine and ^{13}C-phenylalanine (56.0 ± 9.9 $\mu mol.kg^{-1}.h^{-1}$ vs 50.5 ± 8.0 $\mu mol.kg^{-1}.h^{-1}$ respectively, $P < 0.05$) in the fed state. This is potentially relevant given the application of stable isotope tracers for feeding studies when assessing digestibility scores for protein sources.

Another potential limitation of the dual stable isotope tracer technique is that the protein source is often fed as a primed bolus, followed by regular twenty-minute smaller portions to facilitate steady state labelling (Devi et al., 2020; Moughan & Wolfe, 2019). Whilst achieving steady state labelling is essential to the validity of the dual stable isotope tracer techniques, it has been noted that this does not reflect

habitual feeding patterns (Bandyopadhyay et al., 2021). It is possible that this may potentially reduce the accuracy of digestibility estimates when compared to consumption of the same protein source as a single bolus. However, it has also been suggested by Bandyopadhyay et al (2021) that, given the reserve capacity of pancreatic proteases when consuming protein, the rate at which it is digested should not vary much in respect to consumption pattern.

1.2.4- Protein Digestibility Quantification

The ability to quantify protein digestibility using these techniques has resulted in the development of measures by which we can compare the efficacy of protein sources, accounting for their digestibility. Historically, the most widely used scale to differentiate between the quality of protein sources was the Protein Digestibility-Corrected Amino Acid Score (PDCAAS), which considers the EAA content of a test protein against a reference EAA pattern and applies a correction based on the digestibility value of the protein source (Schaafsma, 2005). The PDCAAS has some utility when applied to healthy individuals or to score the quality of single proteins, but there are several limitations which prevent its more widespread use. Most notably, this includes the truncation of all protein quality scores to a value of 100. This results in high quality proteins, which provide more EAAs than can be accounted for by PDCAAS scoring system, being given a score of 100, which is not reflective of their true EAA content and utility for supplementing lower quality protein sources in mixed meals (Schaafsma, 2012). Beyond this, there are also limitations associated with the use of faecal sampling for determination of protein digestibility which can result in overestimation of true protein quality. Additionally, there is no consideration for antinutritional factors which may inhibit protein digestion, or the potential need for corrections related to amino acid bioavailability (Boye et al., 2012).

Given the widely accepted limitations of the PDCAAS, there has been more recent support for another system which attempts to address some of these problems. The Digestible Indispensable Amino Acid Score (DIAAS) was proposed as an alternative method of assessment of protein quality in 2013 following a meeting of the Joint Food and Agriculture Organisation of the United Nations (FAO, 2013). The design of the DIAAS varies from the PDCAAS in several ways to address many of the central issues facing its predecessor (Leser, 2013; Rutherfurd et al., 2015). This includes removal of the truncation system to fully differentiate between the standard of higher quality protein sources which would have previously been limited to a score of 100 using PDCAAS. The DIAAS uses measures of true ileal protein digestibility rather than fecal protein digestibility, improving the accuracy of this measure by preventing over-estimation associated with gut microbial metabolism in fecal measures (Moughan, 2003). Given these factors, the DIAAS will be a more accurate alternative to the PDCAAS moving forward and should therefore be adopted as the new system by which protein digestibility is quantified.

As has already been established, the quality of a protein source depends both upon its amino acid composition and how efficiently these amino acids are made available through the circulation to the muscle based on the digestibility of the protein. Many of these protein sources have already been examined and reported on using DIAAS calculations to determine their quality (Bailey et al., 2020; Fanelli et al., 2021; Han et al., 2019; Herreman et al., 2020; Mathai et al., 2017; Rutherfurd et al., 2015). Whilst there has been a clear drive to expand the DIAAS database and include many protein sources, there are still numerous factors which need to be considered when applying these digestibility scores to target populations. Additionally, it has been established that there are changes in the digestive capacity of individuals with ageing. This may limit the application of the DIAAS or other similar scoring criteria which have typically been tested in young, healthy populations, highlighting the need for further research to assess the digestibility of protein sources more comprehensively across all populations.

1.3- Factors Affecting Protein Digestibility

1.3.1- Protein Blends

One of the immediate challenges facing the quantification of protein digestibility is that, within a free-living environment, most proteins consumed as part of mixed meals will contain a blend of different types of protein sources. Though it is useful to quantify the digestibility of protein sources individually using dual stable isotope tracer techniques in combination with the DIAAS, there is currently no investigation in the literature into the effects of protein blends on digestibility scores. Given the relatively recent introduction of this technique for quantifying protein digestibility, it is understandable that studies have focused on specific key protein sources which are widely consumed. However, this may provide a potential area for future research to focus on the digestibility of blends of protein sources.

1.3.2- GIT Function

Changes to the ageing GIT function have a significant impact on the digestibility and utilisation of a protein source. It has already been established that there is a reduced drive for food consumption in older individuals attributed to changes in the secretion of peripheral hormones (Moss et al., 2012), declining oral health (Walls & Steele, 2004), chemosensory changes (Rolls, 1999), and more (Rémond et al., 2015). These problems are compounded by reductions in GIT function as this reduces the digestive efficiency of protein sources that are consumed, alongside reductions in the overall protein quantity within the diet. In vitro research has demonstrated the effects of different levels of GIT functional decline on protein digestibility of various fish protein sources (Hernández-Olivas et al., 2020). This study found that there is as much as a 50% decline in the quantity of amino acids made freely available following consumption of a fish protein source when comparing a healthy adult control to conditions simulating elderly oral, gastric and intestinal conditions. Importantly, when assessing the effect of GIT status on individual amino acid profiles following

digestion, leucine was found to be one of the amino acids most affected by declines in age related GIT function (Hernández-Olivas et al., 2020), which is particularly noteworthy given the importance of leucine in regulating MPS. Whilst these findings are insightful regarding the role of the ageing GIT on reductions in protein digestibility, it is important to consider that the study by Hernández-Olivas et al (2020) used an in vitro simulation of ageing GIT conditions. Given the introduction of the non-invasive dual stable isotope tracer assessment of protein digestibility, future research could look to apply this technique to obtain an in vivo comparison of the digestibility of a protein source under different extents of GIT decline to validate the in vitro findings of this study.

1.3.3- Splanchnic Amino Acid Sequestration

Elevated splanchnic sequestration of amino acids during the digestive process may be somewhat responsible for reduced bioavailability of amino acids following protein consumption in older individuals, consequently lowering net muscle protein turnover. The splanchnic bed, consisting of the liver, stomach, small intestines, pancreas, and spleen, receive amino acids from protein digestion first, meaning that the requirements of these tissues are met before any remaining amino acids are made available to skeletal muscle (Hickson, 2015). In older rats, it has been demonstrated that leucine sequestration by the gut and liver is doubled relative to young controls (Jourdan et al., 2011). This finding has also been observed in humans following a standardized meal, where the splanchnic sequestration of leucine was significantly greater in older men than younger men (50 ± 11% vs 23 ± 2%, $P < 0.05$) (Boirie et al., 1997). These findings were accompanied by a significant negative correlation between the splanchnic extraction of leucine and the plasma appearance of leucine in both studies. This is an issue for older individuals as it means there is a reduction in the amount of amino acids being made available to the muscle. It has been well established that plasma hyperaminoacidemia is an important factor in determining muscle protein synthetic response (Bohé et al., 2003), particularly in older individuals who have a blunted anabolic sensitivity to protein feeding (Breen &

Phillips, 2011; Volpi et al., 2000). Consequently, reducing the extent of this hyperaminoacidemia by increasing first pass extraction of amino acids may be another contributing detrimental factor of protein digestion in the elderly which contributes to conditions of sarcopenia.

1.3.4- Antinutritional Factors (ANFs)

Another factor affecting protein digestibility is the presence of ANFs. These ANFs can either be naturally occurring or introduced through processing but have been found to be most common in plant and cereal proteins, which is partly responsible for the reduced digestibility of plant-based sources compared to animal sources (Tomé, 2013). Highly prevalent naturally occurring ANFs observed in these protein sources include trypsin inhibitors, haemagglutinins, tannins, phytates, glucosinolates, gossypol and uricogenic nucleic acid bases (Gilani et al., 2012). ANFs have been shown to negatively affect the digestibility of protein sources, with significant decreases up to 49% and 23% in amino acid digestibility caused by trypsin inhibitors (table 1.2) and tannins (table 1.3) respectively (Jansman et al., 1995; S. Li et al., 1998). The various ANFs work via different mechanisms to reduce the digestibility of a protein source and the subsequent bioavailability of amino acids. The insights into the mechanisms behind all the ANFs is beyond the scope of this review, but readers are directed to the following papers for further information (Gilani et al., 2012; Thakur et al., 2019). Importantly, it should be considered that the current research into the effects of ANFs has largely been carried out in animal studies using the ileal sampling technique. Whilst this is more reliable than fecal sampling, future studies could potentially look to determine the impacts of ANFs using the dual stable isotope tracer method in humans, as this would eliminate the need for pig-based models in favour of human studies which, despite the similarities between human and pig digestive systems, provide a truer representation of human physiology.

Table 1.2. Amino acid digestibility scores for six barrow pigs fed a maize starch-based diet with incorporation of either Nutrisoy (high in trypsin inhibitors) or autoclaved nutrisoy (low in trypsin inhibitors). Values are mean percentage digestibility scores. * Autoclaved Nutrisoy significantly greater than Nutrisoy ($P < 0.01$). (S. Li et al., 1998).

Amino Acid	Ileal Digestibility (%)	
	Nutrisoy	Autoclaved Nutrisoy
Essential		
Histidine	43.9	82.5 *
Isoleucine	40.4	86.3 *
Leucine	37.1	86.3 *
Lysine	40.8	79.6 *
Methionine	58.9	85.9 *
Phenylalanine	39.1	87.8 *
Threonine	36.5	73.3 *
Valine	38.2	83.6 *
Non-Essential		
Alanine	43.4	81.1 *
Aspartic Acid	42.4	72.6 *
Cysteine	35.5	67.7 *
Glutamic Acid	48.6	83.7 *
Glycine	29.4	70.2 *
Serine	36.8	80.6 *
Tyrosine	34.1	84.9 *
Arginine	45.4	90.0 *

Table 1.3. Amino acid digestibility scores for five pigs fed experimental diets. Experimental diets were high solubility control diet (HSC), high solubility high tannin diet (HSHT), low solubility control diet (LSC), low solubility high tannin diet (LSHT) and low solubility low tannin diet (LSLT). Values are mean percentage digestibility scores. * Significant difference compared to HSC ($P < 0.05$). † Significant difference compared to HSHT ($P < 0.05$). ‡ Significant difference compared to LSC ($P < 0.05$). ǂ Significant difference compared to LSHT ($P < 0.05$). + Significant difference compared to LSLT ($P < 0.05$). (Jansman et al., 1995).

	Ileal Digestibility (%)				
Amino Acid	**HSC**	**HSHT**	**LSC**	**LSHT**	**LSLT**
Essential					
Histidine	84.2†‡ǂ+	74.0*ǂ	74.5*ǂ	64.6*†‡+	71.5*ǂ
Isoleucine	85.6†‡ǂ+	77.2*ǂ	80.0*ǂ	72.4*†‡+	77.7*ǂ
Leucine	88.9†‡ǂ+	81.7*ǂ	80.1*ǂ	72.8*†‡+	78.9*ǂ
Lysine	85.1†‡ǂ+	76.9*‡ǂ	68.8*†	63.4*‡+	72.9*ǂ
Methionine	84.0†‡ǂ+	72.3*+	76.2*ǂ	68.2*†+	78.1*‡ǂ
Phenylalanine	87.7†‡ǂ+	81.2*ǂ	81.1*ǂ	73.2*†‡+	80.3*ǂ
Threonine	73.2†‡ǂ	62.7*	66.6*ǂ	59.7*†+	67.6ǂ
Valine	85.3†‡ǂ+	78.4*ǂ	77.0*ǂ	69.0*†‡+	75.6*ǂ
Non-Essential					
Alanine	77.7†ǂ	63.3*‡+	73.8†ǂ	63.1*†+	72.5‡ǂ
Aspartic Acid	83.6†‡ǂ+	75.1*ǂ+	72.0*	67.7*‡	70.2†ǂ
Cysteine	31.6†ǂ	2.5*‡ǂ+	43.3†ǂ	20.4†‡+	43.7‡ǂ
Glutamic Acid	89.9‡ǂ+	85.9‡ǂ+	80.2*†ǂ	74.7*†‡+	80.7*‡ǂ
Glycine	65.7†ǂ	43.9*‡+	57.9†	49.6*+	62.4‡ǂ
Proline	84.1‡ǂ+	71.4ǂ	56.3*	41.3*‡+	66.3*ǂ
Serine	80.7†‡ǂ+	71.4*ǂ	71.6*ǂ	64.0*†‡+	72.6*ǂ
Tyrosine	88.7†‡ǂ+	79.2*ǂ	79.7*ǂ	70.0*†‡+	76.1*ǂ

Arginine	89.9†‡‡	82.6$^{*\ddagger+}$	84.9*‡	78.0$^{*\dagger\ddagger+}$	87.8‡‡

It has also been suggested that the presence of ANFs is more detrimental to protein source digestibility in combination with ageing (Gilani & Sepehr, 2003). The study by Gilani and Sepher (2003) found a small but significant decrease in digestibility scores of properly processed animal and vegetable protein sources of up to 3% and 5% respectively when comparing old rats to young rats (both $P < 0.05$). By contrast, when assessing protein sources high in ANFs, there was a greater decline in digestibility scores from young to old rats of between 7-17%. This finding is potentially very important when considering the role of diet in older and sarcopenic populations, as this may further emphasise the need for consideration of the types of protein sources that older individuals are fed in addition to the current focus on protein quantity in the diet. Furthermore, this would also highlight the importance of processing strategies to remove the presence of ANFs, particularly when preparing food sources for older populations. However, these findings do require further validation using current techniques, as this data was collected using fecal sampling in rats and the PDCAAS scoring system. This limits the applicability to human populations, and the results were also confounded by contributions of microbiota, reducing their accuracy. The authors did report this potential limitation within their study design but given the lack of validation of ileal sampling techniques at the time, the non-existence of the DIASS scoring system, and the introduction of the dual stable isotope tracer method for protein digestibility coming over a decade after this publication, the study was somewhat limited by the available techniques of the time. The findings do however warrant potential interest from future studies using more novel, accurate techniques which can be carried out in humans to assess the significance of the combination of deleterious effects caused by ANFs and ageing on the digestibility of protein sources.

1.4- Improving Protein Digestibility

1.4.1- Increased Protein Intake

The elevated splanchnic sequestration of amino acids in the elderly has been a target of strategies to improve the digestibility of protein sources. Evidently, one way to tackle this challenge would be to increase the protein intake of older people to ensure greater levels of plasma hyperaminoacidemia. It has previously been suggested that the current recommended dietary allowance of 0.8g/kg/day of protein should be considered as the minimum requirement to prevent malnutrition, rather than the optimal requirement which should not be exceeded (Wolfe & Miller, 2008). Importantly, the recommended dietary allowance is meant as general guidance for the entire population and does not consider individual differences, particularly those related to age and health. Consequently, it has been suggested that the recommended protein intake of 0.8g/kg/day is insufficient to meet the needs of older populations, with a more suitable target being in the region of 1.5g/kg/day (Wolfe et al., 2008).

In support of this, there has been evidence published demonstrating greater function in older women with protein intakes greater than 1.2g/kg/day compared to older women with moderate (0.8-1.19g/kg/day) and low (< 0.8g/kg/day) protein intakes (Isanejad et al., 2016). These include measures of muscle strength, namely hand grip strength/body mass, knee extension strength/body mass, and squats, as well as functional measures such as chair rise, one leg stance, 10m walking speed and greater physical performance battery scores, as can be seen in table 1.4. Other studies have shown that greater physical function has been found to be significantly correlated to elevated quality of life and reductions in mortality risk (Bjerk et al., 2018; Cesari et al., 2008). If applied at a population level, the benefits of increased protein intake on function may translate to improvements in quality of life and mortality based on these correlations. However, future studies using high protein

nutritional interventions and assessing quality of life and morbidity should be considered to elucidate the potential benefits of higher protein diets in the elderly. It should also be noted that the study by Isanejad et al (2016) used a very large sample size, with the high, moderate, and low protein intake groups having sample sizes of 112, 269 and 171 respectively. This means that some of the significant differences observed between groups in the functional measures have relatively small effect sizes, further reinforcing the need for randomised control studies to determine the potential benefits of increases in habitual protein intake.

Table 1.4. Physical performance test outcomes for 552 women separated into low (< 0.8g/kg/day), medium (0.8–1.19g/kg/day) and high (> 1.2g/kg/day) protein intakes. Results shown are at baseline and at three years follow up. All values are mean ± SD. * Significant different from medium and high intake groups (P < 0.05). † Significantly different from high intake group (P < 0.05). (Isanejad et al., 2016).

Physical Performance Measure	Timepoint	< 0.8g/kg/day protein intake	0.8–1.19g/kg/day protein intake	> 1.2g/kg/day protein intake
Hand-grip strength/body mass	Baseline	0.32 ± 0.08*	0.37 ± 0.06†	0.40 ± 0.01
	Change	-1.51 ± 6.70*	-0.79 ± 3.68†	-0.68 ± 1.32
Knee extension/body mass	Baseline	3.71 ± 1.13*	4.34 ± 1.25†	4.47 ± 1.32
One Leg Stance for 30 seconds	Baseline	15.79 ± 10.90*	19.31 ± 10.28†	21.54 ± 9.42
	Change	-1.64 ± 10.02*	-1.50 ± 10.89†	-0.96 ± 10.48
Chair rises	Baseline	7.87 ± 6.97*	7.84 ± 2.86†	8.41 ± 2.20
	Change	0.12 ± 6.07*	0.83 ± 2.82†	1.15 ± 2.68
10m walk speed	Baseline	1.53 ± 0.31*	1.67 ± 0.32†	1.72 ± 0.28
	Change	-0.11 ± 0.24	-0.10 ± 0.33	-0.11 ± 0.29
	Baseline	94.1*	95.6†	97.0

Standing with eyes closed for 10 seconds	Change	-5.54	-5.19	-4.94
Squat ability (%)	Baseline	91.1*	94.3†	97.0
	Change	-0.08*	0.32†	0.21
Squat to the ground ability (%)	Baseline	58.0*	69.8†	78.7
	Change	-0.02	-0.01	-0.06
Short physical performance battery score	Baseline	5.52 ± 1.82*	6.28 ± 1.87†	6.51 ± 1.77
	Change	1.35 ± 0.21	1.55 ± 0.14	1.57 ± 0.24

Whilst there is some consensus in the literature that the recommended dietary allowance for protein intake should be revisited, particularly for elderly individuals who may require greater protein intake to maintain muscle mass, there has also been some focus on the potential deleterious effects of increasing protein intake too much. Numerous adverse health effects, including renal function disorders, increased risk of cancer, reduced liver function and coronary artery disease progression have been linked to high protein intakes, particularly animal source proteins (Delimaris, 2013). The mechanisms by which high protein intakes may contribute to these various diseases and disorders are beyond the scope of this review, but readers are directed to the comprehensive review article by Delimaris (2013) for further information. By comparison to the aforementioned health complications, there has been a greater focus in the literature on the effects of high protein intakes on bone quality. Given the focus that has been directed towards bone health in high protein diets in the literature, as well as the evident functional impacts of reductions in bone quality, this will be focused on in greater detail within this review.

A major concern associated with highly elevated protein intakes is dysregulation of calcium homeostasis which may negatively affect bone quality (Delimaris, 2013). This is caused by the need for greater acid excretion associated with increased protein intake, resulting in resorption of bone to provide a buffer for the excess acid (Barzel & Massey, 1998). Urinary N-telopeptide excretion has been used as a biochemical marker for bone quality and has been applied in the assessment of osteoporosis (Ganesan & Vijayaraghavan, 2019). Previous research assessing the effects of a low protein (0.7g/kg/day) versus a high protein (2.1g/kg/day) diet on bone quality demonstrated a significant increase in urinary N-telopeptides over the four days of the dietary intervention, with a mean of 32.7 ± 5.3 compared to 48.2 ± 7.2 for the low and high protein diets respectively (Kerstetter et al., 1999). Whilst this does highlight the negative effects of a high protein diet on bone resorption, it should be noted that the quantity of protein in the high protein diet is reflective of the upper end of protein intakes for young elite weightlifters (Lemon, 1991). This far exceeds the increase in protein intake that would be recommended for elderly individuals looking to prevent sarcopenia. Notably, the study by Kerstetter et al (1999) also included a medium protein intake group (1.0g/kg/day) which is somewhat more reflective of the elevation in protein intake that would be recommended for most elderly individuals. Crucially, the increase in urinary N-telopeptides from the low to the medium protein intake groups in this study were found to be nonsignificant.

In addition to the biochemical assessment of bone quality, clinical outcomes including hip and forearm fractures across a range of protein intakes have been assessed using prospective cohort studies (Feskanich et al., 1996). Interestingly, this study found that women who had protein intakes greater than 90g per day had a significantly increased risk of forearm fracture compared to those with intakes less than 68g. Whilst this evidence is a contraindication for the use of high protein diets, it has been highlighted that, within the same prospective cohort study, women with diets high in both protein and calcium did not have increased forearm fracture relative risk (RR) compared to women with moderate protein intakes, ranging from 68-95g protein per day (de Souza Genaro & Martini, 2010; Feskanich et al., 1996).

Women who had a high protein and low calcium intake had a RR of 1.15 compared to a RR of 1.31 in women with a high protein, low calcium intake. This would suggest that the high protein intake itself is not the determining factor in adverse health outcomes such as fractures, but it is instead the combination of a high protein diet with low calcium intake that elevates risk. This belief has been further supported by other prospective cohort studies which have reported this finding when assessing the association between protein intake and fracture risk (Dargent-Molina et al., 2008; Meyer et al., 1997). To further counteract the argument that high protein intakes negatively impact bone health, it has been shown in older women (58 ± 6 years) that an increase in dietary protein content concomitantly increases calcium absorption at the small intestine which almost entirely compensates for the elevated calcium loss in urine, when fed a lower calcium diet (Hunt et al., 2009). From this evidence, the use of high protein diets in the elderly to increase muscle mass and prevent sarcopenia should not be discredited based on potential deleterious effects on bone health, as these are largely mitigated with adequate calcium intake.

1.4.2- Pulse Feeding

As has been established, it is crucial to induce plasma hyperaminoacidemia to elevate MPS above MPB and induce a state of anabolism. However, this is more challenging in older individuals who have greater first pass splanchnic amino acid extraction (Boirie et al., 1997), resulting in the balance between MPS and MPB being shifted in favour of catabolism. Increasing habitual protein intake has already been proposed as a solution to induce greater hyperaminoacidemia, though there are alternative strategies which do not require increasing protein intake. One such strategy is the use of a pulse feeding diet, where 80% of the daily protein intake is contained within a single meal rather than being spread evenly across meals throughout the day (Arnal et al., 1999). This feeding pattern overcomes the elevated splanchnic amino acid sequestration to ensure that sufficient plasma hyperaminoacidemia is achieved in older individuals. Pulse feeding has been utilised effectively in hospitalised elderly patients to produce significantly greater increases

in amino acid bioavailability compared to a spread protein feeding pattern (Bouillanne et al., 2014). Importantly, this study also demonstrated that there is still a greater plasma hyperaminoacidemia after six weeks of consuming the experimental diets, suggesting that there is no adaptation to the pulse feeding diet reducing its effectiveness in the long term. Pulse feeding diets have also been shown to produce significantly greater increases in lean mass in hospitalised elderly patients compared to spread feeding diets over a six-week period, with the pulse diet producing a 0.38 kg/m^2 increase (95% confidence interval, 0; 0.60) compared to a -0.21 kg/m^2 change (95% confidence interval, -0.61; 0.20) in the spread diet. These findings in hospitalised elderly patients are extremely promising for the use of pulse feeding diets in elderly individuals, but future studies should look to assess the efficacy of this nutritional strategy in a free-living environment to further validate its use.

1.4.3- Type of Protein Source

As well as manipulating the pattern of protein consumption, the type of protein source can also potentially be altered to further elevate plasma hyperaminoacidemia to promote greater MPS increases in older individuals. Different protein sources are digested at different rates, with some being digested rapidly such as whey protein, and others being digested more slowly such as casein protein. Previous research in young people has found that, despite a greater initial aminoacidemia following whey protein digestion, slower digesting casein proteins result in a higher leucine balance in the postprandial period because this hyperaminoacidemia persists for longer compared to the transient effect observed with whey protein (Dangin et al., 2001). Interestingly, the opposite effect has been observed in older individuals, where whey protein feeding produces greater increases in mixed muscle fractional synthesis rates than casein protein (Pennings et al., 2011). This has been attributed to the importance of overcoming the increased splanchnic sequestration in older individuals to ensure that hyperaminoacidemia is induced following the protein feed. This further reinforces the efficacy of manipulating nutritional practices in older

individuals to optimise the protein synthetic response, which could serve as a potential alternative to simply increasing daily protein intake.

1.4.4- Food Processing

As discussed previously, a potential challenge of protein digestibility in the elderly is reductions in the quality of the GIT health of this population which limits the availability of plasma amino acids following digestion. Some research has highlighted the efficacy of various food processing techniques which may improve the digestibility of protein sources in these more vulnerable target populations (Lee et al., 2021).

One such common technique is the thermal processing of protein sources. The cooking of protein sources has been found to be effective in improving protein digestibility of both meat (Bax et al., 2012; Lee et al., 2021) and vegetable (Kataria et al., 1989) proteins respectively. This has largely been attributed to the unfolding of proteins as cooking takes place which increases the accessibility of enzymes to cleavage sites, facilitating more complete hydrolysis. Additionally, in plant-based protein sources, cooking can improve protein digestibility by reducing levels of ANFs, including trypsin inhibitors, lectins and phytic acids (Habiba, 2002). As mentioned previously, ANFs can significantly reduce protein digestibility, particularly in older individuals, making them a central target of processing techniques for older, sarcopenic people. Whilst there are well documented benefits of thermal processing for protein digestibility, it is also possible to reduce the digestibility of protein sources by cooking them at temperatures that are too high or for too long. This is caused by protein aggregation from the formation of disulphide bonds and this aggregation subsequently limits proteolysis rates (Gomes Almeida Sá et al., 2019; Joye, 2019). It is important to note that different types of protein sources respond to different cooking temperatures and techniques differently (Gomes Almeida Sá et al., 2019). This may make it difficult to apply the appropriate cooking techniques to the

specific protein source in a real-world setting, given that many older individuals will be preparing their own meals and may not have access to the appropriate information for thermal processing of specific protein sources.

When discussing the effectiveness of thermal processing, it should also be considered that there are various cooking techniques which can have different impacts on the overall digestibility of a protein source. Many of these techniques have been applied to a range of plant protein sources to quantify their effects on digestibility, including soaking, boiling, roasting, autoclaving, microwaving, fermentation and micronization (Khattab, Arntfield and Nyachoti, 2009). The study by Khattab et al (2009), demonstrated that in vitro protein digestibility was significantly increased across the six plant protein sources relative to raw digestibility following soaking (mean increase = 5.27 ± 0.12%) , boiling (mean increase = 16.01 ± 0.44%), microwaving (mean increase = 10.71 ± 0.22) , fermentation (mean increase = 2.84 ± 0.09%) and autoclaving (mean increase = 8.15 ± 0.15) but not for roasting (mean decrease = 5.14 ± 0.31%) or micronization (mean decrease = 2.43 ± 0.15%). Whilst this highlights the importance of thermal processing of protein sources to increase their digestibility, it should be noted that some of these techniques may not be readily available on a population level, limiting their applicability in a real-world setting. Additionally, there is also a need to evaluate the potential benefits of these increases in protein digestibility on muscle mass and functional outcomes if these techniques are applied for a prolonged period.

Germination is a commonly used processing technique for plant protein sources whereby seeds are typically soaked in water until sprouting occurs (Khetarpaul and Chauhan, 1990), during which time the seed becomes much more metabolically active. Germination has been found to significantly improve the digestibility of various legume protein sources, including lentils, chickpea, cowpea, and green gram (all $P < 0.05$) (Ghavidel & Prakash, 2007). The mechanisms behind the benefits of germination on protein digestibility have mostly been attributed to its effects on

ANFs. Germination has been shown to reduce the levels of various ANFs, including trypsin inhibitors, phytic acids, lectins and more (Bau et al., 1997), which would result in an improvement in the digestibility of protein sources being treated. Other research has also suggested that germination may contribute to the unfolding of the protein structure based on an increase in the surface hydrophobicity of sesame proteins after two days of germination treatment (Di et al., 2022). This is particularly important to consider in developing regions of the world where protein intake is lower than in developed regions and is also comprised of a greater proportion of vegetable protein sources (Grigg, 1995). Consequently, it is imperative to maximise the digestibility of the most readily available protein sources to these populations, with techniques such as germination providing a valuable means by which to enhance the digestibility of vegetable protein sources. It should be considered that the effects of germination can be variable based on the seed species being treated, the temperature at which seeds undergo germination and the duration of the treatment, so future research should assess the optimal treatment conditions for some of the most consumed plant protein sources.

1.5- Summary and Future Directions

Within the context of a globally ageing population, strategies to minimise the prevalence of conditions such as sarcopenia are becoming increasingly important. There are inherent age-related declines in physical function associated with reductions in muscle mass, irrespective of contributing factors such as physical activity levels and protein intake. Whilst this decline cannot be prevented, it can be slowed to ensure a healthy ageing process, maximising function and quality of life into old age. One such strategy to achieve this is through optimising nutritional strategies related to protein intake to ensure that, following protein feeding, MPS is being stimulated beyond the rate of MPB, ensuring a shift in favour of muscle protein anabolism. Many factors contribute to the effectiveness of a protein source for stimulating MPS in the elderly, with the digestibility of a protein source being

critical to ensuring that amino acids are made available through the circulation for uptake and utilisation at the muscle.

The determination and quantification of protein digestibility has been an evolving field, with new techniques looking to enhance the accuracy of measurements by removing the effects of confounding factors which may have previously impacted digestibility estimates. To achieve the desired level of accuracy, previous techniques required highly invasive procedures to be able to sample from the terminal ileum. However, the introduction of the dual stable isotope tracer technique has provided a non-invasive alternative to assess protein digestibility for all the amino acids simultaneously. The lack of invasiveness of the dual stable isotope tracer technique is critical as this results in excellent potential for the assessment of numerous protein sources. With the relatively recent introduction of this technique, there is still a need for validation against other techniques, such as the relatively more invasive sampling of the terminal ileum, across individual protein sources. Once there has been categorisation of the digestibility of many commonly consumed protein sources across a variety of diets using the new DIAAS categorisation, the dual stable isotope tracer technique will also allow for research that may be more applicable for a free-living environment. One such field to explore could be the effects of different blends of protein sources on their digestibility, given that many mixed meals contain various types of protein sources in this setting.

This review also explored some of the key factors affecting protein digestibility. This includes the role of ageing, which is particularly pertinent to consider given the importance of protein digestibility and utilisation on the reduction of sarcopenia prevalence and improvement of function and quality of life in the elderly. There should be emphasis on the role of the ageing GIT which has been shown to reduce protein digestibility, as well as the elevated splanchnic sequestration of amino acids that occurs in older individuals. The combination of these effects may be particularly deleterious in older individuals, given that reductions in GIT function limit plasma

amino acid availability following digestibility whilst older individuals require greater levels of hyperaminoacidemia than younger individuals to stimulate MPS. Additionally, beyond the effects of the individual on reductions in protein digestibility, there are also the effects of the protein source which should be considered. ANFs have been found to play a significant role in the reduced digestibility of a protein source and are particularly prevalent in vegetable protein sources, largely explaining the widely reported reduced digestibility scores of vegetable sources compared to animal protein sources.

Numerous strategies have been highlighted which can improve the digestibility of protein sources, ranging from increasing habitual protein intake to increase hyperaminoacidemia and overcome increased splanchnic sequestration of amino acids, to manipulating the pattern and type of protein source consumption. Importantly, these strategies would be viable within a free-living environment and could be recommended to target populations to promote improved protein digestibility. Additionally, other techniques, such as various processing methods for protein sources, have been examined. Evidence in the literature has highlighted the efficacy of some of these processing methods and cooking techniques for improving protein digestibility. However, there should be some consideration for the capacity of certain processing methods outside of a research setting, as some techniques require specialist equipment or long cooking times. Evidently, any technique which can produce a significant increase in protein digestibility is extremely promising for application to older populations. Given the overarching aim of limiting sarcopenia and improving function in older individuals, future research should focus on using randomised control trials to assess the capacity of these techniques when they are used for a prolonged period to bring about significant improvements in muscle mass and functional outcomes in older individuals.

Chapter 2- Investigating the Digestibility and Bioavailability of Selected Protein Sources in Older Adults

2.1- Introduction

The importance of inducing plasma hyperaminoacidemia following protein feeding has already been well established in the prior review, including a particular focus on overcoming the increased splanchnic uptake of amino acids in older individuals to ensure that the necessary hyperaminoacidemia is still achieved. This is because the consumption of protein and subsequent utilisation of amino acids in skeletal muscle provides the anabolic stimulus to elevate MPS above MPB, which is necessary to offset the net loss of amino acids to oxidation and gluconeogenesis that occurs in the postprandial state (Ruderman, 1975; Wagenmakers, 1998), achieving a so-called dynamic equilibrium (Mitchell et al., 2016). Notably, previous research has highlighted that this increase in MPS is transient irrespective of remaining plasma amino acid concentrations and, in the absence of external stimuli such as exercise training, is only capable of maintaining muscle mass, an effect which has been labelled as 'muscle full' (Atherton & Smith, 2012). Given the myriad of challenges facing older individuals in relation to consumption of adequate protein and inducing the necessary hyperaminoacidemia to facilitate increases in MPS as discussed in the '*Factors Affecting Protein Digestibility*' section, it is essential to ensure that protein sources are optimised to mitigate this anabolic resistance that naturally occurs with ageing (Breen & Phillips, 2011). This anabolic resistance is further compounded by inactivity, which is particularly common in older individuals because of worsening physical function and loss of independence (Cunningham et al., 2020). Inactivity has been found to result in muscle reaching a full state prematurely, characterised by blunted pre and postprandial MPS (de Boer et al., 2007; Drummond et al., 2012), which is particularly pertinent when considering the development and prevalence of sarcopenia in older populations (C. S. Deane et al., 2021). This further reinforces the need to optimise the digestibility of protein intakes in older individuals to counteract

the combination of natural age-related anabolic resistance and the amplified resistance that occurs in sedentary older individuals.

Plasma hyperaminoacidemia is essential to stimulating MPS (Bohé et al., 2003), but it is important to acknowledge that not all amino acids are equally potent in achieving this. BCAAs, EAAs, and of these, particularly leucine, can stimulate MPS more effectively than non-essential amino acids (NEAAs) for instance. Indeed, it has been shown that only 10g of EAAs alone is enough to maximally stimulate MPS in both young and old individuals, with no further increases in MPS observed with 20 g and 40 g EAA doses (Cuthbertson et al., 2005). Expectedly, the study by Cuthbertson et al (2005), also showed that there existed a blunted maximal MPS response in older individuals compared to young. By comparison, a study which performed similar analysis with different doses of whey protein ingestion found that MPS rates were maximally stimulated at 20 g of whey protein, with no further increases in MPS occurring with ingestion of a 40 g bolus (Witard et al., 2014). The lower quantity of EAAs required for maximal MPS stimulation compared to whey protein ingestion demonstrates the greater efficiency of EAAs compared to a protein source with a mixed amino acid profile. Additionally to the reported benefits of EAAs alone, enrichment of EAAs with leucine has been found to further increase the efficiency of these sources for stimulating MPS, with a dose as low as 1.5 g of EAAs enriched with 0.6 g of leucine inducing maximal MPS rates at rest (Wilkinson et al., 2018). This increase in MPS following 1.5 g of leucine enriched EAA consumption was equally as robust as 6 g of leucine enriched EAA, as well as 40 g of whey protein, indicating that maximal MPS rates were indeed achieved by all three treatments, none of which were significantly different from each other at 2 and 4 hours post feeding. Based on all of this, research should focus on nutritional interventions which are able to elucidate an improved bioavailability of BCAAs, particularly leucine.

The plasticity of MPS in response to protein feeding has made this a central focus of strategies to increase muscle mass. However, it should be noted that, whilst

increases in MPS do play the most integral role in promoting anabolism, this is not to say that there is no contribution of reductions in MPB. Insulin only plays a permissive role in stimulating MPS and is instead arguably more potent for affecting protein balance through an important anti-proteolytic role (Abdulla et al., 2016). The clamping of insulin at 15 μU/ml, which is equivalent to approximately three times greater than postabsorptive insulin concentrations, was able to inhibit MPB by approximately 50% (Wilkes et al., 2009). Importantly, this same reduction in MPB was not achieved following primed constant infusion of mixed amino acids at a rate of 18 g/hour with an insulin clamp at postabsorptive levels of 5 μU/ml (Greenhaff et al., 2008). This indicates that the reductions in MPB are attributable to insulin itself and are in no way a consequence of surplus amino acid availability. Whilst this 50% depression of MPB is a far smaller magnitude than the three-fold reported increase in MPS following 48 g whey protein isolate (Atherton et al., 2010), the impact that insulin can have on suppressing MPB and promoting anabolism should not be ignored.

One potential strategy by which protein intakes can be optimised is through changing the type of the protein source that is consumed. Whey protein has received much attention in the literature as a particularly effective protein source and has been shown to be more robust than other protein sources such as soy proteins for increases in lean muscle mass following resistance training (Phillips et al., 2013). Regarding older individuals and the prevention of sarcopenia, whey protein has been proposed as a potentially effective protein feeding option, as discussed in the '*Improving Protein Digestibility'* section of the previous review. Whey protein has been shown to produce significantly greater increases in MPS than both casein and casein hydrolysate proteins in older individuals (Pennings et al., 2011). The authors attributed this to a combination of the more rapid digestion rates of whey protein compared with casein which elevates the rate of plasma amino acid appearance, in addition to the greater leucine content of whey protein compared to casein. Similar findings have been reported in other studies (Burd et al., 2012), further reinforcing the importance of utilising fast digesting proteins such as whey to

overcome the increased splanchnic sequestration of amino acids and induce a potent hyperaminoacidemia in elderly individuals which then translates to greater increases in MPS.

In addition to evaluating the effectiveness of individual types of protein, there has been some interest in the literature for the efficacy of protein blends, combining different protein types for enhanced increases in MPS (Olaniyan et al., 2021). This is because it has been well established that animal-based protein sources are superior to plant-based protein sources because of their improved digestibility, facilitating greater increases in MPS. However, plant-based protein sources present a more environmentally sustainable option, meaning that the capacity to induce similar or greater anabolic responses from plant-based sources compared to animal sources would be highly beneficial. Protein blends may be able to achieve this to some extent by combining protein sources with different amino acid profiles, compensating for any potential shortcomings within the amino acid profile of one individual plant protein source and ensuring the complete availability of amino acids, particularly BCAAs, EAAs and leucine (Deane et al., 2020).

Protein blends have been tested to a limited extent in postexercise conditions, with studies reporting similar increases in MPS between whey and protein blends in both young and old men (Borack et al., 2016; Reidy et al., 2013). This should be somewhat expected given that whey protein and the protein blends consumed in these studies provided similar quantities of EAAs and leucine. Despite this, it is still noteworthy that a protein blend containing a mixture of plant and animal protein sources could elicit a similar response to whey protein alone. Beyond these human studies, there has been a relative lack of research in this field, with few attempts to characterize the potential efficacy of different compositions of protein blends or to assess their role in populations outside of post-exercise conditions. One research study carried out in rats claims to support the use of protein blends, finding a significantly higher MPS response at 135 minutes for a 25: 50: 25 whey: caseinate: soy protein blend

compared to whey protein following a 4g feed (Butteiger et al., 2013). Whilst this may support the use of protein blends to enhance and prolong the anabolic effects of protein feeding, it should be considered that there was no significant increase relative to baseline for either whey protein or soy protein isolates. This may suggest that a higher quantity of protein was necessary in the feed to produce more robust increases in MPS, or this may reflect on the low sample size of 5-6 rats per timepoint, resulting in large SEM values and difficulty in detecting significant differences.

The current research does provide promising evidence for the utilisation of protein blends, but it is evident that more robust studies are needed within humans to corroborate these preliminary findings, particularly in resting conditions. To date, research studies investigating the efficacy of protein blends have also focused on blends containing primarily animal-based protein sources, comprised of only 25% plant-based protein sources within the blends (Deane et al., 2020). Given that plant-based sources have lower digestibility scores and reduced leucine and EAA content compared to animal sources, the extent to which the composition of the blend could be shifted in favour of plant-based sources without compromising the anabolic response should be tested. Indeed, with a myriad of different protein sources available, it is evident that far more research is necessary to optimise the use of protein blends and characterise the potential that they could present for alternative sustainable and robust protein sources.

Based on all of this, it is evident that, as a precursor to critical outcomes such as MPS, plasma amino acid bioavailability is of paramount importance. The following study aimed to assess the changes in plasma amino acid concentrations following consumption of three different protein blends, each standardised to 20 g of protein intake, with a particular focus on the BCAAs, EAAs and leucine because of their importance and potency which has been well established within the literature. The protein blends selected covered a range of animal and plant-based protein sources in varying quantities, as well as including both simple blends of only two different

protein sources and complex blends of four protein sources. Plasma insulin was also measured based on the anti-catabolic effects that it has been established to exhibit. It was hypothesised that the different compositions of these protein blends would affect the in vivo digestibility of the protein sources. In turn, this would alter the resulting patterns of increases in plasma total BCAA, total EAA and leucine bioavailability observed in response to each of the three test drinks.

2.2- Methods

2.2.1- Participants and Ethical Approval

Nine healthy older males (aged 65-75) volunteered to take part in this study. Details of the participant characteristics can be found in table 2.1. This study was approved by the University of Nottingham Faculty of Medicine and Health Sciences Research Ethics Committee (116-1120) and conformed with the Declaration of Helsinki. After having the details of the study explained to them, participants provided written informed consent for their involvement in the study. Potential participants were screened for eligibility against pre-determined exclusion criteria, including body mass index (BMI) >18 or <32 $kg \cdot m^2$, active cardiovascular, cerebrovascular, respiratory, renal, or metabolic disease, malignancy, musculoskeletal or neurological conditions. Participants who met all the inclusion criteria were then invited back to the laboratory to take part in the study.

Table 2.1. Descriptive characteristics of participants.

N = 9	Mean ± SD
Age (years)	72.4 ± 3.2
Height (cm)	179.8 ± 6.3

Weight (kg)	82.9 ± 12.2
BMI (kg/m^2)	25.5 ± 2.4

2.2.2- Experimental Design

This study was designed as a repeated measures study design with three independent variables. Consequently, participants were required to come into the lab on three separate occasions separated by approximately a week each. At each visit, the participants were randomly allocated to one of the three test protein drinks in a double-blind manner. The three protein drinks were drink 1 (80: 20, casein: whey), drink 2 (35: 25: 20: 20, whey: casein, soy, pea) and drink 3 (51: 49, casein: soy).

On the day of the study visit, participants reported to the laboratory in the morning in the postabsorptive state. A single cannula was inserted into the antecubital vein of one arm under aseptic conditions to facilitate venous blood sampling. A baseline blood sample was taken both 15 minutes prior to and immediately before consumption of the randomly allocated protein drink. The protein drink was consumed as a single bolus which was standardised to 20 g of protein intake for each of the three drinks. Following this, blood samples were taken every 15 minutes for the first 90 minutes, followed by every 30 minutes until 240 minutes after consumption of the protein bolus, at which point all the blood samples were collected and the cannula was removed. A schematic showing the timepoints for collection of all blood samples can be found in figure 2.1. The same protocol was repeated for all three of the test drinks.

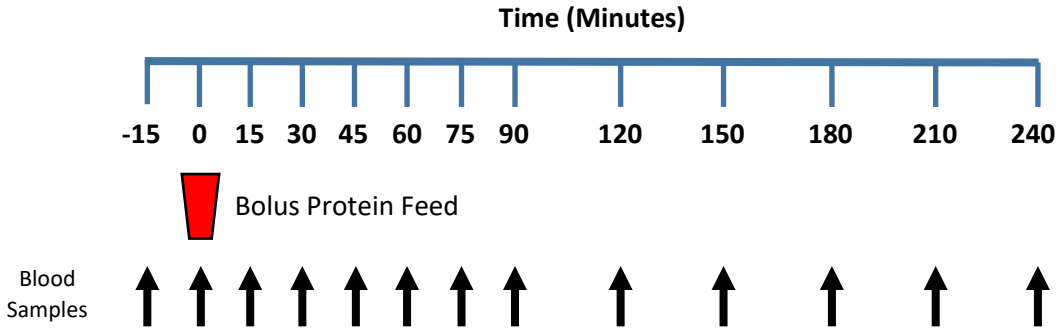

Figure 2.1. Schematic of study protocol.

2.2.3- Plasma Amino Acids

Following collection, blood samples were centrifuged for 20 minutes at 3200 RPM to isolate plasma samples. Plasma samples were then stored at -80 °C after collection ready for analysis.

For analysis of the plasma amino acid concentration, samples were prepared in accordance with our established standard methods (Wilkinson et al., 2013). Plasma samples containing a stable isotopically labelled internal standard were precipitated with 1ml ice cold ethanol and centrifuged at 9600 RPM for five minutes. Following this, the supernatant was removed and dried down under nitrogen at 90 °C. 500 µl of 0.5 M HCL was then added followed by lipid extraction using ethyl acetate. Briefly, this involved adding 2 ml of ethyl acetate and vortexing before removal of the top ethyl acetate layer which contains the lipids. The remaining bottom layer containing the amino acids was then evaporated to complete dryness under nitrogen at 90 °C. The samples were then derivatised using a combination of a 70 µl 1: 1 acetonitrile: dimethylformamide mix and 70 µl N-Methyl-N-(tert-butyldimethylsilyl)trifluoroacetamide (MTBSTFA). Samples were heated to 90 °C for one hour, converting the amino acids to their t-butyldimethylsilyl (tBDMS)

derivatives. Amino acid enrichments were then determined by GC-MS and compared to a reference standard curve which was prepared using a standard amino acid mix of known quantities.

GC-MS analysis was performed using an ISQ Trace 1300 single quadrupole GC-MS (Thermofisher Scientific, Hemel Hempstead, UK). 0.5 µl of sample was injected into the GC-MS using a 1: 10 split injection mode with a starting oven temperature of 100 °C for one minute. The temperature was then increased at a rate of 12 °C/min to 300 °C, at which point the temperature remained constant for five minutes. This analysis required helium as a carrier gas with a flow rate of 1.5 ml/min. Sample separation took place on a 30 m Rxi-5ms fused silica column with 0.25 mm internal diameter and 0.25 µm thickness (Restek, Bellafonte, Pennsylvania). A selected ion monitoring (SIM) scan was used to search for amino acid masses and their corresponding internal standard masses for analysis.

2.2.4- Plasma Insulin

Plasma insulin was measured using an ultrasensitive human insulin ELISA kit (Mercodia, Uppsala, Sweden). All samples were diluted with an eight times dilution and were run in duplicate and in accordance with the manufacturer's instructions as provided with the test kit.

2.2.5- Statistical Analysis

All values are presented as mean ± SEM, unless stated otherwise. Both the plasma amino acid response and the plasma insulin response were assessed using a two-way repeated measures ANOVA (drink * time). Dunnett's post hoc test was used to identify individual significant differences. When performing within-drink post hoc analysis for significant differences over time, baseline was treated as 0 minutes.

Statistical significance was accepted at the $P < 0.05$ threshold. Results which surpassed this level of significance are stated as such including notations as described in figure legends. Analysis was carried out using GraphPad Prism Version 9 (GraphPad Software, CA, USA).

2.3- Results

2.3.1- Plasma BCAAs

A summary of the changes in plasma BCAA concentrations in response to the three test drinks are given in figure 2.2 and table 2.2. Comparisons between drinks at each timepoint are shown in figure 2.2A, with there being a significant main treatment and time effect being observed (both $P < 0.001$). There was also a significant difference between drink 2 and drink 3 at 120 minutes (563.4 ± 29.7 μM/L vs 476.6 ± 22.5 μM/L respectively, $P < 0.05$). Plasma BCAA concentrations normalised to baseline are shown in figure 2.2B with comparisons between drinks at each timepoint. Significant main treatment and time effects were also found for the change in BCAA concentration when normalised to baseline ($P < 0.01$ and $P < 0.001$ respectively). There was a significant difference for the change in plasma amino acid concentration between drink 1 and drink 2 at 240 minutes (57.2 ± 19.9 μM/L vs -40.0 ± 20.8 μM/L respectively, $P < 0.01$). No significant differences were found between the AUCs for the three drinks which are depicted in figure 2.2C.

Comparisons within drinks for each timepoint compared to baseline are demonstrated in table 2.2. All three drinks produced significant increases in plasma BCAA concentration relative to baseline after 30 minutes. This significant increase lasted until 150 minutes for drink 1 and drink 2 but only until 90 minutes in drink 3. P values for the significant increases at all these timepoints can be seen in table 2.2.

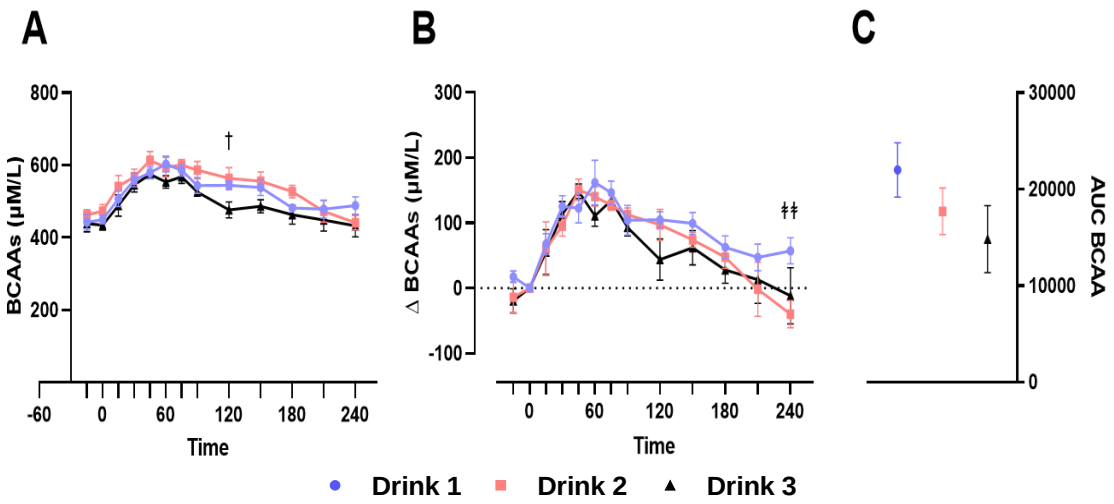

Figure 2.2. A. Plasma BCAA concentrations over time in elderly men before and after ingesting each of the three test drinks. **B.** Change in plasma BCAA concentrations relative to baseline in elderly men before and after ingesting each of the three test drinks. **C.** Area under the curve (AUC) for BCAA concentrations over time in elderly men before and after ingesting each of the three test drinks. † Significant difference between drink 2 and drink 3 at timepoint ($P < 0.05$). †† Significant difference between drink 1 and drink 2 at timepoint ($P < 0.01$).

Table 2.2. Plasma BCAA concentrations (µM/L) over time in elderly men following the ingestion of each of the three test drinks. P values indicate the within drink comparison of each timepoint with the baseline concentration for that drink (0 minutes). Bold P values indicate a significant difference from the respective timepoint to baseline ($P < 0.05$).

	Dr1	Dr1 SEM	P Value	Dr2	Dr2 SEM	P Value	Dr3	Dr3 SEM	P Value
-15	442.2	22.2	1.000	462.3	14.5	1.000	438	22.9	1.000
0	*449.4*	*12.2*		*473.2*	*18.3*		*434.5*	*14.1*	
15	506.7	21.8	0.328	540.8	31	0.165	488.2	29.6	0.500
30	559	18.9	**0.003**	568.6	20.8	**0.009**	545.9	20.2	**0.007**
45	578.9	16.7	**<0.001**	612.9	24.7	**<0.001**	574.5	10.6	**<0.001**
60	602.8	22.5	**<0.001**	594.1	27.6	**0.002**	553.4	17.5	**0.004**

75	585.4	16	**<0.001**	600.5	15.7	**<0.001**	567.4	18.3	**<0.001**
90	543.5	21.9	**0.014**	586.1	24.4	**<0.001**	525.7	11.6	**0.044**
120	544	12.6	**0.013**	563.4	29.7	**0.022**	476.6	22.5	0.771
150	538.6	22.2	**0.023**	555.4	25.5	**0.048**	486.5	17.9	0.574
180	481.9	7	0.951	526.8	17.6	0.464	463	26.2	0.979
210	478.1	25	0.972	472.4	30.4	>0.999	448	31.2	0.999
240	488	24	0.837	441.2	20.5	0.925	432.2	30.5	>0.999

2.3.2- Plasma EAAs

The changes in plasma EAA concentration following the three drinks are shown in figure 2.3 and table 2.3. Significant main treatment and time effects were found for the change in plasma EAA concentration shown in figure 2.3A ($P < 0.01$ and $P < 0.001$ respectively). A significant main time effect was also found for the change in EAA concentration normalised to baseline which is shown in figure 2.3B ($P < 0.001$). There was no significant difference between drinks for the AUC analysis in figure 2.3C.

Time comparisons for the changes in plasma EAA concentrations are shown in table 2.3. Significant increases in plasma EAA concentrations compared to baseline occurred in all three drinks. For drink 1, the EAA concentration was significantly greater from 45-75 minutes, whilst for drink 2 and drink 3 the increase was significant from 75-90 minutes and 30-75 minutes respectively. P values for all these significant increases are available in table 2.3.

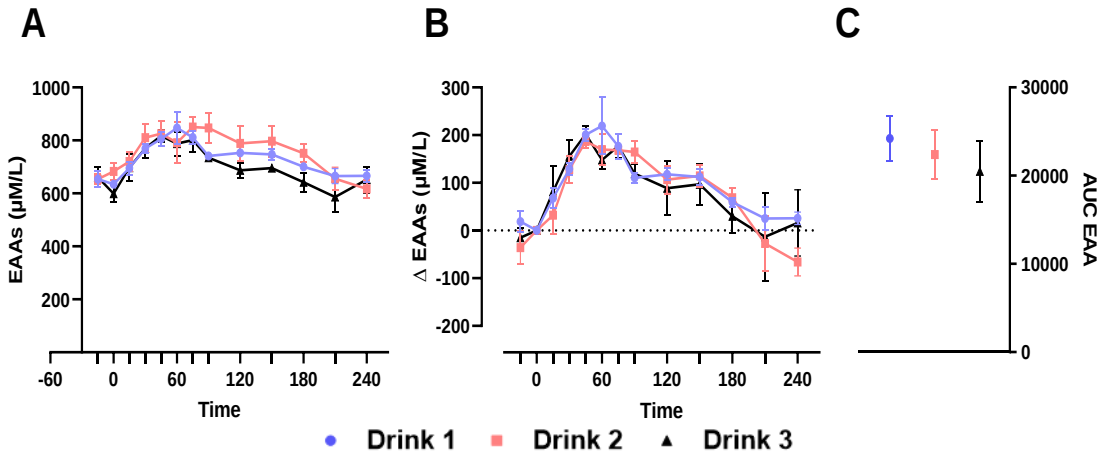

Figure 2.3. A. Plasma EAA concentrations over time in elderly men before and after ingesting each of the three test drinks. **B.** Change in plasma EAA concentrations relative to baseline in elderly men before and after ingesting each of the three test drinks. **C.** Area under the curve (AUC) for EAA concentrations over time in elderly men before and after ingesting each of the three test drinks.

Table 2.3. Plasma EAA concentrations (µM/L) over time in elderly men following the ingestion of each of the three test drinks. P values indicate the within drink comparison of each timepoint with the baseline concentration for that drink (0 minutes). Bold P values indicate a significant difference from the respective timepoint to baseline ($P < 0.05$).

	Dr1	Dr1 SEM	P Value	Dr2	Dr2 SEM	P Value	Dr3	Dr3 SEM	P Value
-15	653.8	28.9	1.000	653.6	18.7	0.999	661.4	38.0	0.926
0	*634.8*	*9.0*	-	*682.5*	*33.3*	-	*598.4*	*31.6*	-
15	695.7	24.2	0.877	720.6	34.4	0.992	697.1	50.8	0.334
30	769.5	15.9	0.103	810.5	49.7	0.080	770.7	38.6	**0.008**
45	805.7	26.3	**0.009**	825.4	48.0	0.109	817.0	21.6	**<0.001**
60	847.1	59.7	**<0.001**	791.1	77.1	0.497	787.9	45.4	**0.007**
75	811.3	25.9	**0.004**	850.7	37.1	**0.007**	801.2	43.8	**0.002**
90	741.6	8.3	0.259	847.4	56.2	**0.009**	734.4	14.5	0.067

120	752.8	14.4	0.131	788.7	65.6	0.264	687.1	28.8	0.509
150	746.9	20.6	0.173	796.9	58.2	0.189	695.4	14.3	0.398
180	700.2	8.0	0.864	751.2	33.2	0.739	642.2	35.7	0.988
210	665.6	28.2	0.999	655.1	43.4	0.999	585.9	57.2	1.000
240	666.4	21.5	0.999	616.7	35.5	0.782	651.7	49.6	0.962

2.3.3- Plasma Leucine

Plasma leucine concentrations following the three drinks are displayed in figure 2.4 and table 2.4. Figure 2.4A demonstrates the absolute plasma leucine concentrations throughout the study, for which there were significant main treatment and time effects (both $P < 0.001$). Significant differences occurred between drink 1 and drink 3 at 60 minutes (230.3 ± 16.5 µM/L vs 168.4 ± 7.2 µM/L respectively, $P < 0.01$), 120 minutes (189.3 ± 15.3 µM/L vs 145.2 ± 12.8 µM/L respectively, $P < 0.05$), 180 minutes (177.2 ± 21.3 µM/L vs 130.4 ± 7.4 µM/L respectively, $P < 0.05$) and 240 minutes (166.5 ± 14.2 µM/L vs 115.3 ± 9.6 µM/L respectively, $P < 0.05$). A significant difference also occurred between drink 2 and drink 3 at 180 minutes (182.4 ± 10.0 µM/L vs 130.4 ± 7.4 µM/L respectively, $P < 0.05$). Figure 2.4B shows the change in leucine concentration when normalised to baseline following consumption of each of the three drink boluses. Significant main treatment and time effects were observed for the change in plasma leucine concentration relative to baseline ($P < 0.01$ and $P < 0.001$ respectively). There was a significant difference in the change in leucine concentration between drink 1 and drink 3 at 60 minutes (79.7 ± 18.0 µM/L vs 42.3 ± 4.3 µM/L respectively, $P < 0.05$). No significant differences were found for the comparison of AUCs between the three test drinks as shown in figure 2.4C.

Table 2.4 demonstrates the changes in plasma leucine concentration over time following the consumption of each of the three test drinks. All three drinks produced a significant increase in plasma leucine concentration relative to baseline, with this

increase occurring from 45-75 minutes for drink 1, at 75 minutes for drink 2 and 45 minutes for drink 3. The P values for these significant increases are highlighted in table 2.4.

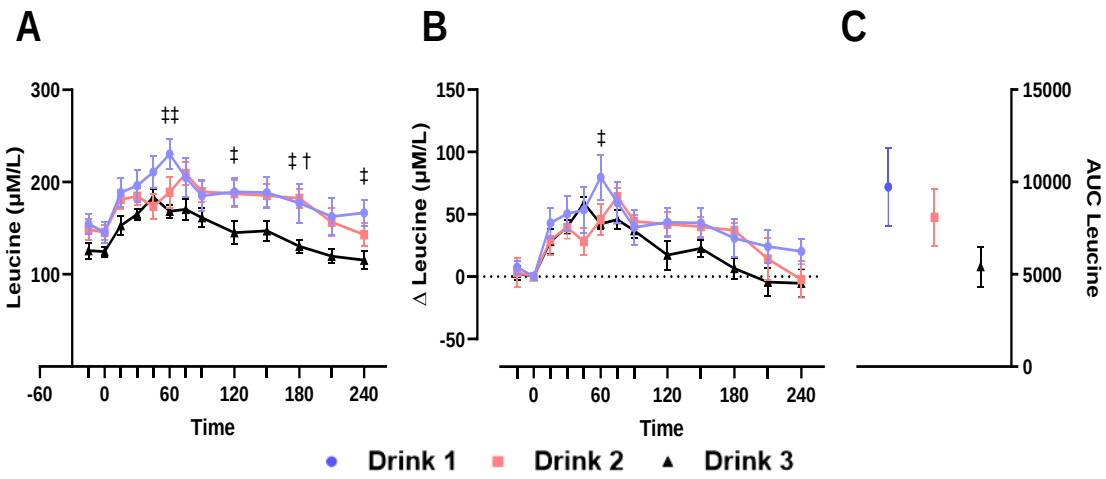

Figure 2.4. A. Plasma leucine concentrations over time in elderly men before and after ingesting each of the three test drinks. **B.** Change in plasma leucine concentrations relative to baseline in elderly men before and after ingesting each of the three test drinks. **C.** Area under the curve (AUC) for leucine concentrations over time in elderly men before and after ingesting each of the three test drinks. ‡ Significant difference between drink 1 and drink 3 at timepoint ($P < 0.05$). ‡‡ Significant difference between drink 1 and drink 3 at timepoint ($P < 0.01$). † Significant difference between drink 2 and drink 3 at timepoint ($P < 0.05$).

Table 2.4. Plasma leucine concentrations (µM/L) over time in elderly men following the ingestion of each of the three test drinks. P values indicate the within drink comparison of each timepoint with the baseline concentration for that drink (0 minutes). Bold P values indicate a significant difference from the respective timepoint to baseline ($P < 0.05$).

	Dr1	Dr1 SEM	P Value	Dr2	Dr2 SEM	P Value	Dr3	Dr3 SEM	P Value
-15	154.6	10.9	0.999	148.7	11.3	1.000	125.7	8.5	1.000

0	*145.6*	*11.4*		*145.3*	*8.3*		*124.5*	*5.6*	
15	188.6	16.0	0.142	181.2	10.2	0.349	152.8	10.4	0.614
30	196.1	17.2	0.051	184.5	9.0	0.221	165.2	6.0	0.217
45	210.9	17.4	**0.009**	173.7	13.5	0.680	183.5	8.6	**0.013**
60	230.3	16.5	**<0.001**	188.8	16.6	0.155	168.4	7.2	0.150
75	204.9	21.5	**0.012**	209.3	12.5	**0.005**	170.3	11.7	0.099
90	185.0	17.0	0.219	189.6	11.8	0.119	161.4	10.2	0.288
120	189.3	15.3	0.130	187.4	14.5	0.157	145.2	12.8	0.916
150	188.7	17.3	0.141	185.3	12.7	0.202	147.1	10.7	0.843
180	177.2	21.3	0.512	182.4	10.0	0.277	130.4	7.4	1.000
210	162.7	20.3	0.982	156.6	15.5	0.999	119.5	7.5	1.000
240	166.5	14.2	0.910	142.8	12.6	1.000	115.3	9.6	1.000

2.3.4- Plasma Insulin

Changes in plasma insulin concentrations after each of the three test drinks are shown in figure 2.5 and table 2.5. A significant main time effect was observed for plasma insulin concentrations which are depicted in figure 2.5A ($P < 0.0001$). No significant differences in the insulin concentrations at any individual time points were found between each of the three drinks ($P < 0.05$). There were also no significant differences observed for the AUC analysis between each of the three drinks for plasma insulin concentrations as shown in figure 2.5B.

The within-drink comparisons over time for each of the three test drinks are shown in table 2.5. A significant increase in plasma insulin was observed in all three of the drinks. For the drink 1, this significant increase was observed between 45 and 120 minutes, whilst for drink 2 and drink 3, the increase was significant between 30 and 90 minutes. The P values for all these significant increases are highlighted in bold in table 2.5.

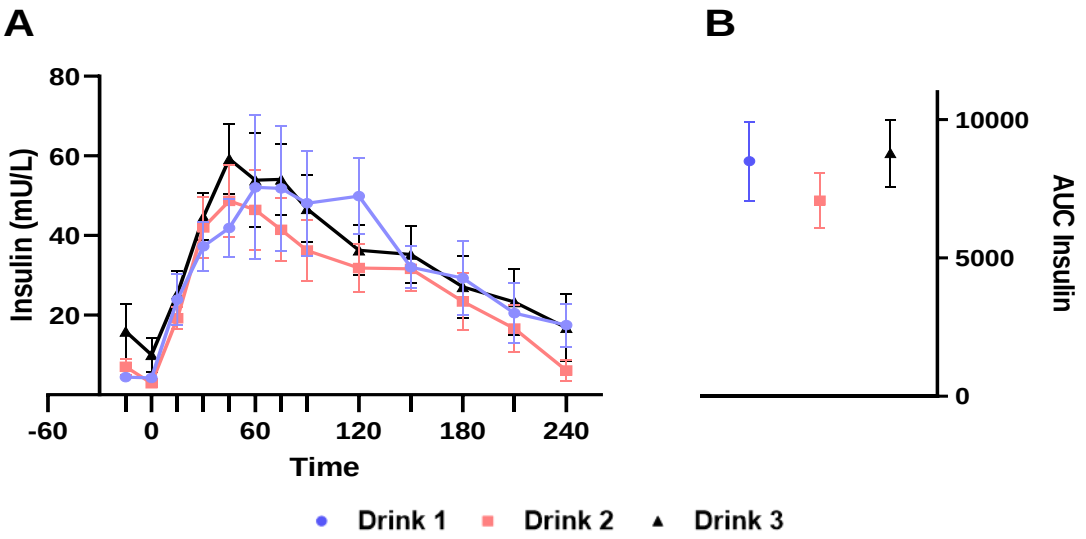

Figure 2.5. A. Plasma insulin concentrations over time in elderly men before and after ingesting each of the three test drinks. **B.** Area under the curve (AUC) for insulin concentrations over time in elderly men before and after ingesting each of the three test drinks.

Table 2.5. Plasma insulin concentrations (mU/L) over time in elderly men following the ingestion of each of the three test drinks. P values indicate the within drink comparison of each timepoint with the baseline concentration for that drink (0 minutes). Bold P values indicate a significant difference from the respective timepoint to baseline ($P < 0.05$).

	Dr1	Dr1 SEM	P Value	Dr2	Dr2 SEM	P Value	Dr3	Dr3 SEM	P Value
-15	4.4	0.9	0.9999	7.0	2.1	0.9995	15.9	6.8	0.999
0	4.2	0.9		2.9	0.7		10.0	4.2	
15	23.9	6.4	0.5364	19.2	2.8	0.7094	25.3	5.7	0.772
30	37.3	6.2	0.0624	42.0	7.7	**0.0077**	44.7	5.9	**0.026**
45	41.9	7.3	**0.0225**	48.7	9.0	**0.0009**	59.2	8.9	**0.001**
60	52.1	18.1	**0.0014**	46.4	10.2	**0.0020**	53.9	11.9	**0.002**
75	51.8	15.7	**0.0016**	41.4	7.9	**0.0091**	54.1	8.9	**0.002**
90	48.1	13.2	**0.0046**	36.2	7.6	**0.0363**	46.7	8.4	**0.015**

120	49.9	9.5	**0.0028**	31.8	6.0	0.0993	36.3	6.3	0.166
150	32.0	5.3	0.1696	31.6	5.7	0.1036	35.2	7.3	0.204
180	29.3	9.3	0.2623	23.4	7.3	0.4339	27.1	7.8	0.654
210	20.5	7.6	0.7456	16.6	5.8	0.8641	23.3	8.4	0.882
240	17.5	5.4	0.8999	6.1	2.6	0.9996	16.8	8.4	0.999

2.4- Discussion

The main finding of this study was that the magnitude and pattern of changes in plasma hyperaminoacidemia varied in response to feeding of protein blends with different compositions. It was found that the significant increase from baseline observed in plasma BCAA concentrations for all three drinks was more prolonged in drink 1 and drink 2 than drink 3, as shown in table 2.2. This finding could potentially be explained by differences in the compositions of the drinks, with both drink 1 and drink 2 containing whey protein whilst drink 3 does not. Indeed, previous research has highlighted that whey protein is better able to stimulate hyperaminoacidemia than other protein sources, including casein (Hall et al., 2003) and soy (Morifuji et al., 2010). It should be noted that the doses of whey protein were different in the studies by Hall et al (2003) and Morifuji et al (2010) than the present study, and the study by Hall et al (2003) also assessed a greater range of amino acids than the BCAAs. However, given that drink 3 was comprised entirely of a combination of casein and soy protein, the findings of these previous studies in combination with the results of this study provide compelling evidence for the importance of whey protein for maintaining a significant hyperaminoacidemia at 120 and 150 minutes, observed in drink 1 and drink 2 compared to drink 3.

Another interesting finding when assessing the differences in BCAA concentration responses between the three drinks was the significantly greater increase in plasma BCAA concentration at 240 minutes for drink 1 compared to drink 2. It is possible

that this was related to the composition of the different drinks, with drink 2 containing a lower proportion of casein protein than drink 1. Previous research has already illuminated the slower digestion profile of casein protein, caused by the coagulation and formation of a protein network during digestion which does not occur in faster digesting proteins such as whey, which remains in solution (Lambers et al., 2013). Therefore, it is possible that the higher proportion of slower digesting casein protein in the drink 1 resulted in sustained elevated levels of BCAAs relative to baseline at 240 minutes which were significantly greater than that achieved by drink 2. This may also indicate why there were no significant differences reported at this timepoint compared to the drink 3, which contained an intermediate proportion of casein at 51%.

Somewhat surprisingly, there were no significant differences found in the plasma EAA response at any given time point between the three test drinks. It should be considered that there was a significant main treatment effect observed for plasma EAAs ($P < 0.01$), but this did not translate into significant differences at any time points. This was unexpected given that there were significant differences reported for BCAA concentrations at specific timepoints, with the three BCAAs; leucine, isoleucine, and valine, also contributing to the total EAA concentration. One potential explanation for this finding could be differences in the amino acid composition of each of the test drinks, as it has been found that the pattern of plasma amino acid response to protein feeding reflects the amino acid composition of the protein source consumed (Bos et al., 2003; Liu et al., 2019). Consequently, it is possible that the significant differences observed for BCAA concentrations, such as at 120 minutes between drink 2 and drink 3, were no longer significant when considering all the EAAs, as drink 3 may have been comprised of more non-BCAA EAAs.

Plasma leucine concentration was significantly different between the three test drinks at numerous time points, namely when comparing either drink 1 or drink 2

with drink 3. It should be noted that this can partially be explained by the non-significantly lower baseline leucine concentration for drink 3 compared to the other two drinks, though the reason for this is unclear as a repeated measures study design was used. However, even when normalising concentrations to baseline, there was still a significant difference reported between drink 1 and drink 3 at 60 minutes. These findings are perhaps unsurprising given that both drink 1 and drink 2 contain whey protein whilst drink 3 does not. Previous research has highlighted that whey protein is a particularly rich source of protein for leucine compared to other plant and animal-based protein sources, including casein and soy proteins which make up drink 3 (Gorissen et al., 2018). Consequently, the presence of whey protein, which is typically richer in leucine, in combination with the fact that plasma amino acid responses reflect the amino acid composition of the protein source (Bos et al., 2003; Liu et al., 2019), likely explains this finding.

In addition to the observed plasma hyperaminoacidemia, all three drinks induced a plasma hyperinsulinemia. Notably, there was no significant main treatment effect and no significant differences observed between any of the three drinks at any time points for plasma insulin and the time course of changes were very similar between each of the three drinks, with drink 1 producing significant increases from 45 to 120 minutes and both drink 2 and drink 3 resulting in significant increases from 30 to 90 minutes. The finding that there were no significant differences in insulin response between drinks despite the differences in protein composition is somewhat surprising, given that previous research has shown significant differences in plasma insulin with either casein, soy, or whey protein feeding, though notably not all studies have been consistent in which protein type produces greater increases in plasma insulin concentration (Hoppe et al., 2009; Veldhorst et al., 2009). One potential explanation for the disparity between the findings of the present study and those of previous studies could be that the present study used protein blends rather than assessing each type of protein individually. The use of blends means that the composition of each of the drinks, whilst being distinct from each other, do somewhat overlap with certain types of protein such as whey and casein being

present in multiple drinks, albeit in varying quantities. This overlapping in composition may have been somewhat responsible for reducing the variability in insulin response between the drinks. Additionally, the present study, being primarily concerned with plasma amino acid changes, matched the three protein drinks to 20 g of protein, resulting in small differences in the carbohydrate content for drink 1, drink 2 and drink 3 at 67.1 g, 61.0 g and 72.6 g of carbohydrates respectively. These differences in carbohydrate composition may have slightly affected the insulin response between drinks, meaning that the lack of variation cannot solely be attributed to differences in the protein composition of the drinks. Further research investigating the roles of protein blends on plasma insulin responses would need to be carried out with blends containing the same quantity of both protein and carbohydrates to more accurately assess this, but this could be an interesting area of research considering that this has not been tested in protein blends to date.

2.5- Conclusion and Future Directions

The present study showed that the plasma amino acid changes in older men differs in response to different drinks of varying protein compositions. In particular, the bioavailability of BCAAs and leucine were significantly different, which is likely attributable to the affects of different protein blend compositions on protein source digestibility. Notably, most significant differences were reported between drink 1 or drink 2 with drink 3, which may indicate that the presence of whey protein within the blend is critical to achieving greater hyperaminoacidemia. Even though whey appears to play an integral role within the protein blend, there may also be an argument for the importance of including casein in high proportions alongside whey, as a slower digesting protein may help to attenuate the decline in plasma amino acid concentration back to basal levels following its peak. Despite the differences in plasma amino acid response between the three test drinks in the present study, there were no differences between drinks in the pattern of insulin response, which could be a result of the matching of the three test drinks for total protein intake and

the fact that there is some overlap between the protein source compositions within the three different protein blends.

The findings of the present study are particularly important given the established importance of inducing plasma hyperaminoacidemia, particularly in older populations, to produce robust increases in MPS. Whilst the present study has highlighted that different protein blends can produce significant differences in plasma amino acid bioavailability, future studies should look to extend these findings to MPS measures, using muscle biopsy techniques in combination with protein feeding, to assess how effectively these differences in hyperaminoacidemia responses translate into changes in MPS. Alongside this, studies could also seek to utilise techniques such as the dual stable isotope tracer measurements, as discussed in the *'Determining Protein Digestibility'* section of the prior review, to quantify the differences in digestibility of these different protein blends. If performed together within a single study, this would provide a full quantitative story from digestive capacity to plasma amino acid bioavailability, and then into the muscle for utilisation, which would further assist in illuminating the potential benefits of protein blends, particularly in older adults.

References

Abdulla, H., Smith, K., Atherton, P. J., & Idris, I. (2016). Role of insulin in the regulation of human skeletal muscle protein synthesis and breakdown: a systematic review and meta-analysis. *Diabetologia*, *59*(1), 44–55. https://doi.org/10.1007/S00125-015-3751-0/TABLES/2

Arango-Lopera, V. E., Arroyo, P., Gutiérrez-Robledo, L. M., Perez-Zepeda, M. U., & Cesari, M. (2013). Mortality as an adverse outcome of sarcopenia. *The Journal of Nutrition, Health & Aging 2012 17:3*, *17*(3), 259–262. https://doi.org/10.1007/S12603-012-0434-0

Arnal, M. A., Mosoni, L., Boirie, Y., Houlier, M. L., Morin, L., Verdier, E., Ritz, P., Antoine, J. M., Prugnaud, J., Beaufrère, B., & Mirand, P. P. (1999). Protein pulse feeding improves protein retention in elderly women. *The American Journal of Clinical Nutrition*, *69*(6), 1202–1208. https://doi.org/10.1093/AJCN/69.6.1202

Atherton, P. J., Etheridge, T., Watt, P. W., Wilkinson, D., Selby, A., Rankin, D., Smith, K., & Rennie, M. J. (2010). Muscle full effect after oral protein: time-dependent concordance and discordance between human muscle protein synthesis and mTORC1 signaling. *The American Journal of Clinical Nutrition*, *92*(5), 1080–1088. https://doi.org/10.3945/AJCN.2010.29819

Atherton, P. J., & Smith, K. (2012). Muscle protein synthesis in response to nutrition and exercise. *The Journal of Physiology*, *590*(5), 1049–1057. https://doi.org/10.1113/JPHYSIOL.2011.225003

Bailey, H. M., Mathai, J. K., Berg, E. P., & Stein, H. H. (2020). Pork Products Have Digestible Indispensable Amino Acid Scores (DIAAS) That Are Greater Than 100 When Determined in Pigs, but Processing Does Not Always Increase DIAAS. *The Journal of Nutrition*, *150*(3), 475–482. https://doi.org/10.1093/JN/NXZ284

Bandyopadhyay, S., Kashyap, S., Calvez, J., Devi, S., Azzout-Marniche, D., Tomé, D., Kurpad, A. v, & Gaudichon, C. (2021). Evaluation of Protein Quality in Humans

and Insights on Stable Isotope Approaches to Measure Digestibility – A Review. *Advances in Nutrition*. https://doi.org/10.1093/ADVANCES/NMAB134

Barzel, U. S., & Massey, L. K. (1998). Excess Dietary Protein Can Adversely Affect Bone. *The Journal of Nutrition*, *128*(6), 1051–1053. https://doi.org/10.1093/JN/128.6.1051

Baumgartner, R. N., Koehler, K. M., Gallagher, D., Romero, L., Heymsfield, S. B., Ross, R. R., Garry, P. J., & Lindeman, R. D. (1998). Epidemiology of Sarcopenia among the Elderly in New Mexico. *American Journal of Epidemiology*, *147*(8), 755–763. https://doi.org/10.1093/OXFORDJOURNALS.AJE.A009520

Bax, M. L., Aubry, L., Ferreira, C., Daudin, J. D., Gatellier, P., Rémond, D., & Santé-Lhoutellier, V. (2012). Cooking temperature is a key determinant of in vitro meat protein digestion rate: Investigation of underlying mechanisms. *Journal of Agricultural and Food Chemistry*, *60*(10), 2569–2576. https://doi.org/10.1021/JF205280Y/ASSET/IMAGES/LARGE/JF-2011-05280Y_0001.JPEG

Bjerk, M., Brovold, T., Skelton, D. A., & Bergland, A. (2018). Associations between health-related quality of life, physical function and fear of falling in older fallers receiving home care. *BMC Geriatrics*, *18*(1), 1–8. https://doi.org/10.1186/S12877-018-0945-6/TABLES/3

Bohé, J., Low, A., Wolfe, R. R., & Rennie, M. J. (2003). Human Muscle Protein Synthesis is Modulated by Extracellular, Not Intramuscular Amino Acid Availability: A Dose-Response Study. *The Journal of Physiology*, *552*(1), 315–324. https://doi.org/10.1113/JPHYSIOL.2003.050674

Boirie, Y., Gachon, P., & Beaufrère, B. (1997). Splanchnic and whole-body leucine kinetics in young and elderly men. *The American Journal of Clinical Nutrition*, *65*(2), 489–495. https://doi.org/10.1093/AJCN/65.2.489

Borack, M. S., Reidy, P. T., Husaini, S. H., Markofski, M. M., Deer, R. R., Richison, A. B., Lambert, B. S., Cope, M. B., Mukherjea, R., Jennings, K., Volpi, E., & Rasmussen, B. B. (2016). Soy-Dairy Protein Blend or Whey Protein Isolate

Ingestion Induces Similar Postexercise Muscle Mechanistic Target of Rapamycin Complex 1 Signaling and Protein Synthesis Responses in Older Men. *The Journal of Nutrition*, *146*(12), 2468–2475. https://doi.org/10.3945/JN.116.231159

Bos, C., Metges, C. C., Gaudichon, C., Petzke, K. J., Pueyo, M. E., Morens, C., Everwand, J., Benamouzig, R., & Tomé, D. (2003). Postprandial Kinetics of Dietary Amino Acids Are the Main Determinant of Their Metabolism after Soy or Milk Protein Ingestion in Humans. *The Journal of Nutrition*, *133*(5), 1308–1315. https://doi.org/10.1093/JN/133.5.1308

Bouillanne, O., Neveux, N., Nicolis, I., Curis, E., Cynober, L., & Aussel, C. (2014). Long-lasting improved amino acid bioavailability associated with protein pulse feeding in hospitalized elderly patients: A randomized controlled trial. *Nutrition*, *30*(5), 544–550. https://doi.org/10.1016/J.NUT.2013.10.006

Boye, J., Wijesinha-Bettoni, R., & Burlingame, B. (2012). Protein quality evaluation twenty years after the introduction of the protein digestibility corrected amino acid score method. *British Journal of Nutrition*, *108*(S2), S183–S211. https://doi.org/10.1017/S0007114512002309

Breen, L., & Phillips, S. M. (2011). Skeletal muscle protein metabolism in the elderly: Interventions to counteract the “anabolic resistance” of ageing. *Nutrition and Metabolism*, *8*(1), 1–11. https://doi.org/10.1186/1743-7075-8-68/FIGURES/2

Bunout, D., Maza, M. P. de la, Barrera, G., Leiva, L., & Hirsch, S. (2011). Association between sarcopenia and mortality in healthy older people. *Australasian Journal on Ageing*, *30*(2), 89–92. https://doi.org/10.1111/J.1741-6612.2010.00448.X

Burd, N. A., Yang, Y., Moore, D. R., Tang, J. E., Tarnopolsky, M. A., & Phillips, S. M. (2012). Greater stimulation of myofibrillar protein synthesis with ingestion of whey protein isolate v. micellar casein at rest and after resistance exercise in elderly men. *British Journal of Nutrition*, *108*(6), 958–962. https://doi.org/10.1017/S0007114511006271

Butteiger, D. N., Cope, M., Liu, P., Mukherjea, R., Volpi, E., Rasmussen, B. B., & Krul, E. S. (2013). A soy, whey and caseinate blend extends postprandial skeletal

muscle protein synthesis in rats. *Clinical Nutrition*, *32*(4), 585–591. https://doi.org/10.1016/J.CLNU.2012.10.001

Cesari, M., Onder, G., Zamboni, V., Manini, T., Shorr, R. I., Russo, A., Bernabei, R., Pahor, M., & Landi, F. (2008). Physical function and self-rated health status as predictors of mortality: Results from longitudinal analysis in the ilSIRENTE study. *BMC Geriatrics*, *8*(1), 1–9. https://doi.org/10.1186/1471-2318-8-34/FIGURES/1

Cunningham, C., O' Sullivan, R., Caserotti, P., & Tully, M. A. (2020). Consequences of physical inactivity in older adults: A systematic review of reviews and meta-analyses. *Scandinavian Journal of Medicine & Science in Sports*, *30*(5), 816–827. https://doi.org/10.1111/SMS.13616

Cuthbertson, D., Smith, K., Babraj, J., Leese, G., Waddell, T., Atherton, P., Wackerhage, H., Taylor, P. M., & Rennie, M. J. (2005). Anabolic signaling deficits underlie amino acid resistance of wasting, aging muscle. *The FASEB Journal*, *19*(3), 1–22. https://doi.org/10.1096/FJ.04-2640FJE

Dangin, M., Boirie, Y., Garcia-Rodenas, C., Gachon, P., Fauquant, J., Callier, P., Ballèvre, O., & Beaufrère, B. (2001). The digestion rate of protein is an independent regulating factor of postprandial protein retention. *American Journal of Physiology - Endocrinology and Metabolism*, *280*(2 43-2). https://doi.org/10.1152/AJPENDO.2001.280.2.E340/ASSET/IMAGES/LARGE/H10210268005.JPEG

Dargent-Molina, P., Sabia, S., Touvier, M., Kesse, E., Bréart, G., Clavel-Chapelon, F., & Boutron-Ruault, M. C. (2008). Proteins, Dietary Acid Load, and Calcium and Risk of Postmenopausal Fractures in the E3N French Women Prospective Study. *Journal of Bone and Mineral Research*, *23*(12), 1915–1922. https://doi.org/10.1359/JBMR.080712

de Boer, M. D., Selby, A., Atherton, P., Smith, K., Seynnes, O. R., Maganaris, C. N., Maffulli, N., Movin, T., Narici, M. v., & Rennie, M. J. (2007). The temporal responses of protein synthesis, gene expression and cell signalling in human

quadriceps muscle and patellar tendon to disuse. *The Journal of Physiology*, *585*(1), 241–251. https://doi.org/10.1113/JPHYSIOL.2007.142828

de Souza Genaro, P., & Martini, L. A. (2010). Effect of protein intake on bone and muscle mass in the elderly. *Nutrition Reviews*, *68*(10), 616–623. https://doi.org/10.1111/J.1753-4887.2010.00321.X

Deane, C., Bass, J., Crossland, H., Nutrients, B. P.-, & 2020, undefined. (n.d.). Animal, plant, collagen and blended dietary proteins: Effects on musculoskeletal outcomes. *Nutrients, 12*(9), 2670. https://doi.org/10.3390/nu12092670

Deane, C. S., Ely, I. A., Wilkinson, D. J., Smith, K., Phillips, B. E., & Atherton, P. J. (2021). Dietary protein, exercise, ageing and physical inactivity: interactive influences on skeletal muscle proteostasis. *Proceedings of the Nutrition Society*, *80*(2), 106–117. https://doi.org/10.1017/S0029665120007879

Deglaire, A., Bos, C., Tomé, D., & Moughan, P. J. (2009). Ileal digestibility of dietary protein in the growing pig and adult human. *British Journal of Nutrition*, *102*(12), 1752–1759. https://doi.org/10.1017/S0007114509991267

Delimaris, I. (2013). *Adverse Effects Associated with Protein Intake above the Recommended Dietary Allowance for Adults*. *2013*. https://doi.org/10.5402/2013/126929

Devi, S., Varkey, A., Dharmar, M., Holt, R. R., Allen, L. H., Sheshshayee, M. S., Preston, T., Keen, C. L., & Kurpad, A. v. (2020). Amino Acid Digestibility of Extruded Chickpea and Yellow Pea Protein is High and Comparable in Moderately Stunted South Indian Children with Use of a Dual Stable Isotope Tracer Method. *The Journal of Nutrition*, *150*(5), 1178–1185. https://doi.org/10.1093/JN/NXAA004

Devi, S., Varkey, A., Sheshshayee, M. S., Preston, T., & Kurpad, A. v. (2018). Measurement of protein digestibility in humans by a dual-tracer method. *The American Journal of Clinical Nutrition*, *107*(6), 984–991. https://doi.org/10.1093/AJCN/NQY062

Di, Y., Li, X., Chang, X., Gu, R., Duan, X., Liu, F., Liu, X., & Wang, Y. (2022). Impact of germination on structural, functional properties and in vitro protein digestibility

of sesame (Sesamum indicum L.) protein. *LWT*, *154*, 112651. https://doi.org/10.1016/J.LWT.2021.112651

Drummond, M. J., Dickinson, J. M., Fry, C. S., Walker, D. K., Gundermann, D. M., Reidy, P. T., Timmerman, K. L., Markofski, M. M., Paddon-Jones, D., Rasmussen, B. B., & Volpi, E. (2012). Bed rest impairs skeletal muscle amino acid transporter expression, mTORC1 signaling, and protein synthesis in response to essential amino acids in older adults. *American Journal of Physiology - Endocrinology and Metabolism*, *302*(9). https://doi.org/10.1152/AJPENDO.00603.2011

Fanelli, N. S., Bailey, H. M., Guardiola, L. v., & Stein, H. H. (2021). Values for Digestible Indispensable Amino Acid Score (DIAAS) Determined in Pigs Are Greater for Milk Than for Breakfast Cereals, but DIAAS Values for Individual Ingredients Are Additive in Combined Meals. *The Journal of Nutrition*, *151*(3), 540–547. https://doi.org/10.1093/JN/NXAA398

Feskanich, D., Willett, W. C., Stampfer, M. J., & Colditz, G. A. (1996). Protein Consumption and Bone Fractures in Women. *American Journal of Epidemiology*, *143*(5), 472–479. https://doi.org/10.1093/OXFORDJOURNALS.AJE.A008767

Fujita, S., & Volpi, E. (2006). Amino Acids and Muscle Loss with Aging. *The Journal of Nutrition*, *136*(1), 277S-280S. https://doi.org/10.1093/JN/136.1.277S

Ganesan, G. R., & Vijayaraghavan, P. V. (2019). Urinary N-telopeptide: The New Diagnostic Test for Osteoporosis. *The Surgery Journal*, *5*(1), e1. https://doi.org/10.1055/S-0038-1677483

Ghavidel, R. A., & Prakash, J. (2007). The impact of germination and dehulling on nutrients, antinutrients, in vitro iron and calcium bioavailability and in vitro starch and protein digestibility of some legume seeds. *LWT - Food Science and Technology*, *40*(7), 1292–1299. https://doi.org/10.1016/J.LWT.2006.08.002

Gilani, G. S., & Sepehr, E. (2003). Protein Digestibility and Quality in Products Containing Antinutritional Factors Are Adversely Affected by Old Age in Rats. *The Journal of Nutrition*, *133*(1), 220–225. https://doi.org/10.1093/JN/133.1.220

Gilani, G. S., Xiao, C. W., & Cockell, K. A. (2012). Impact of Antinutritional Factors in Food Proteins on the Digestibility of Protein and the Bioavailability of Amino Acids and on Protein Quality. *British Journal of Nutrition*, *108*(S2), S315–S332. https://doi.org/10.1017/S0007114512002371

Gomes Almeida Sá, A., Maria Franco Moreno, Y., Augusto Mattar Carciofi, B., Augusto, B., & Gomes Almeida, A. S. (2019). Food processing for the improvement of plant proteins digestibility. *Https://Doi.Org/10.1080/10408398.2019.1688249*. https://doi.org/10.1080/10408398.2019.1688249

Gorissen, S. H. M., Crombag, J. J. R., Senden, J. M. G., Waterval, W. A. H., Bierau, J., Verdijk, L. B., & van Loon, L. J. C. (2018). Protein content and amino acid composition of commercially available plant-based protein isolates. *Amino Acids*, *50*(12), 1685–1695. https://doi.org/10.1007/S00726-018-2640-5/TABLES/2

Greenhaff, P. L., Karagounis, L. G., Peirce, N., Simpson, E. J., Hazell, M., Layfield, R., Wackerhage, H., Smith, K., Atherton, P., Selby, A., & Rennie, M. J. (2008). Disassociation between the effects of amino acids and insulin on signaling, ubiquitin ligases, and protein turnover in human muscle. *American Journal of Physiology - Endocrinology and Metabolism*, *295*(3). https://doi.org/10.1152/AJPENDO.90411.2008

Grigg, D. (1995). The pattern of world protein consumption. *Geoforum*, *26*(1), 1–17. https://doi.org/10.1016/0016-7185(94)00020-8

Habiba, R. A. (2002). Changes in anti-nutrients, protein solubility, digestibility, and HCl-extractability of ash and phosphorus in vegetable peas as affected by cooking methods. *Food Chemistry*, *77*(2), 187–192. https://doi.org/10.1016/S0308-8146(01)00335-1

Hall, W. L., Millward, D. J., Long, S. J., & Morgan, L. M. (2003). Casein and whey exert different effects on plasma amino acid profiles, gastrointestinal hormone

secretion and appetite. *British Journal of Nutrition*, *89*(2), 239–248. https://doi.org/10.1079/BJN2002760

Han, F., Han, F., Wang, Y., Fan, L., Song, G., Chen, X., Jiang, P., Miao, H., & Han, Y. (2019). Digestible indispensable amino acid scores of nine cooked cereal grains. *British Journal of Nutrition*, *121*(1), 30–41. https://doi.org/10.1017/S0007114518003033

Hernández-Olivas, E., Muñoz-Pina, S., Andrés, A., & Heredia, A. (2020). Impact of elderly gastrointestinal alterations on in vitro digestion of salmon, sardine, sea bass and hake: Proteolysis, lipolysis and bioaccessibility of calcium and vitamins. *Food Chemistry*, *326*, 127024. https://doi.org/10.1016/J.FOODCHEM.2020.127024

Herreman, L., Nommensen, P., Pennings, B., & Laus, M. C. (2020). Comprehensive overview of the quality of plant- And animal-sourced proteins based on the digestible indispensable amino acid score. *Food Science & Nutrition*, *8*(10), 5379–5391. https://doi.org/10.1002/FSN3.1809

Hickson, M. (2015). Nutritional interventions in sarcopenia: a critical review. *Proceedings of the Nutrition Society*, *74*(4), 378–386. https://doi.org/10.1017/S0029665115002049

Holmes, J. H. G., Bayley, H. S., Leadbeater, P. A., & Horney, F. D. (1974). Digestion of protein in small and large intestine of the pig. *British Journal of Nutrition*, *32*(3), 479–489. https://doi.org/10.1079/BJN19740102

Hoppe, C., Mølgaard, C., Dalum, C., Vaag, A., & Michaelsen, K. F. (2009). Differential effects of casein versus whey on fasting plasma levels of insulin, IGF-1 and IGF-1/IGFBP-3: results from a randomized 7-day supplementation study in prepubertal boys. *European Journal of Clinical Nutrition 2009 63:9*, *63*(9), 1076–1083. https://doi.org/10.1038/ejcn.2009.34

Hunt, J. R., Johnson, L. A. K., & Roughead, Z. K. F. (2009). Dietary protein and calcium interact to influence calcium retention: a controlled feeding study. *The*

American Journal of Clinical Nutrition, *89*(5), 1357–1365. https://doi.org/10.3945/AJCN.2008.27238

Isanejad, M., Mursu, J., Sirola, J., Kröger, H., Rikkonen, T., Tuppurainen, M., & Erkkilä, A. T. (2016). Dietary protein intake is associated with better physical function and muscle strength among elderly women. *British Journal of Nutrition*, *115*(7), 1281–1291. https://doi.org/10.1017/S000711451600012X

Jansman, A. J., Verstegen, M. W., Huisman, J., & van den Berg, J. W. (1995). Effects of hulls of faba beans (Vicia faba L.) with a low or high content of condensed tannins on the apparent ileal and fecal digestibility of nutrients and the excretion of endogenous protein in ileal digesta and feces of pigs. *Journal of Animal Science*, *73*(1), 118–127. https://doi.org/10.2527/1995.731118X

Janssen, I., Shepard, D. S., Katzmarzyk, P. T., & Roubenoff, R. (2004). The Healthcare Costs of Sarcopenia in the United States. *Journal of the American Geriatrics Society*, *52*(1), 80–85. https://doi.org/10.1111/J.1532-5415.2004.52014.X

Jourdan, M., Deutz, N. E. P., Cynober, L., & Aussel, C. (2011). Features, Causes and Consequences of Splanchnic Sequestration of Amino Acid in Old Rats. *PLOS ONE*, *6*(11), e27002. https://doi.org/10.1371/JOURNAL.PONE.0027002

Joye, I. (2019). Protein Digestibility of Cereal Products. *Foods 2019, Vol. 8, Page 199*, *8*(6), 199. https://doi.org/10.3390/FOODS8060199

Kashyap, S., Shivakumar, N., Sejian, V., Deutz, N. E. P., Preston, T., Sreeman, S., Devi, S., & Kurpad, A. v. (2021). Goat milk protein digestibility in relation to intestinal function. *The American Journal of Clinical Nutrition*, *113*(4), 845–853. https://doi.org/10.1093/AJCN/NQAA400

Kashyap, S., Shivakumar, N., Varkey, A., Duraisamy, R., Thomas, T., Preston, T., Devi, S., & Kurpad, A. v. (2018). Ileal digestibility of intrinsically labeled hen's egg and meat protein determined with the dual stable isotope tracer method in Indian adults. *The American Journal of Clinical Nutrition*, *108*(5), 980–987. https://doi.org/10.1093/AJCN/NQY178

Kashyap, S., Varkey, A., Shivakumar, N., Devi, S., Reddy B H, R., Thomas, T., Preston, T., Sreeman, S., & Kurpad, A. v. (2019). True ileal digestibility of legumes determined by dual-isotope tracer method in Indian adults. *The American Journal of Clinical Nutrition*, *110*(4), 873–882. https://doi.org/10.1093/AJCN/NQZ159

Kataria, A., Chauhan, B. M., & Punia, D. (1989). Antinutrients and protein digestibility (in vitro) of mungbean as affected by domestic processing and cooking. *Food Chemistry*, *32*(1), 9–17. https://doi.org/10.1016/0308-8146(89)90003-4

Katsanos, C. S., Kobayashi, H., Sheffield-Moore, M., Aarsland, A., & Wolfe, R. R. (2006). A high proportion of leucine is required for optimal stimulation of the rate of muscle protein synthesis by essential amino acids in the elderly. *American Journal of Physiology - Endocrinology and Metabolism*, *291*(2), 381–387. https://doi.org/10.1152/AJPENDO.00488.2005/ASSET/IMAGES/LARGE/ZH10080645870007.JPEG

Kerstetter, J. E., Mitnick, M. E., Gundberg, C. M., Caseria, D. M., Ellison, A. F., Carpenter, T. O., & Insogna, K. L. (1999). Changes in Bone Turnover in Young Women Consuming Different Levels of Dietary Protein. *The Journal of Clinical Endocrinology & Metabolism*, *84*(3), 1052–1055. https://doi.org/10.1210/JCEM.84.3.5552

Khattab, R. Y., Arntfield, S. D., & Nyachoti, C. M. (2009). Nutritional quality of legume seeds as affected by some physical treatments, Part 1: Protein quality evaluation. *LWT - Food Science and Technology*, *42*(6), 1107–1112. https://doi.org/10.1016/J.LWT.2009.02.008

Khetarpaul, N., & Chauhan, B. M. (1990). Effect of Germination and Fermentation on in vitro Starch and Protein Digestibility of Pearl Millet. *Journal of Food Science*, *55*(3), 883–884. https://doi.org/10.1111/J.1365-2621.1990.TB05261.X

Kilavuz, A., Meseri, R., Savas, S., Simsek, H., Sahin, S., Bicakli, D. H., Sarac, F., Uyar, M., & Akcicek, F. (2018). Association of sarcopenia with depressive symptoms

and functional status among ambulatory community-dwelling elderly. *Archives of Gerontology and Geriatrics*, *76*, 196–201. https://doi.org/10.1016/J.ARCHGER.2018.03.003

Krempf, M., Hoerr, R. A., Marks, L., & Young, V. R. (1990). Phenylalanine flux in adult men: Estimates with different tracers and route of administration. *Metabolism*, *39*(6), 560–562. https://doi.org/10.1016/0026-0495(90)90018-8

Lambers, T. T., van den Bosch, W. G., & de Jong, S. (2013). Fast and slow proteins: Modulation of the gastric behavior of whey and casein in vitro. *Food Digestion*, *4*(1), 1–6. https://doi.org/10.1007/S13228-012-0028-7/FIGURES/5

Landi, F., Liperoti, R., Russo, A., Giovannini, S., Tosato, M., Capoluongo, E., Bernabei, R., & Onder, G. (2012). Sarcopenia as a risk factor for falls in elderly individuals: Results from the ilSIRENTE study. *Clinical Nutrition*, *31*(5), 652–658. https://doi.org/10.1016/J.CLNU.2012.02.007

Lee, S., Choi, Y. S., Jo, K., Yong, H. I., Jeong, H. G., & Jung, S. (2021). Improvement of meat protein digestibility in infants and the elderly. *Food Chemistry*, *356*, 129707. https://doi.org/10.1016/J.FOODCHEM.2021.129707

Lemon, P. W. (1991). Protein and Amino Acid Needs of the Strength Athlete. *International Journal of Sport Nutrition and Exercise Metabolism*, *1*(2), 127–145. https://doi.org/10.1123/IJSN.1.2.127

Leser, S. (2013). The 2013 FAO report on dietary protein quality evaluation in human nutrition: Recommendations and implications. *Nutrition Bulletin*, *38*(4), 421–428. https://doi.org/10.1111/NBU.12063

Li, J., Han, X., Zhang, X., & Wang, S. (2019). Spatiotemporal evolution of global population ageing from 1960 to 2017. *BMC Public Health 2019 19:1*, *19*(1), 1–15. https://doi.org/10.1186/S12889-019-6465-2

Li, S., Sauer, W. C., & Caine, W. R. (1998). Response of Nutrient Digestibilities to Feeding Diets with Low and High Levels of Soybean Trypsin Inhibitors in Growing Pigs. *J Sci Food Agric*, *76*, 357–363. https://doi.org/10.1002/(SICI)1097-0010(199803)76:3

Liu, J., Klebach, M., Visser, M., & Hofman, Z. (2019). Amino Acid Availability of a Dairy and Vegetable Protein Blend Compared to Single Casein, Whey, Soy, and Pea Proteins: A Double-Blind, Cross-Over Trial. *Nutrients 2019, Vol. 11, Page 2613*, *11*(11), 2613. https://doi.org/10.3390/NU11112613

Marchini, J. S., Castillo, L., Chapman, T. E., Vogt, J. A., Ajami, A., & Young, V. R. (1993). Phenylalanine conversion to tyrosine: Comparative determination with l-[ring-2H5]phenylalanine and l-[1-13C]phenylalanine as tracers in man. *Metabolism*, *42*(10), 1316–1322. https://doi.org/10.1016/0026-0495(93)90131-7

Mathai, J. K., Liu, Y., & Stein, H. H. (2017). Values for digestible indispensable amino acid scores (DIAAS) for some dairy and plant proteins may better describe protein quality than values calculated using the concept for protein digestibility-corrected amino acid scores (PDCAAS). *British Journal of Nutrition*, *117*(4), 490–499. https://doi.org/10.1017/S0007114517000125

Maughan, P. J., & Smith, W. C. (2012). Determination and assessment of apparent ileal amino acid digestibility coefficients for the growing pig. *Http://Dx.Doi.Org/10.1080/00288233.1985.10430440*, *28*(3), 365–370. https://doi.org/10.1080/00288233.1985.10430440

Mesinovic, J., Zengin, A., Courten, B. de, Ebeling, P. R., & Scott, D. (2019). Sarcopenia and type 2 diabetes mellitus: a bidirectional relationship. *Diabetes, Metabolic Syndrome and Obesity: Targets and Therapy*, *12*, 1057. https://doi.org/10.2147/DMSO.S186600

Meyer, H. E., Pedersen, J. I., Løken, E. B., & Tverdal, A. (1997). Dietary Factors and the Incidence of Hip Fracture in Middle-aged NorwegiansA Prospective Study. *American Journal of Epidemiology*, *145*(2), 117–123. https://doi.org/10.1093/OXFORDJOURNALS.AJE.A009082

Millward, D. J., Garlick, P. J., Nnanyelugo, D. O., & Waterlow, J. C. (1976). The relative importance of muscle protein synthesis and breakdown in the regulation of

muscle mass. *Biochemical Journal*, *156*(1), 185–188. https://doi.org/10.1042/BJ1560185

Mitchell, W. K., Wilkinson, D. J., Phillips, B. E., Lund, J. N., Smith, K., & Atherton, P. J. (2016). Human Skeletal Muscle Protein Metabolism Responses to Amino Acid Nutrition. *Advances in Nutrition*, *7*(4), 828S-838S. https://doi.org/10.3945/AN.115.011650

Morifuji, M., Ishizaka, M., Baba, S., Fukuda, K., Matsumoto, H., Koga, J., Kanegae, M., & Higuchi, M. (2010). Comparison of different sources and degrees of hydrolysis of dietary protein: Effect on plasma amino acids, dipeptides, and insulin responses in human subjects. *Journal of Agricultural and Food Chemistry*, *58*(15), 8788–8797. https://doi.org/10.1021/JF101912N/ASSET/IMAGES/LARGE/JF-2010-01912N_0004.JPEG

Morley, J. E., Baumgartner, R. N., Roubenoff, R., Mayer, J., & Nair, K. S. (2001). Sarcopenia. *Journal of Laboratory and Clinical Medicine*, *137*(4), 231–243. https://doi.org/10.1067/MLC.2001.113504

Moss, C., Dhillo, W. S., Frost, G., & Hickson, M. (2012). Gastrointestinal hormones: the regulation of appetite and the anorexia of ageing. *Journal of Human Nutrition and Dietetics*, *25*(1), 3–15. https://doi.org/10.1111/J.1365-277X.2011.01211.X

Moughan, P. J. (2003). Amino acid availability: aspects of chemical analysis and bioassay methodology. *Nutrition Research Reviews*, *16*, 127–141. https://doi.org/10.1079/NRR200365

Moughan, P. J., Smith, W. C., Kies, A. K., & James, K. A. C. (2012). Comparison of the ileal digestibility of amino acids in ground barley for the growing rat and pig. *Http://Dx.Doi.Org/10.1080/00288233.1987.10430478*, *30*(1), 59–66. https://doi.org/10.1080/00288233.1987.10430478

Moughan, P. J., & Wolfe, R. R. (2019). Determination of Dietary Amino Acid Digestibility in Humans. *The Journal of Nutrition*, *149*(12), 2101–2109. https://doi.org/10.1093/JN/NXZ211

Olaniyan, E. T., O'Halloran, F., & McCarthy, A. L. (2021). Dietary protein considerations for muscle protein synthesis and muscle mass preservation in older adults. *Nutrition Research Reviews*, *34*(1), 147–157. https://doi.org/10.1017/S0954422420000219

Pap, F. C.-F. F. Nutr., & 2011, undefined. (2013). Dietary protein quality evaluation in human nutrition. *Sochob.Cl*. https://www.sochob.cl/pdf/libros/Dietary%20protein%20quality%20evaluation%20in%20human%20nutrition.pdf

Pennings, B., Boirie, Y., Senden, J. M. G., Gijsen, A. P., Kuipers, H., & van Loon, L. J. C. (2011). Whey protein stimulates postprandial muscle protein accretion more effectively than do casein and casein hydrolysate in older men. *The American Journal of Clinical Nutrition*, *93*(5), 997–1005. https://doi.org/10.3945/AJCN.110.008102

Phillips, S. M., Tang, J. E., & Moore, D. R. (2013). The Role of Milk- and Soy-Based Protein in Support of Muscle Protein Synthesis and Muscle Protein Accretion in Young and Elderly Persons. *Http://Dx.Doi.Org/10.1080/07315724.2009.10718096*, *28*(4), 343–354. https://doi.org/10.1080/07315724.2009.10718096

Plotkin, D. L., Delcastillo, K., van Every, D. W., Tipton, K. D., Aragon, A. A., & Schoenfeld, B. J. (2021). Isolated Leucine and Branched-Chain Amino Acid Supplementation for Enhancing Muscular Strength and Hypertrophy: A Narrative Review. *International Journal of Sport Nutrition and Exercise Metabolism*, *31*(3), 292–301. https://doi.org/10.1123/IJSNEM.2020-0356

Reidy, P. T., Walker, D. K., Dickinson, J. M., Gundermann, D. M., Drummond, M. J., Timmerman, K. L., Fry, C. S., Borack, M. S., Cope, M. B., Mukherjea, R., Jennings, K., Volpi, E., & Rasmussen, B. B. (2013). Protein Blend Ingestion Following

Resistance Exercise Promotes Human Muscle Protein Synthesis. *The Journal of Nutrition*, *143*(4), 410–416. https://doi.org/10.3945/JN.112.168021

Rémond, D., Shahar, D. R., Gille, D., Pinto, P., Kachal, J., Peyron, M. A., dos Santos, C. N., Walther, B., Bordoni, A., Dupont, D., Tomás-Cobos, L., & Vergères, G. (2015). Understanding the gastrointestinal tract of the elderly to develop dietary solutions that prevent malnutrition. *Oncotarget*, *6*(16), 13858. https://doi.org/10.18632/ONCOTARGET.4030

Rieu, I., Balage, M., Sornet, C., Giraudet, C., Pujos, E., Grizard, J., Mosoni, L., & Dardevet, D. (2006). Leucine supplementation improves muscle protein synthesis in elderly men independently of hyperaminoacidaemia. *The Journal of Physiology*, *575*(1), 305–315. https://doi.org/10.1113/JPHYSIOL.2006.110742

Robinson, S. M., Reginster, J. Y., Rizzoli, R., Shaw, S. C., Kanis, J. A., Bautmans, I., Bischoff-Ferrari, H., Bruyère, O., Cesari, M., Dawson-Hughes, B., Fielding, R. A., Kaufman, J. M., Landi, F., Malafarina, V., Rolland, Y., van Loon, L. J., Vellas, B., Visser, M., Cooper, C., … Rueda, R. (2018). Does nutrition play a role in the prevention and management of sarcopenia? *Clinical Nutrition*, *37*(4), 1121–1132. https://doi.org/10.1016/J.CLNU.2017.08.016

Rolls, B. J. (1999). Do Chemosensory Changes Influence Food Intake in the Elderly? *Physiology & Behavior*, *66*(2), 193–197. https://doi.org/10.1016/S0031-9384(98)00264-9

Rowan, A. M., Moughan, P. J., Wilson, M. N., Maher, K., & Tasman-Jones, C. (1994). Comparison of the ileal and faecal digestibility of dietary amino acids in adult humans and evaluation of the pig as a model animal for digestion studies in man. *British Journal of Nutrition*, *71*(1), 29–42. https://doi.org/10.1079/BJN19940108

Ruderman, N. B. (1975). Muscle amino acid metabolism and gluconeogenesis. *Annual Review of Medicine*, *26*, 245–258. https://doi.org/10.1146/ANNUREV.ME.26.020175.001333

Rutherfurd, S. M., Fanning, A. C., Miller, B. J., & Moughan, P. J. (2015). Protein Digestibility-Corrected Amino Acid Scores and Digestible Indispensable Amino Acid Scores Differentially Describe Protein Quality in Growing Male Rats. *The Journal of Nutrition*, *145*(2), 372–379. https://doi.org/10.3945/JN.114.195438

Schaafsma, G. (2005). The Protein Digestibility-Corrected Amino Acid Score (PDCAAS)—A Concept for Describing Protein Quality in Foods and Food Ingredients: A Critical Review. *Journal of AOAC INTERNATIONAL*, *88*(3), 988–994. https://doi.org/10.1093/JAOAC/88.3.988

Schaafsma, G. (2012). Advantages and limitations of the protein digestibility-corrected amino acid score (PDCAAS) as a method for evaluating protein quality in human diets. *British Journal of Nutrition*, *108*(S2), S333–S336. https://doi.org/10.1017/S0007114512002541

Shivakumar, N., Kashyap, S., Kishore, S., Thomas, T., Varkey, A., Devi, S., Preston, T., Jahoor, F., Sheshshayee, M. S., & Kurpad, A. v. (2019). Protein-quality evaluation of complementary foods in Indian children. *The American Journal of Clinical Nutrition*, *109*(5), 1319–1327. https://doi.org/10.1093/AJCN/NQY265

Thakur, A., Sharma, V., Stud, A. T.-Int. J. Chem., & 2019, undefined. (2019). An overview of anti-nutritional factors in food. *Researchgate.Net*. https://www.researchgate.net/profile/Dr-Thakur-11/publication/336983167_An_overview_of_anti-nutritional_factors_in_food/links/5dbd3666a6fdcc2128f92574/An-overview-of-anti-nutritional-factors-in-food.pdf

Tomé, D. (2013). Digestibility issues of vegetable versus animal proteins: Protein and amino acid requirements-functional aspects. *Food and Nutrition Bulletin*, *34*(2), 272–274. https://doi.org/10.1177/156482651303400225

Trommelen, J., Tomé, D., & van Loon, L. J. C. (2021). Gut amino acid absorption in humans: Concepts and relevance for postprandial metabolism. *Clinical Nutrition Open Science*, *36*, 43–55. https://doi.org/10.1016/J.NUTOS.2020.12.006

Veldhorst, M. A. B., Nieuwenhuizen, A. G., Hochstenbach-Waelen, A., van Vught, A. J. A. H., Westerterp, K. R., Engelen, M. P. K. J., Brummer, R. J. M., Deutz, N. E. P., & Westerterp-Plantenga, M. S. (2009). Dose-dependent satiating effect of whey relative to casein or soy. *Physiology & Behavior*, *96*(4–5), 675–682. https://doi.org/10.1016/J.PHYSBEH.2009.01.004

Vetrano, D. L., Pisciotta, M. S., Laudisio, A., lo Monaco, M. R., Onder, G., Brandi, V., Fusco, D., di Capua, B., Ricciardi, D., Bernabei, R., & Zuccalà, G. (2018). Sarcopenia in Parkinson Disease: Comparison of Different Criteria and Association With Disease Severity. *Journal of the American Medical Directors Association*, *19*(6), 523–527. https://doi.org/10.1016/J.JAMDA.2017.12.005

Villaume, H.-M. C., Nicolas, J.-P., & Me Jean Bau, L. (1997). Effect of Germination on Chemical Composition, Biochemical Constituents and Antinutritional Factors of Soya Bean max) Seeds (Glycine. *J Sci Food Agric*, *73*, 1–9. https://doi.org/10.1002/(SICI)1097-0010(199701)73:1

Volpi, E., Kobayashi, H., Sheffield-Moore, M., Mittendorfer, B., & Wolfe, R. R. (2003). Essential amino acids are primarily responsible for the amino acid stimulation of muscle protein anabolism in healthy elderly adults. *The American Journal of Clinical Nutrition*, *78*(2), 250–258. https://doi.org/10.1093/AJCN/78.2.250

Volpi, E., Mittendorfer, B., Rasmussen, B. B., & Wolfe, R. R. (2000). The Response of Muscle Protein Anabolism to Combined Hyperaminoacidemia and Glucose-Induced Hyperinsulinemia Is Impaired in the Elderly. *The Journal of Clinical Endocrinology & Metabolism*, *85*(12), 4481–4490. https://doi.org/10.1210/JCEM.85.12.7021

Wagenmakers, A. J. M. (1998). Muscle amino acid metabolism at rest and during exercise: role in human physiology and metabolism. *Exercise and Sport Sciences Reviews*, *26*(1), 287–314. https://doi.org/10.1249/00003677-199800260-00013

Wall, B. T., Gorissen, S. H., Pennings, B., Koopman, R., Groen, B. B. L., Verdijk, L. B., & van Loon, L. J. C. (2015). Aging Is Accompanied by a Blunted Muscle Protein

Synthetic Response to Protein Ingestion. *PLOS ONE*, *10*(11), e0140903. https://doi.org/10.1371/JOURNAL.PONE.0140903

Walls, A. W. G., & Steele, J. G. (2004). The relationship between oral health and nutrition in older people. *Mechanisms of Ageing and Development*, *125*(12), 853–857. https://doi.org/10.1016/J.MAD.2004.07.011

Wilkes, E. A., Selby, A. L., Atherton, P. J., Patel, R., Rankin, D., Smith, K., & Rennie, M. J. (2009). Blunting of insulin inhibition of proteolysis in legs of older subjects may contribute to age-related sarcopenia. *The American Journal of Clinical Nutrition*, *90*(5), 1343–1350. https://doi.org/10.3945/AJCN.2009.27543

Wilkinson, D. J., Bukhari, S. S. I., Phillips, B. E., Limb, M. C., Cegielski, J., Brook, M. S., Rankin, D., Mitchell, W. K., Kobayashi, H., Williams, J. P., Lund, J., Greenhaff, P. L., Smith, K., & Atherton, P. J. (2018). Effects of leucine-enriched essential amino acid and whey protein bolus dosing upon skeletal muscle protein synthesis at rest and after exercise in older women. *Clinical Nutrition*, *37*(6), 2011–2021. https://doi.org/10.1016/J.CLNU.2017.09.008

Wilkinson, D. J., Hossain, T., Hill, D. S., Phillips, B. E., Crossland, H., Williams, J., Loughna, P., Churchward-Venne, T. A., Breen, L., Phillips, S. M., Etheridge, T., Rathmacher, J. A., Smith, K., Szewczyk, N. J., & Atherton, P. J. (2013). Effects of leucine and its metabolite β-hydroxy-β-methylbutyrate on human skeletal muscle protein metabolism. *Journal of Physiology*, *591*(11), 2911–2923. https://doi.org/10.1113/JPHYSIOL.2013.253203

Witard, O. C., Jackman, S. R., Breen, L., Smith, K., Selby, A., & Tipton, K. D. (2014). Myofibrillar muscle protein synthesis rates subsequent to a meal in response to increasing doses of whey protein at rest and after resistance exercise. *The American Journal of Clinical Nutrition*, *99*(1), 86–95. https://doi.org/10.3945/AJCN.112.055517

Wolfe, R. R., & Miller, S. L. (2008). The Recommended Dietary Allowance of Protein: A Misunderstood Concept. *JAMA*, *299*(24), 2891–2893. https://doi.org/10.1001/JAMA.299.24.2891

Wolfe, R. R., Miller, S. L., & Miller, K. B. (2008). Optimal protein intake in the elderly. *Clinical Nutrition*, *27*(5), 675–684. https://doi.org/10.1016/J.CLNU.2008.06.008

Yeung, S. S. Y., Reijnierse, E. M., Pham, V. K., Trappenburg, M. C., Lim, W. K., Meskers, C. G. M., & Maier, A. B. (2019). Sarcopenia and its association with falls and fractures in older adults: A systematic review and meta-analysis. *Journal of Cachexia, Sarcopenia and Muscle*, *10*(3), 485–500. https://doi.org/10.1002/JCSM.12411