# Impact of Multi-Ingredient Formulations to Optimise Exercise Performance and Training Outcomes in Physically Active Adults (>40 to 65 Years Old)

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# DECLARATION

I certify that the work contained in this thesis, or any part of it, has not been accepted in substance for any previous degree awarded to me or any other person, and is not concurrently being submitted for any other degree other than that of MPhil/PhD Student in Sports Nutrition for Healthy Ageing, which has been studied at the University of Greenwich, London, UK.

I also declare that the work contained in this thesis is the result of my own investigations, except where otherwise identified and acknowledged by references. I further declare that no aspects of the contents of this thesis are the outcome of any form of research misconduct.

I declare any personal, sensitive or confidential information/data has been removed or participants have been anonymised. I further declare that where any questionnaires, survey answers or other qualitative responses of participants are recorded/included in the appendices, all personal information has been removed or anonymised. Where University forms (such as those from the Research Ethics Committee) have been included in appendices, all handwritten/scanned signatures have been removed.

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# COLLABORATIONS:

The following statement specifies the relative personal contribution to each experimental chapter and the key roles assumed from other collaborators:

Firstly, for the Chapter 1 – Introduction, Dr. Fernando Naclerio was the responsible for guiding my steps and writing process, by suggesting a clear structure and the topics to speak about, as well as correcting my spelling and scientific writing mistakes. Apart from that, the final product is the result of my hard work, interest and commitment to the thesis excellence.

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# ABSTRACT

This thesis investigated the short- and long-term effects of protein-based multi-ingredient supplements (MTN) compared the ingestion of isoenergetic carbohydrate-only comparator (COMP) ingested pre- (PREW) or post-workout (POSTW) on functional capacity and body composition in physically active middle-aged and older adults undergoing resistance training (RT). The present thesis includes one systematic review with meta-analysis, two crossover short-term investigations (one with a PREW and one using a POSTW MTN), and two 6-week parallel trials using either a PREW or a POSTW MTN. The systematic review resulted in a lack of significant benefits to maximise muscular hypertrophy, strength gains, and functional capacity for those groups using MTN compared to those consuming carbohydrates alone either before, during or after training. Nonetheless, the ingestion of a PREW caffeine-based MTN was shown to be effective in enhancing the amount of load lifted during three RT workouts as well as in maximising fatty acid oxidation during low-intensity cycling. However, when the effect of a similar PREW was investigated over a 6-week RT intervention no advantages over the COMP were identified to maximise performance or changes in body composition. Similarly, a pea protein-based POSTW MTN did not significantly enhance recovery markers or muscle contractile properties after performing three consecutive RT sessions compared to a COMP condition. Conversely, compared to the ingestion of only carbohydrates, a PREW admixture including whey protein, carbohydrate, creatine, β-HMB, and Vitamin D3 promoted better body composition changes and performance outcomes (e.g., vertical jump height) over a 6-week intervention. In conclusion, PREW MTN may promote some initial benefits on performance and fat metabolism with no subsequent effects across longer interventions. On the other hand, POSTW MTN, composed of plant-based protein with no creatine seem to offer no advantage compared to the ingestion of only carbohydrates. Nonetheless, POSTW MTN containing whey protein and creatine, and fortified with amino acids, seems to be effective in maximising RT outcomes in middle-aged and older adults engaged in RT.

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# ABBREVIATIONS

**5-STS:** 5-times sit-to-stand.
**6-MW:** 6-minutes walking test.
**10-MW:** 10-meters walking test.
**30-STS:** 30-s sit-to-stand test.
***β*-HMB:** *β*-Hydroxy β-Methylbutyrate.
**BC:** Body Composition.
**BM:** Body Mass.
**CHO:** Carbohydrate/s.
**CMBT:** Chest Medicine Ball Throw.
**CMJ:** Countermovement Jump.
**COMP:** Comparator.
**DIAAS:** Digestible Indispensable Amino Acid Score.
**EAA:** Essential Amino Acids.
**FFM:** Fat-Free Mass.
**FM:** Fat Mass.
**LBM:** Lean Body Mass.
**MPS:** Muscle Protein Synthesis.
**MTN:** Multi-ingredient/Multi-nutrient.
**PDCAAS:** Protein Digestibility-Corrected Amino Acid Score.
**POSTW:** Post-workout.
**PREW:** Pre-workout.
**PRISMA:** Preferred Reporting Items for Systematic reviews and Meta-Analyses.
**RDA:** Recommended Dietary Allowances.
**RM:** Repetition Maximum.
**RT:** Resistance Training.
**TMG:** Tensiomyography.
**TUG:** Timed-up-and-go test.

# CHAPTER 1

# INTRODUCTION

Protein-based multi-ingredient formulations have garnered significant attention in the field of sports nutrition, particularly for their potential to enhance exercise performance, training adaptations, and overall wellbeing in physically active middle-aged and older adults (Puente-Fernández et al., 2020). As the global population ages, there is an increasing emphasis on strategies to mitigate age-associated declines in muscle mass, strength, and functional capacity (Cruz-Jentoft et al., 2019). Dietary supplementation presents a viable approach to support these objectives, especially when tailored to the unique physiological needs of this demographic (Phillips, 2015). These formulations typically combine high-quality protein sources, such as whey protein with other ergogenic aids such as creatine, beta-alanine, omega-3 fatty acids, vitamins, and minerals (Harty et al., 2018). The synergistic effects of these ingredients are hypothesised to optimise muscle protein synthesis, improve recovery times, and enhance physical performance beyond what is achievable with protein supplementation alone (Kreider et al., 2017). The convenience of consuming multiple beneficial nutrients in a single product also adds to their appeal among consumers.

In the United Kingdom and many other countries, dietary supplements are regulated under food safety laws rather than pharmaceutical regulations, which means they are subject to less stringent pre-market efficacy and safety evaluations (H&SC, 2020). This regulatory framework allows manufacturers to introduce new products to the market without exhaustive clinical testing, potentially leading to variability in product quality and effectiveness (Geyer et al., 2011). Consequently, consumers and healthcare professionals may face challenges in making informed decisions regarding the use of these supplements.

Research on dietary supplements has traditionally focused on younger athletic populations, but there is a growing body of literature examining their effects in middle-aged and older adults (Tieland et al., 2012; Bauer et al., 2013; Finger et al., 2015; Burd et al., 2019a; Moore, 2019). Aging is associated with anabolic resistance, a reduced sensitivity of muscle protein synthesis to dietary protein and exercise stimuli (Moore et al., 2015). This condition has been considered a multifactorial phenomenon driven by a complex interplay of physiological, molecular and behavioural factors (Aragon, Tipton

& Schoenfeld, 2023), characterised by a diminished sensitivity to muscle skeletal anabolic stimuli such as daily dietary amino acids (AA) or proteins and resistance exercise (Moore et al., 2015). In a deeper analysis of anabolic resistance, it has been observed to be the result of several causes (Aragon, Tipton & Schoenfeld, 2023) such as an impaired mTORC1 signalling, AA delivery to musculoskeletal tissue, exhibiting a greater splanchnic extraction of these AA, leading to lower AA availability for muscle protein synthesis (Breen & Phillips, 2012). These reasons, together with a chronic low-grade inflammation (currently known as *inflammaging* by elevated circulatory pro-inflammatory cytokines) which blunts muscle anabolic response and stimulates catabolic physiological pathways, a mitochondrial dysfunction combined with greater oxidative stress, satellite cell function impairment (with reduced activity, compromising the regenerative capacity and the ability to respond to exercise-induced muscle damage and remodelling), and a potential emerging insulin resistance by a decreased insulin sensitivity produced by Type II muscle fibres loss, frequently lead to perpetuate a lower impact of different efficient strategies to counteract muscle loss and functional capacity while ageing, such as resistance exercise or specific dietary modifications as well as nutritional supplementation protocols (Aragon, Tipton & Schoenfeld, 2023).

Protein-based multi-ingredient supplements may help overcome this resistance by providing a more potent stimulus for muscle anabolism (Witard et al., 2016; Lim et al., 2021). Moreover, the inclusion of ingredients like creatine has been shown to improve muscle strength and cognitive function in older individuals (Rawson & Venezia, 2011; Xu, Zhang & Luo, 2024). These potential mechanisms, synergistically working with the anti-inflammatory effects attributed to Omega-3 fatty acids (Bischoff-Ferrari et al., 2025), anticatabolic properties linked to HMB (Costa Riela et al., 2021), and the anabolic stimuli suggested to leucine-enriched beverages (Ely et al., 2023) might induce a more effective physiological environment to promote enhanced functional capacity and body composition outcomes in ageing populations.

Despite the promising potential of these formulations, their efficacy and safety profiles in older adults remain underexplored (Liu et al., 2024). Factors such as dosage, timing, and the specific combination of ingredients can significantly influence outcomes (Jäger et al., 2017). Additionally, concerns about interactions with medications commonly used by older adults necessitate careful consideration (de Castro et al., 2022).

The adoption of dietary supplements is widespread among physically active individuals, including middle-aged and older adults seeking to maintain or improve their physical fitness (Denison et al., 2012). However, the motivations for supplement use often do not align with evidence-based recommendations, highlighting a gap between consumer practices and scientific research (Dickinson & MacKay, 2014). Misinformation, marketing claims, and a lack of professional guidance contribute to this discrepancy (Maughan, 2013).

To maximize the benefits of protein-based multi-ingredient formulations, supplementation strategies should be individualized, taking into account the specific inter-individual goals, health status, and nutritional needs (Paddon-Jones & Rasmussen, 2009). For example, endurance athletes may require different nutrient ratios compared to those engaged in resistance training (Stokes et al., 2018). Understanding the nuances of how these supplements interact with exercise modalities is crucial for optimising training outcomes and promoting long-term wellbeing.

While certain studies have reported non-significant alterations in body composition following 5 months of consistent resistance training in post-menopausal women, despite observed enhancements in functional capacity (Coelho-Júnior et al., 2019), resistance training is universally acknowledged as the preferred exercise modality for preserving and stimulating muscle mass accrual, augmenting strength, and ultimately enhancing functional capacity to mitigate the onset of sarcopenia in older adults (Messier et al., 2011; Orsatti et al., 2022).

Dietary patterns, as modifiable habits, have been proposed to play a significant role in the management and prevention of sarcopenia (Cruz-Jentoft et al., 2020). The Recommended Dietary Allowances (RDAs) are established as "the levels of essential nutrient intake that are considered adequate, based on scientific knowledge, to meet the known nutrient requirements of nearly all healthy individuals" (NAS, 1989). However, the current RDA of 0.8 $g \cdot kg \cdot day^{-1}$ (WHO, 2007) has remained unchanged for over 15 years. Concerns have been raised regarding the adequacy of these protein intake recommendations for maintaining or increasing muscle mass in adults, including older populations (Bauer et al., 2013; Deutz et al., 2014; Deer & Volpi, 2015; Phillips, Chevalier & Leidy, 2016; Lonnie et al., 2018; Morton et al., 2018; Putra et al., 2021; Ribeiro et al., 2022). Numerous studies have suggested that higher absolute (total amount) and relative (grams per kilogram of body mass) protein intake levels (>1.2 to 1.6 $g \cdot kg^{-1}$ BM) may be necessary to maintain or promote muscle mass accretion and support exercise-induced outcomes, such as strength gains, in both malnourished individuals (Cramer et al., 2016) and healthy older adults (Maltais, Desroches & Dionne, 2009; Phillips & Martinson, 2019; Ribeiro et al., 2022).

Protein is widely recognized as a crucial macronutrient essential for the preservation of normal physiological functions (Putra et al., 2021). Recent findings from an interventional study involving healthy, untrained older women suggest that body recomposition, characterized by greater increases in fat-free mass (FFM) and maintenance of fat mass (FM), is optimized with a dietary protein intake exceeding 0.91 g·kg·day$^{-1}$ after 24 weeks of resistance training (RT) intervention (Ribeiro et al., 2022). The Recommended Dietary Allowance (RDA) for protein may not be adequate for populations engaged in physical activity, clinical settings, or older adults aiming to maximize muscle protein synthesis (MPS), muscle mass, physical function, and overall health (Deutz et al., 2014; Putra et al., 2021). Conversely, considerable scientific evidence in the past decade supports the notion of higher daily protein intake, ranging from 1.2 to 2.0 g·kg$^{-1}$ of body mass, to mitigate age-related declines in musculoskeletal mass quality (muscle strength relative to muscle mass) and functional capacity (Bauer et al., 2013; Deutz et al., 2014; Phillips, Chevalier & Leidy, 2016; Morton et al., 2018; Putra et al., 2021). Notably, the International Society of Sports Nutrition recommends a range of 1.4 to 2.0 g·kg·day$^{-1}$ for most individuals engaged in exercise to promote muscle mass maintenance through positive muscle protein balance, while a range of 2.3 to 3.1 g·kg·day$^{-1}$ might be necessary during hypocaloric periods in resistance-trained individuals aiming to maximize muscle mass retention (Jäger et al., 2017). However, a study by Morton et al. (2018) reported no additional benefits of protein supplementation on RT-induced increases in FFM when protein intake exceeded 1.62 g·kg·day$^{-1}$, suggesting diminished efficacy of protein supplementation in older populations and heightened effectiveness in those experienced in resistance training (Morton et al., 2018). Optimal MPS response in older adults has been observed with the ingestion of 35-40 g of protein immediately following a resistance training session (Churchward-Venne et al., 2016). Additionally, Paddon-Jones & Rasmussen (2009) proposed a target of 25-30 g of high-quality protein per meal during periods of rest to maximize MPS in both young and older individuals. However, achieving the recommended daily protein intake can be challenging for older adults due to decreased appetite, increased satiety from higher protein consumption, and therefore denser meals (Paddon-Jones et al., 2008; Pilgrim et al., 2015; Lonnie et al., 2018; Wolfe et al., 2018). Anabolic resistance, characterized by a diminished muscle protein synthetic response to dietary AA ingestion at rest and after resistance exercise, is observed in sedentary older individuals (Moore, 2021). Consequently, it is recommended to consume higher amounts of high-quality protein per meal to overcome anabolic resistance and maintain muscle mass in untrained adults (Wall et al., 2015).

A suggested dietary approach to meet protein needs in older adults involves incorporating protein-based supplements, such as protein powders, into regular meals. Numerous studies have demonstrated that protein supplements in the form of isolates or hydrolysates are readily consumed, digested, and absorbed by the aging population, despite the diminished absorption kinetics and heightened anabolic resistance associated with aging (Dangin et al., 2003; Koopman et al., 2009; Gorissen et al., 2014; Gorissen et al., 2020).

## 1.1. Protein quality and sources

The functional potential of protein in muscle health is greatly influenced by its AA profile, which is considered a crucial determinant of protein quality (Baum & Wolfe, 2015). There is a wide range of diverse protein sources available, and the choice of protein source consumed can vary significantly among individuals, influencing the rates of muscle protein synthesis (MPS) (Tang & Phillips, 2009; Churchward-Venne et al., 2016). The overall quantity of protein consumed, as well as the composition of essential amino acids (EAAs), have been identified as crucial factors determining the extent of the post-exercise MPS response (Churchward-Venne et al., 2016). Furthermore, the ingestion of different protein types can impact the magnitude and duration of MPS following consumption, with resistance training potentially amplifying this effect (Tang & Phillips, 2009). This influence may be attributed to variations in the EAAs composition and the rate of protein digestion (Phillips, Tang & Moore, 2009). Specifically, it has been observed that rapidly digested protein sources rich in leucine may promote higher rates of MPS after a workout compared to other protein sources, as leucine serves as a key anabolic signal for mRNA translation (Breen & Phillips, 2012).

EAAs encompass nine AA that the body cannot synthesize internally and must be obtained from the diet, including phenylalanine, valine, tryptophan, threonine, isoleucine, methionine, histidine, leucine, and lysine (Lonnie et al., 2018; Lopez & Mohiuddin, 2022). The term "complete protein" signifies that the selected protein source contains all the EAAs (Leser, 2013). However, "high-quality proteins" are characterised by containing all EAAs in the necessary amounts to fulfil the body's requirements (Wu, 2009; Wu, 2013; Naclerio & Seijo, 2019). Non-essential amino acids (NEAAs), on the other hand, can be synthesised by the body in sufficient quantities (Wu, 2009; Wu, 2013; Naclerio & Seijo, 2019) and are not essential for stimulating net balance. The ingestion of EAAs has a dose-dependent effect on muscle protein synthesis (Børsheim, 2002). In 2013, the Food and Agriculture Organization (FAO) of the World Health Organization (WHO) introduced the Digestible Indispensable Amino Acid Score (DIAAS) as a method to evaluate protein quality (Leser, 2013). The DIAAS expands on the principles of the protein digestibility-corrected amino acid score (PDCAAS) established by the same organization in 1990 (FAO, 1991). The DIAAS of a specific protein is determined by its true ileal digestibility and its content and profile of EAA in relation to dietary requirements (Leser, 2013; Wolfe et al., 2016). Postprandial EAA availability is regulated by a series of physiological processes, including protein digestion, AA absorption, splanchnic AA retention, and skeletal muscle perfusion (Groen et al., 2015), along with various nutritional factors such as AA composition, EAA content, and the presence of anti-nutritional factors (Gorissen et al., 2018). Moreover, a DIAAS score greater than 1.0 is defined as "high quality" (Moore et al., 2008; Wolfe et al., 2016). In general, many plant-based foods are deficient in one or more EAAs, known as "limiting amino acids," and are thus referred to as "incomplete protein food sources" (Hoffman & Falvo, 2004). However, certain extracts from plant protein sources, such as rice (Joy et al., 2013), soy (Wolfe, 2015), or pea (Babault, 2015), have demonstrated scores close to or greater than 1.0.

The superiority of animal proteins over plant proteins in terms of Digestible Indispensable Amino Acid Score (DIAAS) is widely acknowledged, often exhibiting values that are twice as high (Wolfe et al., 2018). The evaluation of protein quality suggests that animal proteins may more efficiently and readily meet the daily EAAs requirements compared to plant proteins (Wolfe et al., 2018). Among animal-derived proteins, particular attention has been given to dairy proteins (whey and casein), meat (beef, pork), poultry (chicken, turkey), eggs, and fish. Notably, milk proteins, especially whey protein, have received extensive support and research attention in the literature (Phillips, Tang & Moore, 2009; Naclerio & Larumbe-Zabala, 2016; Sepandi et al., 2022; Srinivasaraghavan et al., 2022).

Houston et al. (2008) conducted a longitudinal study spanning three years in an older adult population, observing that the consumption of animal protein, which had a higher content of EAAs, was significantly associated with the preservation of lean mass (LM) and appendicular LM. Lower EAA content, particularly insufficient leucine, lysine, and/or methionine, may account for the reduced anabolic potential of plant-based proteins compared to their animal-based counterparts (Gorissen et al., 2018). However, the metabolic equivalence of these diverse protein sources has yet to be established (Park et al., 2021).

In recent years, there has been a growing interest in plant-based protein sources, such as soy, rice, hemp, and pea (Putra et al., 2021). This surge in popularity can be attributed to the increasing number of vegetarian and vegan consumers (Bakaloudi et al., 2021), which has reached up to 10% in Europe in recent years (Allès et al., 2017). The perceived higher sustainability of plant-based protein production compared to animal-based sources (Melina, Craig & Levin, 2016; Hemler & Hu, 2019; Segovia-Siapco, Rajaram & Sabaté, 2019*)* and emerging scientific evidence supporting the benefits of plant-based proteins, such as soy (Candow et al., 2006a; Messina et al., 2018; Putra et al., 2021) and pea (Babault, 2015; Banaszek et al., 2019), have captured the attention of both consumers and researchers. However, studies have shown that soy protein elicits lower muscle protein synthesis (MPS) responses than whey protein at rest and after exercise in both young (Tang et al., 2009) and older men (Yang et al., 2012a). Additionally, whey protein has been demonstrated to significantly enhance gains in lean body mass when combined with resistance training over a 9-month period (Volek et al., 2013). A recent study conducted on healthy young males found that the consumption of either 30g of potato protein concentrate or milk protein resulted in similar increases in muscle protein synthesis (MPS) rates at rest and during recovery from exercise (Pinckaers et al., 2022). There is substantial variability between animal and plant-based foods and food products in terms of protein and AA content, as well as the interaction between nutrients and the food matrix, including differences in absorption kinetics (Burd et al., 2019b). Plant-based proteins generally exhibit lower digestibility compared to animal-based proteins due to the presence of anti-nutritional factors (van Vliet, Burd & van Loon, 2015). However, when these protein supplements are provided in isolated forms such as pea, soy, or whey, they have been shown to equally enhance muscle growth, strength (Babault et al., 2015), and body composition (Banaszek et al., 2019) in young and healthy adults following a resistance training program. Gorissen et al. (2018) observed that plant-based protein isolates such as oat (21%), lupin (21%), and wheat (22%) had lower EAAs contents compared to animal-based proteins (e.g., whey 43%, milk 39%, casein 34%, and egg 32%) and muscle protein (38%). Furthermore, the AA profiles of plant-based proteins differed significantly, with leucine content ranging from 5.1% for hemp to 13.5% for corn protein, compared to 9.0% for milk, 7.0% for egg, and 7.6% for muscle protein. However, limited research is available on vegan protein, whether in isolated form or as part of a multi-ingredient supplement, particularly in middle-aged and older adults, and there is currently no evidence regarding its effectiveness in preventing age-related muscle loss compared to animal proteins (Messina et al., 2018; Putra et al., 2021). It has been suggested that combinations of plant-based protein isolates or blends with complementary AA profiles could potentially provide protein characteristics that closely resemble those of animal-based proteins (Gorissen et al., 2018).

In recent years, pea protein has gained popularity among consumers. However, limited research has been conducted to assess its long-term effectiveness and efficacy in promoting muscle health, particularly in young individuals (Putra et al., 2021), and no studies have been conducted in middle-aged to older populations. The appeal of pea protein is primarily attributed to its high Protein Digestibility-Corrected Amino Acid Score (PDCAAS) and the cost-effectiveness of its production. Cooked yellow pea has a PDCAAS of 0.67 (Nosworthy et al., 2017; van Vliet, Burd & van Loon, 2015), while pea protein concentrate has a PDCAAS of 0.89 (Rutherfurd et al., 2014), and pea protein isolate has a PDCAAS of 0.93 out of 1.00 (Yang et al., 2012b). Despite being relatively low in methionine and cysteine (Yang et al., 2012b), the high PDCAAS values of pea protein concentrate and isolate make them promising nutritional supplementation options. However, the lack of short-, medium-, and long-term scientific studies hinders the confirmation of their efficacy and actual benefits.

### 1.2. Protein-based supplements: multi-ingredients

The terms "supplement," "dietary supplement," or "ergogenic aid" in the context of sports and physical exercise refer to orally consumed substances such as vitamins, minerals, herbal remedies, carbohydrates, proteins, AA, and other substances like creatine or caffeine (Petróczi et al., 2007). Extensive research has been conducted to investigate the effectiveness of individual nutrients and supplements, including carbohydrates, proteins, AA, creatine, and caffeine. Studies have provided evidence supporting the use of various combinations of supplements for enhancing health and physical performance (Bell et al., 2017; O'Bryan, 2019). While the positive effects of individual supplements such as whey protein, carbohydrates, creatine monohydrate, beta-hydroxy-beta-methylbutyrate, carnitine, and caffeine have been documented, the impact of commercially available blend mixtures or multi-ingredients (MTN) containing different proportions of these nutrients remains a topic of debate (Morton et al., 2018; O'Bryan et al., 2019).

MTN are specialized formulations that combine macronutrients, micronutrients, and other nutritional substances such as AA derivatives or stimulants (Naclerio et al., 2020). These formulations are believed to enhance exercise outcomes compared to exercise alone (Bell et al., 2017). The inclusion of various ingredients in these formulas aims to synergistically target specific goals, offering the convenience of consuming them in a single intake (Cooper et al., 2013). While commercially available MTN formulas are commonly used by healthy individuals engaged in recreational training to optimize their training outcomes, the effectiveness of these supplements remains uncertain when consumed alongside a healthy diet that already provides sufficient protein intake (Morton et al., 2018).

MTN have been administered at various time points, including prior to exercise, during exercise, post-exercise, at breakfast, or before sleep (O'Bryan et al., 2019). Pre- or intra-exercise consumption of MTN is aimed at enhancing motivation and optimizing performance during workouts (Damas et al., 2015; Puente-Fernández et al., 2020). On the other hand, post-workout MTN are expected to accelerate recovery (Naclerio et al., 2020; Naclerio et al., 2021) and potentially maximize the outcomes of training, such as muscle mass gain, strength improvements, and overall functional capacity (Bell et al., 2017).

The most prevalent formulations combine proteins, carbohydrates, and fats with small quantities of EAAs (e.g., leucine) or conditional amino acids (e.g., glutamine) (Candow, 2006a; Naclerio & Larumbe-Zabala, 2016; Davies, Carson & Jakeman, 2018). Furthermore, recent literature suggests that Vitamin D may enhance muscle strength, potentially through the presence of Vitamin D receptors in muscle tissue. Omega-3 fatty acids have been linked to improvements in muscle mass and strength, possibly by modulating cellular signalling and reducing inflammation-related oxidative damage. Low-dose antioxidants, such as Vitamins C and E, have the potential to protect muscle tissue from oxidative damage (Cruz-Jentoft et al., 2020). Additionally, the inclusion of nutrients like creatine (Negro et al., 2019) or caffeine (Ormsbee et al., 2012; Puente-Fernandez et al., 2020) in these formulations has been shown to enhance the effects of each individual nutrient, although the specific types, quantities, and combinations are still not fully understood when combined with isoenergetic carbohydrate-based formulations (Directo et al., 2019; Curtis et al., 2022).

The aim of this thesis was to evaluate the short-term and long-term impact of various commercially available protein-based multi-ingredient formulations vs. isoenergetic and carbohydrates-only supplements ingested pre- or post-workout on exercise-induced adaptations in physically active middle-aged and older adults engaged in resistance training. However, given the difficulties emerging after Covid-19 pandemic, combined with excessive restrictions and lack of volunteering participants, the project was limited to include two 6-week interventional studies only.

By synthesising current research findings and conducting empirical studies, we seek to provide evidence-based recommendations for the use of these supplements in this population. To achieve the proposed aims the following three phases were designed:

(i) To conduct a Systematic Review of the literature aimed at analysing the effectiveness of combining resistance exercise with a multi-ingredient supplement vs. an isocaloric single nutrient containing no protein on body composition, physical function, and performance in physically active middle-aged and older adults. Procedures have been conducted in accordance with PRISMA guidelines 2020 (Moher et al., 2015; Shamseer et al., 2015; Page et al., 2021).

(ii) To perform two acute interventional studies aimed to determine the effects of combining resistance exercise programmes with the ingestion of pre- or post-workout multi-ingredient formulations on training and recovery variables, such as physical performance decline, muscular contractile properties, and willingness to train in healthy recreationally active middle-aged and older female and male adults.

> (ii.i) To carry out a one-week double-blinded randomised controlled crossover intervention comparing the effect of a caffeinated, protein-based multi-ingredient <u>pre-workout</u> supplement including amino acids, against a single-nutrient carbohydrate-only isocaloric comparator.

(ii.ii) To carry out a one-week double-blinded randomised controlled crossover intervention comparing the effect of a pea protein-based multi-ingredient post-workout supplement including amino acids, against a single-nutrient carbohydrate-only isocaloric comparator.

(iii) To perform two interventional studies aimed to determine the effects of combining resistance exercise programmes with the ingestion of pre- or post-workout multi-ingredient formulations on body composition, physical performance, and willingness to train in healthy recreationally active middle-aged and older female and male adults.

(iii.i) To carry out a parallel double-blinded randomised controlled parallel intervention comparing the effect of a caffeinated, protein-based multi-ingredient pre-workout supplement including stimulant amino acids and carbohydrates, against a non-caffeinated single-nutrient carbohydrate-only isocaloric comparator on body composition, performance outcomes and functional capacity.

(iii.i) To carry out a parallel double-blinded randomised controlled parallel intervention comparing the effect of a protein-based multi-ingredient post-workout supplement including HMB, vitamin D, creatine, and carbohydrates, against a non-caffeinated single-nutrient carbohydrate-only isocaloric comparator on body composition, performance outcomes and functional capacity.

It was hypothesised that:

i. Compared to single-nutrient, carbohydrate-only supplementation, a caffeinated multi-ingredient pre-workout supplement will provide acute further stimulant benefits with a cumulative beneficial effect in the long term.
ii. Compared to single-nutrient, carbohydrate-only supplementation a protein-based multi-ingredient post-workout supplement will provide acute further recovery advantages with a cumulative beneficial effect in the long term.

# CHAPTER 2

# STUDY 1 - NO IMPACT OF COMBINING MULTI-INGREDIENT SUPPLEMENTATION WITH EXERCISE ON BODY COMPOSITION AND PHYSICAL PERFORMANCE, IN HEALTHY MIDDLE-AGED AND OLDER ADULTS: A SYSTEMATIC REVIEW AND META-ANALYSIS

## 2.1. Introduction

An active lifestyle combined with appropriate nutritional patterns has proven to attenuate age-related impairment in quality of life by preserving muscular performance and improving functional capacity (von Berens et al., 2018). The term functional capacity has been used to describe a person's ability to perform domestic and self-care activities free of physically related limitations (Seitsamo, Tuomi & Martikainen, 2007). In this regard, resistance training (RT) combined with a daily protein intake higher than 0.8 $g \cdot kg^{-1}$ of body mass (Thomas, Erdman & Burke, 2016) has proven to be an effective strategy for attenuating age-related declines in functional capacity (Beasley et al., 2013; Seesen et al., 2020; Traylor et al., 2018), and improving musculoskeletal function in middle-aged and older adults (Bunout et al., 2004). Indeed, a well-structured RT programme successfully maintained or even increased muscle mass, muscular strength, and functional capacity in sedentary, obese, sarcopenic, active and trained, middle-aged and older adults (Garber et al., 2011; Giallauria et al., 2015; Clark, 2016; Fragala et al., 2019). The observed health-related benefits are maximised when RT-based interventions are combined with appropriate eating behaviours. For instance, adding whey protein-based admixtures, including carbohydrates, small amounts of fats and some essential (e.g., leucine) or conditional (e.g., glutamine) amino acids has been proven to maximise exercise outcomes in young adults (Candow et al., 2006; Naclerio & Larumbe-Zabala, 2016; Davies, Carson & Jakeman, 2018). However, conflicting results regarding the advantage of combining resistance exercise with protein-based multi-ingredients (MTN) supplements in healthy and pre-conditioned middle-aged and older adults have been published (Eliot et al., 2008; Sugihara Junior et al., 2018). MTN are specialised forms of supplements combining macronutrients, micronutrients, and other nutritional substances (AA derivatives, or stimulants) that may optimise exercise outcomes compared to exercise alone (Bell et al., 2017). Most MTN admixtures contain a proprietary blend of ingredients expected to promote benefits when taken as described. For instance, based on their specific formulation, MTN are recommended to be ingested prior, during, and or post-exercise, at breakfast or before sleep (O'Bryan et al., 2019). The ingestion of MTN before and during workouts has been recommended to enhance motivation to train and to maximise exercise performance (Damas et al., 2015; Puente-Fernández et al., 2020). On the other hand, ingesting MTN after exercise has been proposed to speed up recovery (Naclerio et al., 2020, 2021) and eventually maximise training-induced outcomes (Bell et al., 2017), e.g., muscle mass gain, strength, and general functional capacity.

Older sedentary individuals experience anabolic resistance, which is characterised by a blunted muscle protein synthetic response to dietary AA ingestion at rest and after resistance exercise (Moore, 2021). Therefore, ingesting more protein per meal has been recommended to overcome anabolic resistance and maintain muscle mass in untrained adults (Wall et al., 2015). However, it is still unclear whether older trained persons need higher daily protein intake to benefit from exercise-induced muscle protein synthesis as observed in young individuals (Moore, 2021). Nonetheless, when the appropriate amount of daily protein (i.e., 1.6 $g \cdot kg^{-1}$) is consumed, it seems that adding protein-based supplements would not induce significant improvement in training outcomes in old individuals (Morton et al., 2018).

Two previous systematic reviews conducted in adults observed beneficial effects of adding protein-based supplementation to optimise resistance training outcomes for middle-aged >45 years old (O'Bryan et al., 2019) and ≥60 years old individuals (Liao et al., 2017). However, these reviews included studies using healthy, obese, and patient individuals that were meta-analysed together without considering the impact of health or body composition status on the adaptations induced by training and nutrition combined interventions (Hita-Contreras et al., 2018; Martínez-Amat et al., 2018). For instance, (Liao et al., 2017) included overweight and obese participants while (O'Bryan et al., 2019) included participants diagnosed with sarcopenia. Consequently, an analysis including only healthy individuals accounting for their particular response to combining exercise with MTN supplementation is still necessary. Therefore, the aim of the present systematic review and meta-analysis was to analyse the effects of MTN supplementation on training-induced improvements in body composition and physical performance (i.e., muscular strength, and functional capacity), on healthy and recreationally active middle-aged and older adults (>45 years old).

## 2.2. Methods

The analysis method and inclusion criteria were specified in advance and documented in a protocol registered at the International Prospective Register of Systematic Reviews, PROSPERO (CRD42020200336).

### *2.2.1. Search Strategy*

A systematic review of the literature was conducted in accordance with the recommended criteria provided in the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement (Liberati et al., 2009; Shamseer et al., 2015; Page et al., 2021) and the guidelines described for systematic reviews in the nutrition field (Moher & Tricco, 2008). The procedures incorporated for the current meta-analysis included: identification, screening, eligibility, and inclusion/exclusion of studies. The search of the literature was systematically reviewed by using PubMed, EBSCOhost (Medline, Academic Search Premier, CINAHL Plus with Full Text), Google Scholar, Web of Science, and SPORTDiscus through June to December 2021 (with no lower date limit).

English and Spanish languages randomized controlled trials (RCT) conducted in human populations were identified and categorised as eligible for review, including articles, abstracts from annual scientific conferences and congress presentations, or doctoral theses as well as retrieved preprinted versions via University Resources, without any assigned DOI. Commentaries, reviews, or duplicate publications from the same study were not included in this analysis. Additional studies were identified by contacting experts in the field as well as reviewing other systematic reviews previously published in similar populations and topics (Luo et al., 2017; Morton et al., 2018; O'Bryan et al., 2019; Labata- Lezaun et al., 2020). The reference lists of the retrieved studies were hand-searched to identify potentially eligible studies not captured by the electronic searches. Two reviewers (JPF and FN) independently screened the title, abstract and reference list of each study to locate potentially relevant studies. Any discrepancies between the two authors were resolved through consensus or by the opinion of a third author (ELZ).

Combinations of the following keywords were used as search terms: "((*Multi* OR Protein* OR Beef Protein OR Soy Protein OR Pea Protein OR Rice Protein OR Whey* OR BCAA* OR Branch*) AND (Supplement* OR Enrich* OR Formula* OR Combin* OR Fortifi*) AND ((Adult* OR Middle-Age* OR Old* OR Elderl* OR Ageing OR Aging OR Master Athlet* OR Senior Athlet*))) AND ((Resist* OR Endur* OR Aerobic Training

OR Anaerobic Training OR Train* OR Exercise OR Strength* OR Power* OR Recov* OR Energ* OR Performance))) AND ((Body Composition OR Body* OR Fat* OR Lean* OR Muscle OR Muscul* OR Skelet* OR Weight OR QoL OR Qual* OR Lif* OR Hypertroph* OR Metaboli* OR Capacity OR Function*))) NOT (=(Ill* OR Canc* OR Frail* OR Sick* OR ICU OR Sclerosis OR Diabet* OR Patient* OR Hospit* OR Rehab* OR Child* OR Kid* OR Toddler* OR Animal* OR Rat* OR Mice OR Mouse))".

### *2.2.2. Eligibility Criteria*

The inclusion and exclusion criteria were the following: (i) the trial was randomized and controlled involving at least two groups: treatment and comparator (using an isoenergetic comparator (COMP) treatment including only one macronutrient or a placebo); (ii) the treatment combined prolonged (≥6 weeks) exercise-training intervention (resistance, endurance, flexibility, or mixing modalities) with a minimum workout frequency of 2 days per week while ingesting a MTN supplement containing protein from animal (e.g., whey, casein, beef, etc) or plant based sources (e.g., soy, rice, pea, etc) combined in a singular admixture with at least one more macro or micronutrient; (iii) the study measured primary outcome variables related to body composition such as lean body mass (LBM) or fat-free mass (FFM), upper and lower body strength estimated from the 1 repetition maximum test (1-RM), and markers of physical performance such as the 10-m walking (10-MW), 6- min walking (6-MW), 5-times sit-to-stand (5-STS), 30-s sit-to-stand (30-STS), or timed up-and-go (TUG) tests; (iv) participants were 45 years old or older, free from health related disorders (e.g., acute or chronic illness, disease, or injury) and not taking any medication. Therefore, interventions in patients affected by obesity, diabetes mellitus, osteoporosis, cancer, HIV, or following a rehabilitation intervention after injury, etc., were not included. Additionally, as sarcopenia has been defined as a muscle disease (Cruz-Jentoft et al., 2019), studies conducted in sarcopenic populations were dismissed; (v) the effects of the treatment were compared to the effects of an isoenergetic comparator (COMP) treatment including only one macronutrient, e.g., only protein or carbohydrate; (vi) data on total calories provided by the multi-ingredient or COMP were available; (vii) dietary intake was monitored or eating pattern advises were provided; (viii) studies including remarkably lower (≥100 kcal) or no energy COMP were not included, as it has previously been demonstrated large differences in caloric intake appears to be one of the most relevant factors affecting training adaptations during middle- to long-term exercise interventions (McLellan et al., 2014). In addition, diet interventions and modifications, as well as interventions with food products, were not considered; (ix) the publication presented sufficient data to calculate the mean differences; and (x) abstract was published. These criteria support the notion that the only difference between the experimental (MTN) and COMP groups was attributed to the supplement intervention, and at least one of the aforementioned outcomes (LBM, FFM, upper or lower body strength, and/or functional capacity) was analysed. There were no restrictions on the number of participants, nor for sex or level of performance (e.g., minimum 1-RM). Studies that included participants with a recent history (<1 month before the intervention) of supplementation consuming protein, creatine, AA, or derivatives such as beta-hydroxymethylbutyrate (HMB) at baseline screening were excluded.

### *2.2.3. Study Records*

#### *2.2.3.1. Data management and selection*

Potentially relevant articles were selected by (i) screening the titles; (ii) screening the abstracts; and (iii) if abstracts did not provide sufficient data, the entire article was retrieved and screened to determine whether it met the inclusion criteria; (iv) when data were not accurately presented (only available from figures or graphs), authors were contacted and asked to provide the appropriate range of values (included the raw data). When no answer was obtained but figures were available, values were estimated by using the PDF Adobe Acrobat Pro measuring application. Thereafter JPF and FN met to decide whether the selected articles matched and fitted the purpose of the review.

#### *2.2.3.2. Data collection process and coding*

The following qualitative and quantitative information was extracted from each included study: (1) authors; (2) publication year; (3) baseline population characteristics; (4) intervention type including the exercise programme configuration [exercise mode (resistance or endurance) volume, intensity, and frequency] (5) control procedures; (6) study duration; (7) blinding; (8) sample size per group; (9) nutrient profile of the administered supplements and comparator treatments; (10) methods of ingestion and dose; (11) study compliance; (12) diet assessment; (13) outcomes measured at pre- and post-intervention; (14) group means and standard deviations (SD) for the following outcomes: (i) LBM or FFM; (ii) 1-RM values for upper body and lower body exercises (iii) scores obtained in the functional tests (e.g., 5 times sit-to-stand or timed-up-and-go). Regarding the effects of MTN supplementation on FFM or LBM, the definition of FFM excludes lipids in the cell membranes, central nervous system, and bone marrow, while LBM is an anatomical term that would include some or all of these (Wang et al., 1998). However, both variables share the muscle mass as the main component that would express changes as a consequence of exercise-related interventions in middle and older adults. Therefore, the outcomes affecting these variables have been analysed together. Regarding 1RM values, to reduce bias caused by different exercise modalities and assessments methods, only resistance exercises executed with free weight or weight machines, e.g., bench press, chest press, leg press, or leg extension were considered as valid outcomes to express changes in strength. Furthermore, isometric strength assessments were also included and analysed separately when appropriate.

### *2.2.4. Risk of bias in individual studies*

Methodological information regarding the potential impact of bias was critically examined. Two reviewers (JPF and FN) ascertained individual study information independently as part of the quality control process. For each study, seven domains from the Cochrane collaboration tool for assessing the risk of bias (Higgins et al., 2019) were scored with high, low, or unclear risk for bias: sequence generation, allocation concealment, blinding of participants and personnel, blinding of outcome assessment, incomplete outcome data, selective outcome reporting and "other" issues (similarity in baseline characteristics and timing of outcome assessment). These seven domains assess the level of risk regarding selection bias, allocation bias, performance bias, detection bias, attrition bias, reporting bias and other biases. The aforementioned two researchers performed the quality assessment independently, and their findings were compared and discussed until consensus was achieved. Each domain was scored as -1 for high risk, 0 for unclear risk, and 1 for low risk. Scores were then summed with a possible range of scores from -7 to 7.

### *2.2.5. Data analysis*

Provided the selected studies demonstrated no significant heterogeneity ($p > 0.10$ and $I^2 < 50$), a meta-analysis was performed using the Comprehensive Meta-Analysis Software, version 2.2.064 (Biostat, Englewood, NJ). The random-effects model was selected based on the assumption of variability of true effects between studies. Four or more studies per outcome were required to generate weighted group mean differences, 95 % confidence intervals (CIs), and corresponding p values for heterogeneity. From the collected data, the pre and post values of mean, standard deviation (SD), and sample size, for both MTN and COMP groups were used. Pre and post SD values were calculated when studies reported standard error instead of SD. The effect size was calculated using the Hedges' g. The primary meta-analysis compared the effects of MTN interventions combined with any type of training protocol, which was considered the experimental treatment (without any distinction between each MTN composition) vs. COMP in the analysed outcomes (LBM or FFM, upper and lower body strength, and functional capacity). When a quantitative analysis was not possible, a summary of the critical facts and results of the observed outcomes was considered. If heterogeneity and sensitivity analyses were considered significantly high, no data were meta-analysed and only individual results have been reported. Additionally, the presence of studies with inflated standardised residual values (above 1.96 or below -1.96), to consider them as outliers, was examined. Publication bias was assessed using funnel plots of effect size (horizontal-axis) by the standard error (vertical-axis), the “Trim and fill” procedure for the random effects, and the Orwin Fail-Safe N analysis.

## 2.3. Results

### *2.3.1. Study Selection*

Figure 2.1 describes the search strategy. The preliminary search identified 3329 relevant references. After examining all the retrieved titles, 806 publications were selected. Of those, 745 were excluded based on the abstract review. The remaining 61 publications were fully read and carefully examined by two reviewers. After this examination, 52 studies were excluded resulting in a total of 9 studies (Candow et al., 2006, 2008; Arnarson et al., 2013; Leenders et al., 2013; Bell et al., 2017; Holwerda et al., 2018; Nabuco et al., 2018, 2019; Krause et al., 2019) included in the meta-analysis.

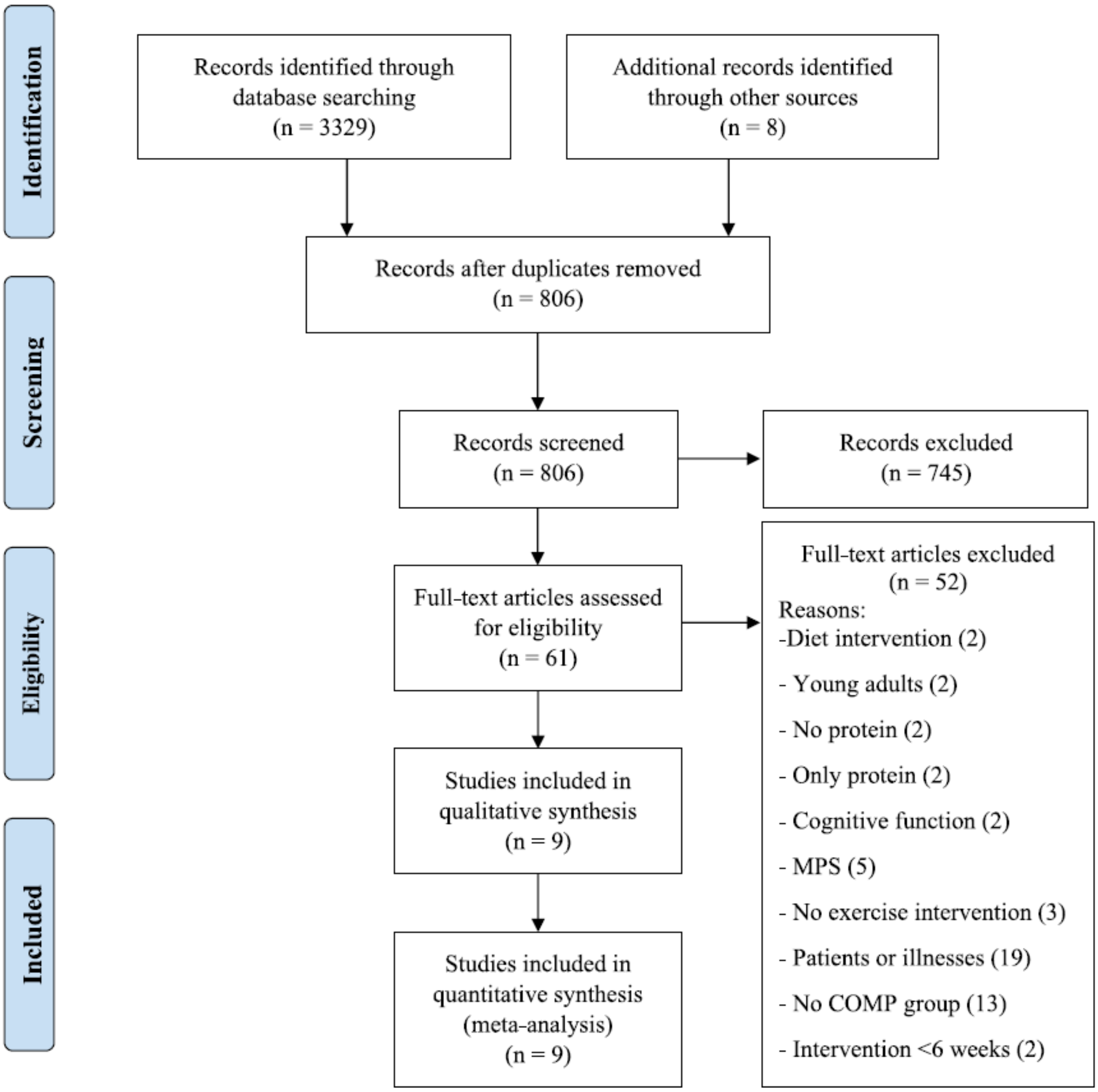


**Fig. 2.1.** PRISMA-P Flow chart diagram of the study selection. MPS: Muscle Protein Synthesis; COMP: Isocaloric Comparator.

### *2.3.2. Characteristics of the included studies*

The overall quality of the included studies was high, with a low risk of bias, scoring between 3 and 6 points in the Cochrane collaboration tool. A table showing the assessment for each study is provided as electronic supplementary material (Appendix Table S1).

Nine studies reported results from 19 valid MTN or COMP groups, including a total of 427 participants, 214 men and 196 women (all postmenopausal) from 59 to 91 years old, met the inclusion criteria (Fig. 2.1). Publications from Nabuco et al. (2018, 2019) were based on the same intervention and with the same participants, but two different manuscripts analysing different outcomes were published. Therefore, those participants were counted only once for the final calculation. The publication dates range from 2006 to 2019. The characteristics and main data of these studies are summarised in Table 2.1.

Sample sizes ranged from 30 to 141 participants, with similar demographic characteristics across the studies. The MTN treatment included 9 to 75 participants while 10 to 66 subjects were assigned to the COMP condition. All selected RCT followed a parallel design. The studies by Bell et al. (2017), Candow et al. (2006), and Nabuco et al. (2018, 2019) included recreationally active or untrained participants not involved in resistance exercise programs for the previous 6 months. Arnarson et al. (2013) and Holwerda et al. (2018) included participants not engaged in regular exercise programmes for 3 years before the beginning of the study, while Leenders et al. (2013) recruited participants with regular physical activity before 5 years of the beginning of the study. Krause et al. (2019) included only sedentary participants. All these studies combined a nutritional intervention with progressive resistance training programmes composed by multi-joint and global exercises as primary movements implemented over 10 (Candow et al., 2008), 12 (Candow et al., 2006; Arnarson et al., 2013; Holwerda et al., 2018; Nabuco et al., 2018, 2019; Krause et al., 2019) and 24 weeks (Leenders et al., 2013) (Table 2.1).

Eight studies (Candow et al., 2006, 2008; Arnarson et al., 2013; Leenders et al., 2013; Bell et al., 2017; Holwerda et al., 2018; Nabuco et al., 2018, 2019), involved resistance training programmes using free weights or machines performed 3 times per week on alternate days, while Krause et al. (2019) combined exercises performed with participants' body mass and elastic bands. Overall, all the included studies used moderate (~60 % to 70 % of the estimated 1-RM) to heavy (~80 % of the estimated 1-RM) overloads, 3 to 4 sets of 15, 10, 8 or 6 repetitions per exercise, and ~ 2 min rest between sets.

All nine studies (Candow et al., 2006, 2008; Arnarson et al., 2013; Leenders et al., 2013; Bell et al., 2017; Holwerda et al., 2018; Nabuco et al., 2018, 2019; Krause et al., 2019) administered MTN providing the three macronutrients (proteins, carbohydrates, and fats), where the protein source was milk concentrate, including whey and casein (Leenders et al., 2013; Krause et al., 2019) or just whey (Candow et al., 2006, 2008; Arnarson et al., 2013; Leenders et al., 2013; Bell et al., 2017; Holwerda et al., 2018; Nabuco et al., 2018, 2019; Krause et al., 2019). Only two studies included MTN with creatine monohydrate (CM), Candow et al. (2008) provided 0.1 $g \cdot kg^{-1}$, while by Bell et al. (2017) administered

2.5 g (~0.03 g·kg$^{-1}$) of CM per dose. Furthermore, the admixture used by Bell et al. (2017) was the only one combining vitamin D, calcium, and n-3 PUFA.

Five studies administered the MTN using the following respective absolute supplement doses and energy content: 20 g and 169 Kcal (Arnarson et al., 2013), ~23 g and 93 Kcal (Leenders et al., 2013), 30 g and 116 Kcal (Bell et al., 2017), 20.7 g and 150 Kcal (Holwerda et al., 2018), and 27.1 g and 131 Kcal Nabuco et al. (2018, 2019). Conversely, the studies conducted by Candow et al. (2006) determined the doses based on the amount of protein per kg of body mass resulting in 0.30 g/ kg with 2.1 kcal/kg and 0.34 g·kg$^{-1}$ with ~4.4 g·kg$^{-1}$, respectively. On the other hand, Krause et al. (2019), classified their participants into 4 categories according to their body mass (i) 45–59.9 kg; (ii) 60–74.9 kg (iii) 75–89.9 and (vi) 90–105 kg. The used MTN contained 72.7 % of proteins and resulted in a consumption of about 8.7; 11.1; 13.6 and 16.1 g·kg$^{-1}$ of protein for the aforementioned four-category groups, respectively.

The supplementation protocol varied across studies. Leenders et al. (2013) and Arnarson et al. (2013) used a singular daily dose of the MTN or COMP administered after breakfast, or immediately post-workout respectively. Candow et al. (2006) and Nabuco et al. (2018, 2019) used a two-daily dose supplementation protocol assigning participants to three groups differentiated by the moment in which the MTN or COMP was consumed: (i) MTN, pre-, and the COMP at post-workout, (ii) the COMP at pre- and the MTN at post-workout, and (iii) COMP at pre- and post-workout. Even though supplements were ingested twice, the MTN treatment involved only one intake per day (pre- or post-workout). No supplementation was administered on non-training days. Furthermore, Candow et al. (2008) tested a 3-dose protocol including supplements (MTN or COMP) at pre-, post-workout and before bedtime. Even though this study included three parallel groups: (i) MTN, (ii) COMP and (iii) creatine with sucrose, the latest was not included because the admixture included almost not protein but creatine and sucrose. Due to combining creatine with carbohydrates supplementation with physical training proven to significantly affect body composition and exercise performance per se (Cooper et al., 2012), no comparator using creatine was included. The studies by Bell et al. (2017), Holwerda et al. (2018), and Krause et al. (2019), used a two-dose supplementation protocol. While Bell et al. (2017) supplemented their participants at breakfast and before bedtime only during training days, Holwerda et al. (2018) included supplements immediately post-workout and prior sleep time during training days while one dose (before bedtime) was implemented on non-training days. On the other hand, Krause et al. (2019) supplemented their participants on a daily basis during breakfast and at midday.

Although no studies modified dietary intake, most of them recorded participants' dietary habits using either a 3-day (Candow et al., 2006, 2008; Arnarson et al., 2013; Bell et al., 2017; Holwerda et al., 2018), a 4- day dietary record intake (Leenders et al., 2013), or a 24-h dietary recall on two non-consecutive days such as in Nabuco et al. (2018, 2019). Data were collected at the beginning, during and final week of the intervention. No detailed information about dietary control was noted, advice to controls was only provided by Krause et al. (2019).

The impact of adding supplements (MTN and COMP) to the diet nutritional composition was reported in four of the selected studies. Candow et al. (2006), observed an increase in energy intake, albeit no changes in protein consumption due to the MTN or comparator ingestion was observed. Bell et al. (2017) reported similar increases in energy for both MTN and COMP groups. Nonetheless, the MTN treatment increased protein intake from 1.1 to 1.6 $g \cdot kg \cdot day^{-1}$. Holwerda et al. (2018) and Nabuco et al. (2018, 2019) reported significant increases in the daily protein intake, from 1.14 to 1.43; 0.92 to 1.38 and 0.94 to 1.49 $g \cdot kg \cdot day^{-1}$, respectively for the participants allocated to the MTN treatment groups.

Body composition was assessed by Dual-energy X-ray absorptiometry (DXA) in seven studies (Arnarson et al., 2013; Leenders et al., 2013; Bell et al., 2017; Holwerda et al., 2018; Nabuco et al., 2018, 2019; Krause et al., 2019), while air-displacement plethysmography using BOD POD method was used by Candow et al. (2006, 2008).

Strength was assessed by the 1-RM bench press (Candow et al., 2006, 2008; Bell et al., 2017; Nabuco et al., 2018), leg press (Candow et al., 2006; Bell et al., 2017; Holwerda et al., 2018; Leenders et al., 2013) and leg extension (Leenders et al., 2013; Bell et al., 2017; Holwerda et al., 2018; Nabuco et al., 2018). Conversely, Bell et al. (2017) added up the 1 RMs values obtained by four upper body (horizontal row, chest press, lateral pull-down, and shoulder press) and two lower body (leg extension and leg press) exercises to assess regional strength changes. Additionally, the sum of all 1-RMs scores was considered a general marker of strength (Bell et al., 2017).

Krause et al. (2019) used an isometric handgrip strength test conducted on both hands while Arnarson et al. (2013) measured changes in quadriceps strength by a maximum voluntary isometric contraction test performed on an isokinetic dynamometer (Kin-Com 500H Chattanooga). The study by Nabuco et al. (2019) did not assess strength outcomes.

Functional capacity was assessed by a variety of testing protocols. All the included studies except those by Candow et al. (2006, 2008) used one of the following five tests to evaluate the ability to move and sustain fast movements involving mainly the lower limb musculature: The 5- times chair stand (Leenders et al., 2013; Holwerda et al., 2018; Nabuco et al., 2018; Krause et al., 2019), the 30-s chair stand (Bell et al., 2017; Krause et al., 2019), timed up and go (Arnarson et al., 2013; Bell et al., 2017), 4-m walking test (Holwerda et al., 2018), 10-m walking test (Nabuco et al., 2018; Krause et al., 2019). Only two studies (Arnarson et al., 2013; Bell et al., 2017) employed the 6-min walk test.

Due to the similar nature of the tests, the 5-times chair stand (5STS) and the timed up-and-go (TUG) were merged with respect to data analysis. All other tests (30-s chair stand, 4-m walking test, the 10-m walking, 6-min walk test) were discussed separately.

Finally, two studies (Candow et al., 2006; Nabuco et al., 2018) conducted a 3-parallel arm design, with two different MTN supplementation protocols in each intervention (MTN intake pre- or post-workout). Consequently, these aforementioned studies were included and counted separately in the meta-analysed outcomes.

**Table 2.1.** Summary of the randomised controlled trials included in the meta-analysis.

| Study | Participants | Supplementation Design[a] | Length | Training protocol | Supplementation protocol | Outcomes |
|---|---|---|---|---|---|---|
| Candow et al. (2006) | Males, n=29; age 59-76; UT | 3PG: (i) MTN-B (n=9); (ii) MTN-A (n=10); (iii) COMP (Maltodextrin/Sucrose, n=10) | 12 wk | RT, three days/wk, 9 ex x 3 sets x 10 reps @70% or 10 RM; with 2 mins rest | Two doses (~2.1 kcal/kg each): one at pre- and one at post-workout. MTN: 0.54 g/kg; COMP: 0.63 g/kg | ↔FFM[b]<br>↔UBS 1RM[b]<br>↔LBS 1RM[b] |
| Candow et al. 2008) | Males, n=35; age 59 to 77; UT | 3PG: (i) MTN (n=10); (ii) Creatine-Sucrose (n=13) EXCLUDED<br>(ii) COMP (Sucrose, n=12) | 10 wk | RT, three days/wk,9 ex, 3 sets of 10 reps @70% or 10 RM; with 2 mins rest | Three Equal doses (~0.4 g/kg each ~1.6 kcal/kg): one at pre-, one post-workout, and one prior to bedtime | ↑ FFM[c]<br>↑ UBS 1RM[c]<br>↔LBS 1RM[b] |
| Arnarson et al. (2013) | Males (n=67) and females (n=94); n=141; age 65 to 91; UT | 2PG: (i) MTN (n=75); (ii) COMP (Carbohydrate and 1 g of fat, n=66) | 12 wk | PRT three days/wk; 2 to 3 sets, 10 reps @60%; 3 sets 6-8 reps @75-80% 1RM. The load increased 5-10% per week | One dose of 51 g (169 kcal) mixed with 250 ml of water at post-workout | ↔FFM[b]<br>↔LBS[b]<br>↔TUG[b] |
| Leenders et al. (2013) | Males (n=29) and Females (n=24); n=53; age 70±1; recreationally active participants | 2PG: (i) MTN (n=21); (ii) COMP (Lactose and Calcium, n=20) | 24 wk | PRT. three days/wk: 6 ex, 3-4 sets, 10-15-8-10 reps @60-75 or 80% 1RM; with 1.5 to 3 mins rest | MTN (93 kcal) and COMP (28 kcal) consumed daily after breakfast. | ↑ FFM[b]<br>↑ %FM[b]<br>↑ LBS 1RM[b]<br>↑ 5ChSt[b] |
| Bell et al. (2017) | Males, n=41; age 73±1;UT | 2PG: (i) MTN (n=21); (ii) COMP (Carbohydrate, n=20) | 12 wk | PRT: two days/wk, 3 sets, 6 ex, 10-12 reps @70-80%. HIIT (1 day): 10 reps of 60 s @90% HRmax with 1 min rest | Two doses of 116 (MTN) and 56 (COMP) kcal: one after breakfast and one prior bedtime | ↔FFM[b]<br>↑ TUG[b]<br>↑ LBS[b]<br>↑ UBS[c] |
| Holwerda et al. (2018) | Males, n=41; age 70±1; UT | 2PG: (i) MTN (n=21); (ii) COMP (Carbohydrate and fat, n=20) | 12 wk | PRT, three days/wk: 4 ex per workout 2-4 sets, 8-10 reps @ 70-80% 1RM with 2-3 mins rest | Training days: Two doses (150 kcal each), one at post-workout, and one prior to bedtime. Non-training days: One dose prior to bedtime | ↔FFM[b]<br>↑ LBS 1RM[b]<br>↑ 5ChSt[b] |
| Nabuco et al. (2018) | Females, n=66; age 67±7; UT | 3PG: (i) MTN-COMP (n=22) (ii) COMP-MTN (n=21); (iii) COMP-COMP (Maltodextrin, n=23) | 12 wk[e] | PRT, three days/wk: 8 ex, 3 sets, 10 to 8-12 RM. The load increased weekly | Training days: two doses (~130 kcal each), one at pre- and one at post-workout | ↑ FFM[c]<br>↑ UBS 1RM[c]<br>↑ LBS 1RM[c] |
| Krause et al. (2019) | Males (n=9) and females (n=12), n=21; age 64±4; UT | 2PG: MTN (n=11); (ii) COMP (Maltodextrin, n=10) | 12 wk | PRT with Elastic Bands: 11 ex, 3 days/wk: 4-6 sets, 8-15 reps with 30 s rest | Two doses of ~0.8 kcal/kg: one at breakfast and one at midday | ↑ FFM[b]<br>↑ %FM[c]<br>↑ 5ChSt[b] |
| Nabuco et al. (2019) | Females, n=66; age 67±7; UT | 3PG: (i) MTN-COMP (n=22)<br>(ii) COMP-MTN (n=21); (iii) COMP-COMP (Maltodextrin, n=23) | 8 wk | PRT, three days/wk: 8 ex, 3 sets, 10 to 8-12 RM. The load increased weekly | Training days: Two doses of 35 g (~140 kcal each), one at pre- and one at post-workout | ↑ FFM[b, d]<br>↑ FM[e] |

**Notes: 5ChSt**: 5-times chair sit to stand test; **CHO**: carbohydrates; **ex**: exercises; **EXCLUDED**: the group consuming creatine monohydrate with negligible amount of proteins and carbohydrate was excluded from the analysis (see text for further explanations); **FFM**: fat-free mass; **FM**: fat mass; **LBS**: Lower Body Strength; **MTN**: multi-ingredient; **MTN-B**: MTN was ingested at pre-workout and comparator at post-workout; **MTN-A**: was ingested at post-workout and comparator at pre-workout; **PG**: parallel groups; **PRT**: progressive resistance training; reps: repetitions; **RM**: maximum number of repetitions per set; **TUG**: timed up-and-go test; **UBS**: Upper Body Strength; **UT**: untrained; **wk**: weeks.

Symbols: [a] Even though all multi-ingredient and comparator supplements matched the inclusion criteria, their composition differs between studies; ↑ = significant improvement to baseline; ↔ = no differences to baseline; [b] No differences ($p > 0.05$) between MTN vs. COMP; [c] Differences ($p < 0.05$) between MTN vs. COMP; [d] Differences ($p < 0.05$) between MTN-PLA vs. PLA-MTN and MTN-PLA vs. COMP; [e] Differences ($p < 0.05$) between PLA-MTN vs. COMP.

### *2.3.3. Changes on the analysed variables*

#### *2.3.3.1. Changes on fat-free mass and lean body mass*

The estimated overall effect of MTN treatment vs. COMP was very small (n = 10, g = 0.044, 95 % CI -0.14 to 0.22). No significant heterogeneity was found within the 10 treatments [Q (9) = 6.025, p = 0.927, $I^2$ = 0]. As shown in Fig. 2 no advantage for increasing FFM or LBM was observed when compared groups ingesting MTN to those groups consuming COMP.

#### *2.3.3.2. Changes in muscular strength*

As described above, three studies measured upper body strength by the 1-RM score in the bench press. However, as the studies by Candow et al. (2006) and Nabuco et al. (2018) used a 3-arms parallel-group design (see Table 1), 5 intervention groups were considered. As described in Fig. 3, the estimated overall effect of MTN vs COMP was very small (n = 5, g = 0.046, 95 % CI -0.24 to 0.33) with no differences between groups. No significant heterogeneity [Q (4) = 0.532, p = 0.752, $I^2$ = 0] between treatments, was observed for changes measured in 1-RM bench press.

Due to the nature of the outcomes, the two exercises used to assess lower-body strength, leg press (multi-joint) and leg extension (single joint) across studies were meta-analysed separately. As previously described, six publications assessed changes in lower-body strength, five (Candow et al., 2006, 2008; Leenders et al., 2013; Bell et al., 2017; Holwerda et al., 2018) used the 1-RM scores from two exercises, leg press (Fig. 2.4) and leg extension (Fig. 2.5). Consequently, five groups from leg press and leg extension were analysed. Furthermore, due to differences in the execution modality (isometric vs. dynamic action), and measurement criteria, data extracted from the study by Arnarson et al. (2013) were not meta-analysed but still discussed separately.

As described by Fig. 2.4, the estimated overall effect of MTN vs. COMP was very small (n = 5, g = 0.025, 95 % CI -0.26 to 0.31). Additionally, no significant heterogeneity [Q (4) = 4.011, p = 0.629, I2 = 0.280] was observed between treatments for the changes measured in 1-RM leg press.

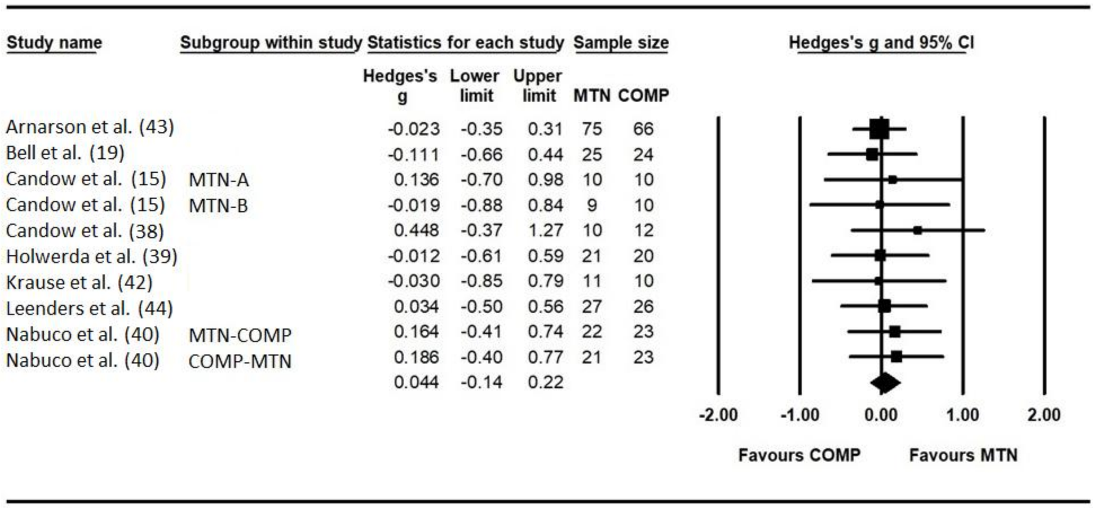
Similarly, Fig. 2.5 shows that the estimated overall effect of MTN vs COMP was very small for the changes measured in 1-RM leg extension (n = 5, g = 0.106, 95 % CI -0.15

to 0.36). Additionally, no significant heterogeneity was observed [Q (4) = 2.341, p = 0.106, I2 = 0] between treatments.

#### *2.3.3.3. Changes in functional capacity*

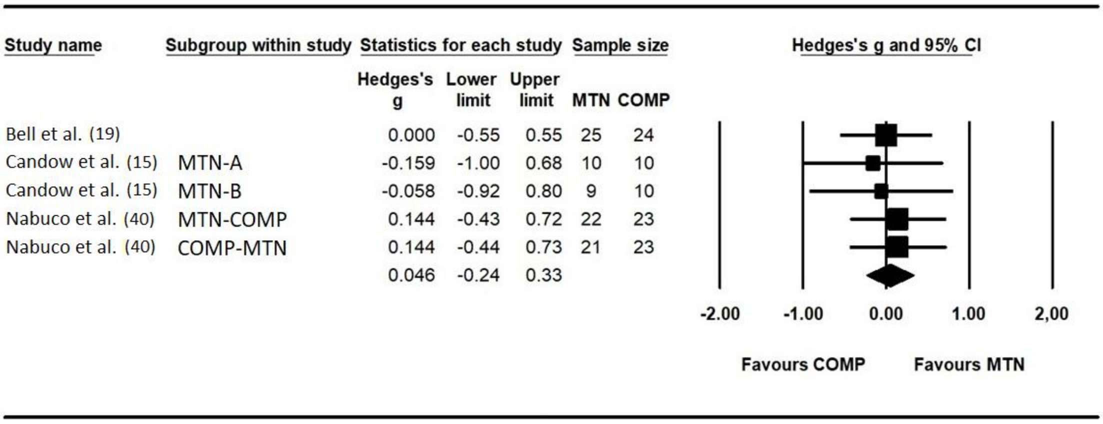
As previously described, the two tests (5STS and TUG) focused on the ability to move and sustain fast movements were analysed together. Although six studies assessed the functional capacity, Nabuco et al. (2018) assessed functional capacity by the 5STS in two different MTN-based intervention groups ingesting the supplement before (MTN-COMP) or after (COMP-MTN) workouts, therefore 7 groups were included in the analysis. As described in Fig. 6, no significant differences between MTN and COMP (g = 0.079, 95 % CI -0.12 to 0.27) were observed. Additionally, no significant heterogeneity [Q (6) = 4.538, p = 0.574, $I^2 = 0$] was observed between treatments.

When considering the impact of using MTN or COMP added to exercise interventions on functional capacity measured by alternative assessment protocols, the observed results are in line with the performed meta-analysis including only the two (5STS and TUG) selected tests. For instance, Holwerda et al. (2018) reported no time (p = 0.604), treatment (p = 0.273) or time interaction (p = 0.877) effects of adding MTN or COMP to a 12-week resistance training protocol on the time to complete the 4-Meters walking test in a group of older men. Similarly, Krause et al. (2019), reported a significant time effect (about 30 % improvement) in both treatments, MTN and COMP but with no significant (p > 0.05) treatment effect after a 12-week intervention period. Furthermore, Bell et al. (2017) did not find a time or treatment effect on the 30-Seconds sit-to-stand test after 12 weeks. Additionally, two studies (Arnarson et al., 2013; Bell et al., 2017) used the 6-min walking test, and although significant post-treatment improvements for both MTN and COMP were identified, none of the studies reported differences between groups. Conversely, Nabuco et al. (2018) was the only study reporting a significant interaction effect, favouring MTN vs. COMP to reduce the time to complete the 10-meter walking test. In this study, the two MTN groups consuming the supplement either before (MTN-pre) or after (MTN-post) workout significantly improved (10.8 % and 11.8 %, respectively) compared to the COMP group.



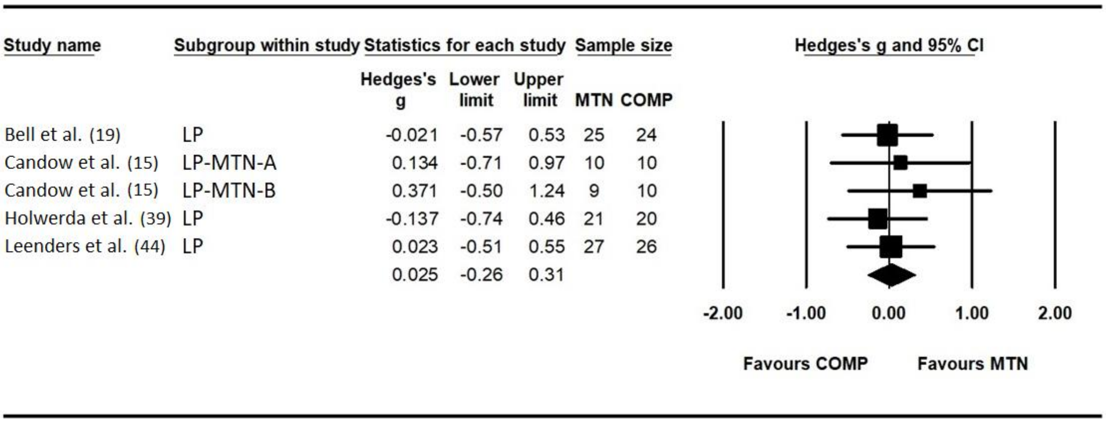
| Study name | Subgroup within study | Hedges's g | Lower limit | Upper limit | MTN | COMP |
|---|---|---|---|---|---|---|
| Arnarson et al. (43) | | -0.023 | -0.35 | 0.31 | 75 | 66 |
| Bell et al. (19) | | -0.111 | -0.66 | 0.44 | 25 | 24 |
| Candow et al. (15) | MTN-A | 0.136 | -0.70 | 0.98 | 10 | 10 |
| Candow et al. (15) | MTN-B | -0.019 | -0.88 | 0.84 | 9 | 10 |
| Candow et al. (38) | | 0.448 | -0.37 | 1.27 | 10 | 12 |
| Holwerda et al. (39) | | -0.012 | -0.61 | 0.59 | 21 | 20 |
| Krause et al. (42) | | -0.030 | -0.85 | 0.79 | 11 | 10 |
| Leenders et al. (44) | | 0.034 | -0.50 | 0.56 | 27 | 26 |
| Nabuco et al. (40) | MTN-COMP | 0.164 | -0.41 | 0.74 | 22 | 23 |
| Nabuco et al. (40) | COMP-MTN | 0.186 | -0.40 | 0.77 | 21 | 23 |
| | | 0.044 | -0.14 | 0.22 | | |

Fig. 2.2. Fat-free mass or lean body mass Forest-plot. Results of a random-effects meta-analysis showed the effect size (g) with 95 % confidence interval. The black diamond represents the pooled (overall) standardised mean difference. CI: confidence interval, *Blank: No subgroup analysis or intervention*; *COMP: Comparator; MTN: Multi-ingredient Supplement; MTN-A: MTN intake after training session; MTN–B: MTN intake before training session*.



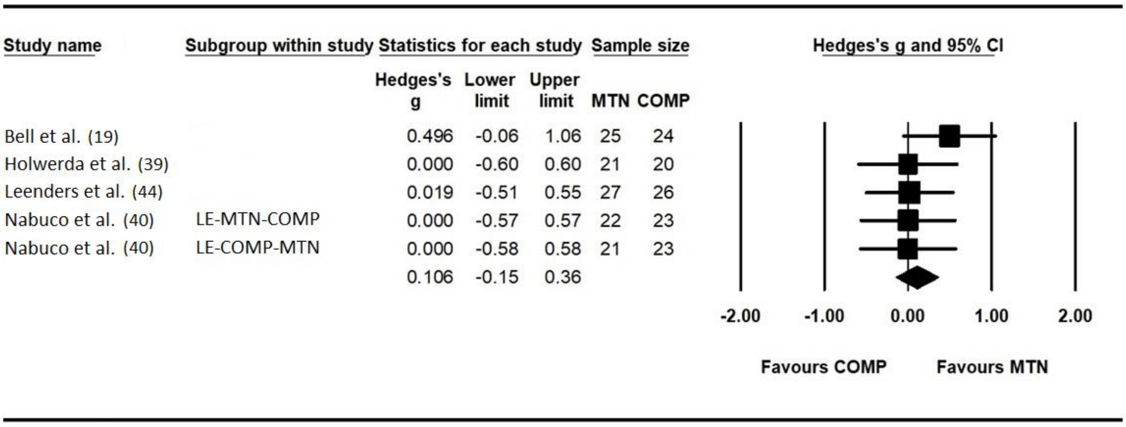
| Study name | Subgroup within study | Hedges's g | Lower limit | Upper limit | MTN | COMP |
|---|---|---|---|---|---|---|
| Bell et al. (19) | | 0.000 | -0.55 | 0.55 | 25 | 24 |
| Candow et al. (15) | MTN-A | -0.159 | -1.00 | 0.68 | 10 | 10 |
| Candow et al. (15) | MTN-B | -0.058 | -0.92 | 0.80 | 9 | 10 |
| Nabuco et al. (40) | MTN-COMP | 0.144 | -0.43 | 0.72 | 22 | 23 |
| Nabuco et al. (40) | COMP-MTN | 0.144 | -0.44 | 0.73 | 21 | 23 |
| | | 0.046 | -0.24 | 0.33 | | |

Fig. 2.3. Upper body Strength Forest-plot. Results of a random-effects meta-analysis showed the effect size (g) with 95 % confidence interval. The black diamond represents the pooled (overall) standardised mean difference. CI: confidence interval, Blank: No subgroup analysis or intervention; COMP: Comparator; MTN: Multi-ingredient Supplement; MTN-A: MTN intake after training session; MTN–B: MTN intake before training session.



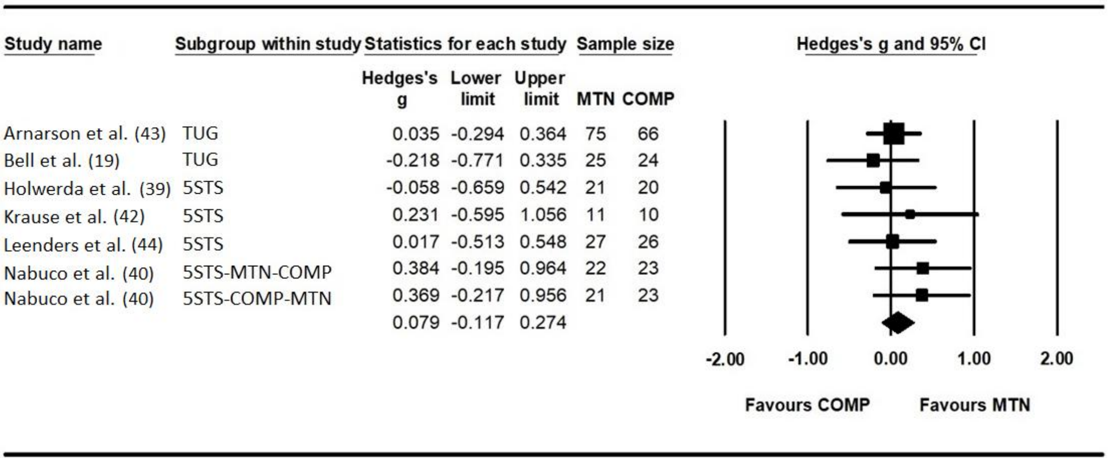
| Study name | Subgroup within study | Statistics for each study<br>Hedges's g | Lower limit | Upper limit | Sample size<br>MTN | COMP |
|---|---|---|---|---|---|---|
| Bell et al. (19) | LP | -0.021 | -0.57 | 0.53 | 25 | 24 |
| Candow et al. (15) | LP-MTN-A | 0.134 | -0.71 | 0.97 | 10 | 10 |
| Candow et al. (15) | LP-MTN-B | 0.371 | -0.50 | 1.24 | 9 | 10 |
| Holwerda et al. (39) | LP | -0.137 | -0.74 | 0.46 | 21 | 20 |
| Leenders et al. (44) | LP | 0.023 | -0.51 | 0.55 | 27 | 26 |
| | | 0.025 | -0.26 | 0.31 | | |

Fig.2. 4. Lower Body (Leg Press) Strength Forest-plot. Results of a random-effects meta-analysis showed the effect size (g) with 95 % confidence interval. The black diamond represents the pooled (overall) standardised mean difference. CI: confidence interval, Blank: No subgroup analysis or intervention; COMP: Comparator; MTN: Multi-ingredient Supplement; MTN-A: MTN intake after training session; MTN–B: MTN intake before training session.



| Study name | Subgroup within study | Statistics for each study<br>Hedges's g | Lower limit | Upper limit | Sample size<br>MTN | COMP |
|---|---|---|---|---|---|---|
| Bell et al. (19) | | 0.496 | -0.06 | 1.06 | 25 | 24 |
| Holwerda et al. (39) | | 0.000 | -0.60 | 0.60 | 21 | 20 |
| Leenders et al. (44) | | 0.019 | -0.51 | 0.55 | 27 | 26 |
| Nabuco et al. (40) | LE-MTN-COMP | 0.000 | -0.57 | 0.57 | 22 | 23 |
| Nabuco et al. (40) | LE-COMP-MTN | 0.000 | -0.58 | 0.58 | 21 | 23 |
| | | 0.106 | -0.15 | 0.36 | | |

Fig. 2.5. Lower Body (Leg Extension) Strength Forest-plot. Results of a random-effects meta-analysis showed the effect size (g) with 95 % confidence interval. The black diamond represents the pooled (overall) standardised mean difference. CI: confidence interval, Blank: No subgroup analysis or intervention; COMP: Comparator; MTN: Multi-ingredient Supplement; MTN-A: MTN intake after training session; MTN–B: MTN intake before training session.



| Study name | Subgroup within study | Hedges's g | Lower limit | Upper limit | MTN | COMP |
|---|---|---|---|---|---|---|
| Arnarson et al. (43) | TUG | 0.035 | -0.294 | 0.364 | 75 | 66 |
| Bell et al. (19) | TUG | -0.218 | -0.771 | 0.335 | 25 | 24 |
| Holwerda et al. (39) | 5STS | -0.058 | -0.659 | 0.542 | 21 | 20 |
| Krause et al. (42) | 5STS | 0.231 | -0.595 | 1.056 | 11 | 10 |
| Leenders et al. (44) | 5STS | 0.017 | -0.513 | 0.548 | 27 | 26 |
| Nabuco et al. (40) | 5STS-MTN-COMP | 0.384 | -0.195 | 0.964 | 22 | 23 |
| Nabuco et al. (40) | 5STS-COMP-MTN | 0.369 | -0.217 | 0.956 | 21 | 23 |
|  |  | 0.079 | -0.117 | 0.274 |  |  |

Fig. 2.6. Functional Capacity (Timed Up-and-go and 5 Times Sit-to-Stand) Forest-plot. Results of a random-effects meta-analysis showed the effect size (g) with 95 % confidence interval. The black diamond represents the pooled (overall) standardised mean difference. CI: confidence interval, 5STS: 5-Times sit-to-stand test; Blank: No subgroup analysis or intervention; COMP: Comparator; MTN: Multi-ingredient Supplement; MTN-A: MTN intake after training session; MTN–B: MTN intake before training session; TUG: Timed up-and-go test.

### *2.3.4. Synthesis of results*

The Grading of Recommendations, Assessment, Development and Evaluation (GRADE) Working Group approach has been followed to summarise the evidence and assess the quality of the evidence factors. The results show a very low level of evidence in the estimated effect for the inclusion of MTN when compared to COMP in healthy middle-aged and older adults in every analysed outcome.

### *2.3.5. Additional analysis*

No subgroup analysis was performed to differentiate between supplementation timing (breakfast, pre- or post-workout, before bedtime,) as insufficient differences between results were observed ($I^2$ = 0). The studies of Candow et al. (2006) and Nabuco et al. (2018) used a three-parallel-group randomized design. From these studies, two different treatment protocols tested the effects of ingesting MTN before and COMP post-workout, compared to the ingestion of COMP pre-workout and MTN post-workout, against the ingestion of COMP at both pre-and post-workout. Thus, two diverse treatments involving the intake of MTN could be considered from each study because of supplement intake and timing variation.

### *2.3.5. Outliers and publication Bias*

No studies were identified as outliers for any of the assessed outcomes. Funnel plots (see Supplementary Material Figures S1, S2, S3, S4 and S5) describe a practically symmetrical plot, the “trim and fill” procedure resulted in an overall treatment effect of -0.04 for the Fat-Free Mass, and when 1 study was added to the left of the mean, it reduced the effect to 0.02. A similar tendency was observed for upper body strength, with a treatment effect of 0.05, which increased up to 0.09 when 2 studies were added to the right of the mean. Also, a positive result of 0.02 was observed for leg press, with no studies added to any side of the mean, and higher values for the leg extension, which resulted in 0.11 and increasing to 0.13 when one study was added to the right of the mean. Finally, the analysis of the functional capacity showed a result of 0.08 turning into 0.04 when one study was added to the left of the mean (Figure S5 appendix).

## 2.4. Discussion

Compared to the ingestion of an isocaloric nutritional comparator, no additional benefits on any of the investigated training outcomes (body composition, muscle strength, and functional capacity) were observed by the administration of a protein-based MTN. Our results differ from two previous systematic reviews including adults ≥ 60 years old (Liao et al., 2017) or adults >45 years old (O’Bryan et al., 2019). Liao et al. (2017) observed beneficial effects of adding protein-based supplementation to optimise gains of lean mass and strength in overweight or obese, ≥ 60-year-old individuals following resistance training programmes. However, though Liao et al. (2017) compared the effects of combining resistance training with either protein-based or non-protein supplements, these authors included groups with no supplementation (merely resistance training). In our review, only studies including isoenergetic non-protein comparators added to any kind of exercise intervention were considered. As previously highlighted, regardless of macronutrient composition, energy intake has proven to be one of the most relevant nutritional factors impacting training-induced outcomes (e.g., strength gains or hypertrophy) (Naclerio and Larumbe-Zabala, 2016). Furthermore, Liao et al. (2017), considered overweight and obese individuals undergoing resistance training, while our analysis was restricted to middle-aged and older healthy non-obese participants. As indicated by the authors, participants with a BMI >30 exhibited substantially greater lean mass and strength gains in response to protein-based supplementation, suggesting that combining resistance exercises with protein supplementation administered in isolation or as part of a MTN formulation elicited a more favourable effect in overweight or obese individuals (Liao et al., 2017). Only high-quality protein sources such as milk protein concentrate (Leenders et al., 2013; Krause et al., 2019) or whey were administered in the MTN formulations in the nine included studies. Except for the study by Bell et al. (2017), which mostly excluded carbohydrates from the MTN, the rest of the included interventions combined high-quality protein and carbohydrates. Even though combining protein and carbohydrates in a single intake has been proposed as an effective strategy to maximise resistance training outcomes in young athletes (Jäger et al., 2017), our results do not show any additional benefit from this combination in middle-aged and older healthy non-obese participants. In fact, the results by Bell et al. (2017) do not seem to impact the lack of effects observed on the analysed variables (Figs. 2.2 to 2.6).

High-quality protein sources with a higher amount of leucine (>6 to 12 %) e.g., whey, have proven to enhance muscle protein synthesis under exercise as well as in resting conditions in both young and elderly individuals (Moore, 2021). Overweight and obese older people, even when physically active, may be at greater risk of losing lean mass compared to normal-weight physically active controls (Liao et al., 2017), and therefore may benefit even greater from adding MTN-based protein supplementation to significantly improve muscle mass and strength from resistance training programmes. It is worth noticing that similar to our results, Liao et al. (2017) failed to observe additional benefits of protein-based supplementation to improve functional capacity assessed by the same two tests used in our review (the timed up-and-go and the chair rise time test). The nature of the physical tasks emphasising a higher neuromuscular activation and movement coordination over a relatively short period of time (<30 s) reduced the relevance of nutrition in supporting the outcomes as observed in strength and muscle mass gain by Liao and colleagues. Nonetheless, it is worth noticing that resistance training provides the most efficient anabolic stimulus for skeletal muscle tissue growth and strength improvement (Morton et al., 2018). Therefore, in well-nourished individuals, as was the case of all studies included in our review, the lack of effect of adding MTN-based protein to maximise lean mass, muscle strength, and functional capacity, supports the notion that energy supply, rather than the macronutrient proportion, is likely the most important nutritional factor impacting performance adaptation (McLellan et al., 2014).

O’Bryan et al. (2019) reported significant beneficial effects of combining resistance training with the ingestion of MTN vs. a comparator group ingesting either placebo, only protein, or performing only resistance training (with no supplement) on improving FFM or LBM and strength performance in older adults (>45 years old). Differences in the inclusion criteria precluded us to include some studies analysed by O'Bryan and colleagues. For instance, the studies by Rondanelli et al. (2016), Seino et al. (2018) and Carter et al. (2005) favoured the effects of MTN over placebo to induce greater gains of FFM or LBM in the review by O’Bryan et al. (2019). These studies were not included in our analysis. Specifically, Rondanelli et al. (2016) studied sarcopenia patients, the study by Seino et al. (2018) was excluded due to the lack of an isocaloric COMP while the study by Carter et al. (2005), besides using non-isocaloric supplements (MTN provided >120 kcal per serving than the COMP) reported incomplete FFM data. Similarly, when looking at upper and lower body strength, two studies (Bemben et al., 2010; Villanueva et al., 2014) impacted the results favouring MTN benefits over COMP in the O’Bryan et al. (2019) review. Both studies were excluded from the present meta-analysis due to the lack of an isocaloric control group.

Another factor influencing our results is the amount of daily protein intake. Except for the study of Bell et al. (2017) where participants in the MTN treatment ingested a significantly higher amount of protein per day with respect to the COMP group [1.7 ± 0.5 (6 weeks) or 1.6 ± 0.5 (19weeks) vs. 1.2 ± 0.3 $g{\cdot}kg^{-1}$), no significant differences were reported for the other eight studies. Indeed, the average daily protein intake of both groups (MTN or COMP) across studies was still inferior to 1.6 $g{\cdot}kg^{-1}$, which is currently considered the optimal protein ingestion in physically active adults and beyond which protein supplementation ceases to provide a measurable benefit in maximising resistance exercise outcomes (Morton et al., 2018). Accordingly, it could have been expected that the addition of MTN-based protein instead of a non-protein isocaloric supplement enhanced training outcomes in the intervention groups. Furthermore, the aforementioned study of Bell and colleagues, the only one providing protein about 1.6 $g{\cdot}kg^{-1}$/day or higher, reported significant improvement in MTN vs. COMP only for upper body strength while no differences were determined in lower body strength and FFM (Table 2.1). Taken together, it seems that as long as the daily energy intake is equated, the addition of an MTN-protein-based supplement without reaching the recommended daily protein intake of 1.6 g·kg$^{-1}$ BM but still approaching the estimated average requirement of 1.24 (Moore, 2021) induced no further benefits in the investigated training outcomes.

Although older trained adults are unlikely to suffer from the typical age-related anabolic resistance (Moore, 2021), most of the participants of the analysed studies were considered untrained or slightly physically active, and therefore it is expected to have a reduced anabolic response requiring higher per-meal protein doses. This would result in a higher daily protein intake to achieve similar rates of muscle protein synthesis compared with younger individuals, as protein is the primary variable regulating changes in skeletal muscle mass (Rennie et al., 2004). In fact, protein-based supplementation seems to be less effective with increasing chronological age (Morton et al., 2018). Thus, the lack of response to MTN supplementation to maximise training outcomes in older individuals suggests they may have an increased need for higher protein intake reaching at least 1.6 g·kg$^{-1}$ and support the notion that training is the most efficient stimulus to increase exercise performance lean mass.

In summary, the pooled estimates from the present study show MTN supplementation during prolonged RT (≥6 weeks) did not promote additional benefits to augment gains in body composition (i.e., FFM or LBM), upper and lower body strength, or functional capacity when compared with isocaloric non-MTN treatments. MTN formulations employed by the nine studies included in the present review included milk protein concentrate and whey protein combined with other synergistic anabolic compounds such as creatine monohydrate (Candow et al., 2008; Bell et al., 2017), or leucine (Holwerda et al., 2018) and additional macronutrients/micronutrients including CHO, vit D and PUFAs (Bell et al., 2017).

### *2.4.1. Limitations and recommendations for future studies*

Several aspects of this review must be considered when attempting to draw evidence-based inferences. The small number of treatments included in this review represent an important limitation. Additionally, some potential sources of heterogeneity across the included studies were identified: (i) the supplement composition (e.g., including creatine), (ii) the timing of the protocol (e.g., at breakfast vs. pre- and post-workout), (iii) dose consumed (absolute vs. relative to body composition intakes), (iv) duration of the intervention (range of 8 to 24 weeks) and (v) the configuration of the training workouts (e.g., using free weights, machines, or elastic bands, 4 to 11 exercises, 2–6 sets per exercise and 30 s to 2 min rest with different patterns of training load progressions across studies), potentially impacting the observed lack of benefits of ingesting MTN vs. an isocaloric non-protein containing comparator. Furthermore, although this review aimed to assess the effectiveness of MTN combined with exercise in middle-aged and older adults (≥45 years old), most of the analysed interventions were conducted with older adults (>65 yrs.). Therefore, there is still a paucity of research on the effectiveness of MTN supplementation in middle-aged physically active adults. Additionally, given the potential benefits of protein supplementation alone to maximise-exercise induced outcomes, particularly lean body mass in older adults (Vieira et al., 2022), studies comparing MTN vs. protein mono-component are necessary. In summary, studies using a broad range of middle-aged participants, longer intervention periods (>24 weeks), stricter control of diet and supplementation protocol comparing MTN vs. non-protein and only protein-containing comparators are necessary to fully understand the role of combining MTN with exercise to optimise physical training adaptation in healthy older adults.

## 2.5. Conclusions

The available evidence from RCTs suggests no additional benefits are obtained by combining exercise intervention with the ingestion of a MTN instead of isocaloric comparator on body composition, strength, and functional capacity outcomes in healthy physically active middle-aged and older adults.

# CHAPTER 3

# STUDY 2 – EFFECTS OF MULTI-INGREDIENT PRE-WORKOUT SUPPLEMENTATION ACROSS A FIVE-DAY RESISTANCE AND ENDURANCE TRAINING MICROCYCLE IN MIDDLE-AGED ADULTS

## 3.1. Introduction

Multi-ingredient pre-workout admixtures have been proposed as a specialised category of dietary supplements to be administered prior to exercise with the aims of increasing motivation to train and maximising exercise performance outcomes (Jagim et al., 2019). Previous studies on pre-workout multi-ingredient admixtures have shown several physical performance-related benefits such as (i) improving muscular endurance (Collins et al., 2017), (ii) increasing peak power output (Ormsbee et al., 2012), (iii) higher training volume capacity (Bergstrom et al., 2018), and (iv) greater fatty acid oxidation (Alkhatib et al., 2015). Furthermore, additional effects on subjective feelings (e.g., increased perceived alertness and focus on the task, and improved energy levels) have been reported (Hoffman et al., 2009a).

Most pre-workout multi-ingredient admixtures contain a proprietary blend of ingredients claiming to produce performance benefits when taken as described. Several pre-workout admixtures contain caffeine mixed with other ingredients in an attempt to produce a synergistic, ergogenic effect that favours fat metabolism during long-lasting activities (Harty et al., 2018). Additionally, the combination of ingredients such as caffeine and AA (e.g., L-Citrulline DL-Malate, L-Tyrosine, L-Taurine, etc.) has been shown to delay fatigue (Ratamess et al., 2007) and improve the overall resistance training volume (Bergstrom et al., 2018). The ergogenic effect of caffeine on exercise performance has been mainly attributed to its action as an adenosine receptor blocker (Davis et al., 2003), which may serve to decrease the perception of fatigue (Hespel, Maughan, & Greenhaff, 2006), stimulate the release of excitatory neurotransmitters, and therefore, increase motor neuron excitability (Rekling et al., 2000). These effects of caffeine may therefore speed up cell's energetic demands and prolong lipolysis (Burke et al., 2000).

Combining caffeine with yerba mate containing caffeoyl derivatives (chlorogenic acid, phytosterols and saponins) promoted fat metabolism (Alkhatib, 2014), increased fatty acid oxidation, and reduced the perception of effort during endurance exercises performed at the intensity associated with the highest fat oxidation (Alkhatib et al., 2015). Additionally, pre-workout admixtures including high-quality protein with added amino acids (e.g., L-leucine, L-arginine, L-tyrosine, or L-taurine) or derivatives (e.g., citrulline-malate, betaine or L-carnitine) may augment the effects of caffeine by increasing muscle efficiency and delaying fatigue (Giannesini et al., 2011). L-taurine is an amino-containing sulfonic acid; its inclusion as an ingredient in pre-workout multi-ingredient admixtures has been associated with higher muscular endurance capacity during resistance exercise (Harty et al., 2018). Betaine is another naturally-occurring derivative of the AA glycine which has been shown to increase muscle blood flow by elevating the levels of nitric oxide and promoting fluid and thermal homeostasis (Harty et al., 2018). L-Carnitine is a conditionally essential amino acid-like molecule found predominantly in skeletal muscle with an essential role in fatty acid metabolism (Rebouche, 2004). Pre-workout L-carnitine supplementation has been associated with increasing vasodilation, favouring oxygen supply, and attenuating exercise-induced hypoxia (Spiering et al., 2007). Additionally, combined with L-leucine, oral L-carnitine supplementation can prevent protein catabolism by stimulating mTOR expression (Evans et al., 2017). Isomaltulose is a disaccharide comprised of glucose and fructose which has been shown to slow rates of hydrolysis and subsequent absorption at the intestinal mucosa, resulting in prolonged glucose delivery to the systemic circulation. Its ingestion before intermittent exercises favoured a more stable glycaemic response in athletes (Stevenson et al., 2017).

It is possible that a combination of both caffeine and yerba mate extract with proteins and slow-release carbohydrate (isomaltulose) admixtures including citrulline-malate, L-leucine, L-tyrosine L-taurine, and betaine may work synergistically to acutely enhance performance beyond what is possible with any one single ingredient.

To the best of the authors' knowledge, no previous studies have investigated the potential ergogenic effect of a similar admixture administered before a workout. Furthermore, a meta-analysis of 35 trials concluded that combining multi-ingredients with resistance training is an effective strategy to induce greater gains of fat-free mass and strength (O'Bryan et al., 2019); however, the observed changes were more evident in untrained and elderly individuals (>45 years; 66±8 years) compared to their trained and younger counterparts (O'Bryan et al., 2019). The aforementioned meta-analysis did not distinguish among studies using different administration protocols, e.g., pre- vs. post-workout or daily multidose intakes. Indeed, none of the included trials conducted with middle-aged and older adult participants considered a pre-workout supplementation alone.

The aim of the present study, therefore, was to compare the acute effects of ingesting a pre-workout multi-ingredient (PREW) admixture over a training week (microcycle) including both resistance and endurance training vs. an isocaloric pre-workout placebo containing carbohydrate alone (CHO) in recreationally trained middle-aged men and women on (i) resistance training performance, (ii) substrate performance, (ii) substrate oxidation during endurance exercise, (iii) post-workout decrease of muscle function, and (vi) subjective measures. The primary outcomes were: (i) the total resistance training volume performed per workout (SVOL) and for the whole week (WVOL), measured in kilograms of lifted load in each exercise and normalised by the fat-free mass, and (ii) the amount of fat and carbohydrate oxidised during the endurance sessions. Secondary outcomes were the estimated decrease of muscle function due to the performed RT on (i) medicine ball throw distance, (ii) jump height, (iii) isometric strength, (iv) the evoked tensiomyography (TMG) contraction velocity (Vc) of vastus medialis (VM), biceps femoris long head (BFLH), and anterior deltoids (AD). In addition, we sought to describe the effects of pre-workout supplementation on the following exploratory variables: (i) subjective feelings of energy, focus and awareness on the task, (ii) the post-workout perceived exertion, and (iii) the rest of the TMG variables [maximum radial muscle displacement (Dm), contraction time (Tc)].

It was hypothesised that compared to the ingestion of carbohydrate alone, a pre-workout multi-ingredient admixture will promote a higher resistance training volume and favour a greater proportion of fatty acid oxidation during endurance exercises. Additionally, it will attenuate the decrease of muscular function after resistance workouts.

## 3.2. Materials and Methods

Following inclusion, familiarisation, baseline assessments, and a five-day recovery period, using a randomised, counterbalanced, cross-over, double-blind, placebo-controlled design, the participants were randomly allocated to receive either PREW or CHO. Thereafter, the participants completed two identical training and testing microcycle periods (five days each) separated by a two-week washout period. The nutritional treatment was switched from the first to the second five-day training and testing period (Figure 3.1).

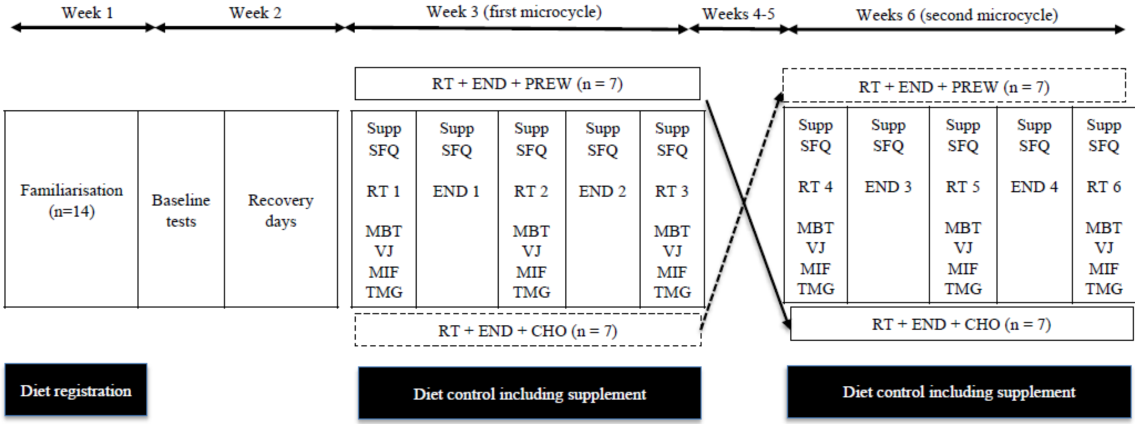


**Figure 3.1.** Overview of the study design. The overall intervention involved six consecutive weeks. First week: Familiarisation; Second week: baseline tests followed by a recovery period; Third week: First microcycle including three resistance workouts (RT 1, RT 2 and RT 3) and two endurance workouts (END 1 and END 2), pre- and post- training assessments; Fourth and Fifth weeks: Recovery/washout period; Sixth week: second microcycle including three resistance workouts (RT 4, RT 5 and RT 6) and two endurance workouts (END 3 and END 4), pre- and post-training assessments. Supp: Ingestion of the corresponding supplement condition [Pre-workout multi-nutrient (PREW) or carbohydrate (CHO)]; SFQ: Participants completed the subjective feeling questionnaire; MBT: medicine ball throw test; VJ: vertical jump test; MIF: maximal isometric force test; TMG: Tensiomyography of vastus medialis, biceps femoris long head and anterior deltoids.

### *3.2.1. Participants*

Fourteen recreationally active, middle-aged adults (seven females) participated in this study. To be eligible, participants were required to have been training regularly two to three times per week, using routines including resistance exercises (e.g., bench press, leg press, squats, or lunges) for a minimum of six months before the beginning of the study. Exclusion criteria included anyone suffering from recent (last six months) or present injuries which may prevent them from performing the required exercises, suffering from current illnesses or chronic diseases (including metabolic syndrome, advanced obesity, or sarcopenia), or taking any medication or supplements that would affect exercise performance (i.e., protein amino-acids supplements, NSAIDs, etc.). All female participants were premenopausal and were randomly tested throughout their menstrual cycle (MacNutt et al., 2012). All participants provided written informed consent in accordance with the Declaration of Helsinki. Procedures were approved by the University of Greenwich Research Ethics Committee (FES-FREC-18-3.04.16) on 23 January 2020.

The project was registered as a clinical trial at the U.S. National Institutes of Health. https://www.clinicaltrials.gov (NCT041477741).

### ***3.2.2. Procedures***

#### *3.2.2.1. Familiarisation*

After confirming their eligibility for the study, the participants completed three sessions of familiarization (week 1) during which the training protocol, exercise techniques, and the assessment procedures were explained.

#### *3.2.2.2. Baseline Assessments*

Environmental conditions kept constant in all testing and training sessions, i.e., mean (SD): 20 (1) ºC, 775.6 (12) mmHg and 51.1 (6.1) % for air temperature, barometric pressure, and relative humidity, respectively. Participants refrained from heavy exercise during 48-h prior to all baseline assessments. Tests were conducted within one day and in the following order: (i) body composition, (ii) tensiomyography, (iii) medicine ball throw, (iii) vertical jump, (iv) maximal isometric mid-thigh pull, and (v) incremental cycling test to exhaustion. A passive 10-min recovery period was provided between each individual test.

#### *3.2.2.3. Body Composition*

The standard measurements were performed following the recommendations for anthropometric assessment (Ross & Marfell-Jones, 1991). To eliminate interobserver variability, one investigator consistently performed all measurements. Height was measured in a stretched stature to the nearest 0.01 m using a wall mounted stadiometer (Seca GmbH, Hamburg, Germany) and body mass (BM) was corrected to the nearest 0.1 kg using a digital scale (Seca GmbH, Hamburg, Germany). Fat mass (FM) and fat-free mass (FFM) were estimated from whole-body densitometry using air displacement via Bod Pod® (Life Measurements, Concord, CA, USA) and following the manufacturer’s instructions as detailed elsewhere (Dempster & Aitkens, 1995).

### *3.2.2.4. Medicine Ball Throw (MBT)*

Three overhead throws were performed using the methodology described by Viitasalo (1988). Based on the distance, the best of three attempts was chosen for the analysis. Males used a five kg (circumference 0.30 m), and females a three-kg (circumference 0.21 m) medicine ball.

### *3.2.2.5. Vertical Jump*

A countermovement jump (CMJ) was performed according to the methodology described by Brown & Weir (2001). A Kistler force platform (9287B, three-component force platform; Kistler, Hook, United Kingdom; dimensions: 900 x 600 x 100 mm) with a sampling rate of 2000 Hz was used to calculate the height from the difference, in meters (m), between maximum height of the centre of mass (apex) and the last contact of the toe on the ground during the take-off. Based on the height, the best of three jumps was chosen for the analysis.

### *3.2.2.6. Maximal Isometric Force (MIF)*

A T.K.K. 5402 dynamometer (Takei Scientific Instruments Co. Ltd., Niigata, Japan) with a base of 31.5 x 31.5 cm, chain (51 cm) and latissimus pulldown bar (120 cm; Perform Better, United Kingdom) was used to assess full-body MIF (Till et al., 2018). The participants were instructed to adopt a position similar to the second pull in the power clean exercise (mid-thigh pull). Participants were positioned by standing on the foot grips and adjusting the chain length to have the bar positioned slightly above the knees. Participants gripped the bar without straps, and before pulling, maintained tension on the chain to avoid jerking movements. Thereafter, participants pulled upwards using as much force as possible (Haff et al., 2005). Three attempts of 5 s with 30 s rest were conducted. The maximum recorded value in kilograms force (kgF) was selected for the analysis.

### *3.2.2.7. Tensiomyography Assessment*

A TMG portable device (TMG Measurement System, 146 TMG-BMC Ltd., Ljubljana, Slovenia) with a maximal stimulation output of 110 mA·ms$^{-1}$ was used to measure the contractile properties of the Vastus Medialis (VM), Biceps Femoris Long Head (BFLH) and Anterior Deltoid (AD) at the dominant limb (Rey et al., 2012; Loturco et al., 2016). Measurements were collected by the same trained researcher, following the methodology described by Rey et al. (2012) and obtained at rest, in supine position for the VM, prone position for BFLH and sitting position for the AD. Changes in the evoked muscular contractile properties were estimated by analysing the following variables (i) maximal radial displacement of the muscle belly (Dm), contraction time between 10 and 90% Dm (Tc), and mean velocity of contraction (Vc), which was calculated by dividing the Dm by the sum of the Tc and the delayed time (Td) (Loturco et al., 2016). These three variables demonstrated high levels of accuracy, reliability, and sensitivity to changes in neuromuscular function by TMG analysis (Martín-Rodríguez et al., 2017). Furthermore, it is not uncommon for Tc and Dm to vary disproportionately relative to one another, and changes in Tc, independent from Dm, can be due to alterations in the rate of contraction, as measured by Vc (Macgregor et al., 2018). The intraclass correlation coefficients (ICC) at 95% confidence intervals (CI) for TMG variables ranged from 0.88 to 0.91, similar to those reported in previous investigations (Rey et al., 2012).

### *3.2.2.8. Incremental Cycling Test to Exhaustion*

Following a standardised warm-up, participants completed a maximal incremental laboratory exercise test to exhaustion on a cyclo-ergometer (Lode Corival®). The test commenced at a work rate of 30 or 50 W (women and men, respectively) and increased 15 W every minute. The participants were encouraged to keep a constant cycling rate between 60 and 90 rpm while remaining in a sitting position. When cadence dropped by more than 10 $rev{\cdot}min^{-1}$ for more than 10 s despite strong verbal encouragement, tests were terminated. The test was designed to avoid long-term muscular fatigue, and every trial lasted <18 min. Expired gases were collected continuously during the test using a Cortex MetaLyzer 3B gas analyzer (Cortex Biophysik, Leipzig, Germany). This device was also used to calculate the respiratory exchange ratio (RER), and thereafter, to estimate the oxidation and relative contributions of carbohydrate and fat across the test (Alkhatib et al., 2015). Additionally, the maximum heart rate (HRmax) and VO2peak (calculated as the highest mean oxygen consumption over a 30-s period (Karsten et al., 2014)) were calculated for descriptive purposes.

### *3.2.3. Dietary and Supplementation*

Each participant completed a three-day food diary report (two weekdays, and one weekend day). The Food Processor Software (Version 11.4.70, London, UK) was used to calculate the energy and nutritional compositions of the reported diets. Participants were instructed to maintain their habitual diet throughout the study, including the washout period. They were asked to report any minimal change regarding food composition and size, ingestion of supplements or compliance with the reported meals, including breakfast, lunch, pre- and post-workout food intake, and dinner. If any change had been detected (i.e., becoming vegetarian, restricting calories, taking additional nutritional supplements, etc.), that participant's data would have been excluded from the analysis.

During the five-day training periods (weeks 3 and 6), all participants consumed either one 40g dose of a commercially available pre-workout multi-ingredient blend (Pre-workout PRO ST, Crown Sport Nutrition, Spain) or an isoenergetic comparator (see Table 3.1).

**Table 3.1.** Nutritional composition of supplements per intake mixing with ~350 mL of plain water.

| Description | Multi-ingredient (40 g dose) | Comparator (27 g dose) |
|---|---|---|
| Energy value (kcal) | 100 | 102 |
| **Macronutrients** | | |
| Total carbohydrates (g) of which | ~16 | 25<br>(maltodextrin) |
| Isomaltulose (g) | (14) | |
| Maltodextrin (g) | (1.9) | |
| Total proteins included added amino acids (g) | 9 | - |
| **Amino acids and other ingredients** | | |
| Betaine Hydrochloride (g) | 2 | - |
| L-Carnitine L-tartrate (g) | 1.5 | - |
| L-Citrulline-DL-malate (g) | 2.5 | - |
| L-Leucine (g) | 3 | - |
| L-Lysine (g) | 2.7 | - |
| L-Arginine Base (g) | 2.5 | - |
| L-Isoleucine (g) | 1.5 | - |
| L-Methionine (g) | 0.7 | - |
| L-Phenylalanine (g) | 1.1 | - |
| Taurine (g) | 1 | - |
| L-Threonine (g) | 1.2 | - |
| L-Tryptophan (g) | 0.3 | - |
| L-Tyrosine (g) | 1 | - |
| L-Valine (g) | 1.5 | - |
| Caffeine (mg) | 400 | |
| Yerba Mate extract (mg) | 300 | |

The two products under study were presented in sachets of vanilla-flavoured powder to be diluted in ~350mL of cold plain water and administered 15 min before each workout-session. The diluted drinks were similar in appearance, texture, and taste. The participants were instructed to ingest the last meal ~3 to 4 h before each training session. They were allowed to drink water but not to ingest any food during the 3-h pre-workout time or after completion of all post-workout assessments. An investigator who was not involved in the data collection prepared and administered both supplements for all participants, providing double-blinding of both the participants and the data collection researcher. No supplements were consumed on non-exercising days (e.g., weekends and weeks 4 and 5). Furthermore, to avoid possible confounding trial order effects, the conditions were tested following a balanced randomised order. Following the preintervention assessments, participants were matched by sex and body mass. Assignment of participants to treatments was performed by block randomization using a block size of two and in a double-blind (PREW or CHO) fashion.

### *3.2.4. Exercise Protocols and Post-workout Assessments*

*Resistance Training (RT)*: Three RT sessions were conducted on alternate days (e.g., Monday, Wednesday, and Friday), and all sessions took place late in the afternoon (4 to 6 pm). Each participant performed a supervised full-body resistance-training protocol involving a standardised warm-up followed by three circuits of one set of the following exercises: (i) parallel squat, (ii) bench press, (iii) alternate 40 cm box step-ups, (iv) shoulder press, (v) alternate lunges, (vi) upright row, (vii) deadlift, and (viii) squat jumps. About 30 sec rest between exercises and 3 min between circuits was allowed. As the workout aimed to create a high level of mechanical and metabolic stress, a muscle endurance training targeting 16 self-determined maximum repetitions (>40 to <60% 1RM) per set was designed (Ratamess et al., 2009). When participants were able to perform more than 16 repetitions per set, the load was increased between 2.5 and 5 kg. If fewer than 16 repetitions were completed, a minimum rest period of 15 s was introduced until the participants were able to reach the targeted number of repetitions per set. The time to complete the workouts was ~55-min. Additionally, the rate of perceived exertion (RPE) for the entire workout was measured by using the OMNI-RES scale (Robertson et al., 2003). To avoid easy or difficult elements toward the end of the sessions from skewing the overall rating of the exertion, the participants were asked to rate their session-RPE by answering the question “How hard was your entire workout?” between 15 to 30 min after the completion of each resistance training workout (Lodo et al., 2012). Within 20 min after the completion of each workout (RT1, RT2 and RT3), assessments of voluntary (MBT, CMJ, MIF) and evoked (TMG).

*Endurance Training (END)*: Two END sessions were conducted twice a week on alternate days (e.g., Tuesday and Thursday). Each session involved 30 min of cycling at an individually determined maximum fatty acid consumption (Fatmax) intensity. Additionally, the RPE using the Borg scale (6–20) was measured every five minutes during exercise. The exercise intensity at which the reliance on fat oxidation reached its maximum (Fatmax) was individually determined and chosen as the target intensity for the endurance training sessions. The metabolic data of fat (FAO) and carbohydrate (CHOox) oxidation were estimated using stoichiometric indirect calorimetry equations (Equations (1) and (2)), assuming minimal protein contribution during exercise.

$$\text{FAO} = 1.695 \text{ x } \text{VO}_2 - 1.701 \text{ x } \text{VCO}_2 \quad (1)$$

$$\text{CHOox} = 4.585 \text{ x } \text{VCO}_2 - 3.226 \text{ x } \text{VO}_2 \quad (2)$$

The FAO during exercise was determined based on averaging the last twenty min of the 30-min session. Fatmax ($g \cdot min^{-1}$) was determined from the incremental test as the highest amount of FAO averaged over 30-s. Fatmax corresponding intensity was determined as the mechanical power output (W) and relative intensity (%) relative to peak power (Ppeak), at which each participant achieved Fatmax (Alkhatib et al., 2015).

Subjective Feelings: Prior to each workout session and after taking the supplement, all participants were asked to complete a four-question questionnaire based on a five-point rating scale. The participants were asked to rate their energy level, fatigue level, feelings of alertness, and feelings of focus on the task using the following verbal anchors: 1 = very low, 2 = low, 3 = average, 4 = high, and 5 = very high, with the average response of the three testing sessions computed for a final “score” (Hoffman et al., 2009a). The same researcher performed all test administrations under controlled conditions (i.e., in a quiet room).

Compliance with the study procedures (e.g., potential changes in dietary intake, ingestion of caffeine or other supplement, resting and training time) were checked by the researchers using an individual interview before starting all training or testing sessions.

### *3.2.5. Statistical Analyses*

Descriptive analyses were performed, and Shapiro-Francia tests were applied to assess normality. Before testing the main hypothesis, the possible treatment order effect, and the effectiveness of the washout phase to rule out any carryover effect was checked. For all the analysed variables, a preliminary test using the sum of all values obtained for each participant at any training sessions (RT1, END1, RT2, END2, RT3) in the two periods was calculated and compared across the two sequenced conditions. An independent samples Student's T-test was used to compare the values measured in the seven participants who started with PREW vs. the results determined for the seven others who started with CHO (Wellek & Blettner, 2012).

Two-way repeated-measure ANOVA (3 RT workouts or two END workouts x two conditions (PREW vs. CHO)) was performed to respectively analyse (i) the SVOL lifted and the session RPE rated in each RT workout, and (ii) the substrate oxidation (FAO and CHOox) and the averaged Borg scale score measured during each END session. A related sample Students T-test was used to analyse differences between conditions (PREW vs. CHO) for the WVOL and the averaged measures of the four questions included in the subjective feeling questionnaire. Raw changes in performance (MBT, CMJ and MIF) and TMG, were calculated by subtracting pre- from post- assessment values, without adjusting for pre-values, since the same participants performed under both conditions acting as their own controls. In order to assess the magnitude of the differences from baseline, confidence intervals (CIs) of the differences were calculated and plotted. Those CIs not crossing zero were considered statistically significant from the baseline performance. Additionally, two-tailed one-sample Student T-tests were used to test for a null effect hypothesis.

To compare differences between conditions (PREW vs. CHO) at post-workout measurements in raw change, an ANOVA with repeated measures was used to examine changes over the three sessions (RT1, RT2, and RT3) for MBT, CMJ, MIF, VOL, GRPE (global rate of perceived exertion after 20 min of the end of the workout), and all TMG variables. Differences over time were compared using Bonferroni-adjusted pairwise comparisons when appropriate.

A previous analysis using sex as an interparticipant factor (i.e., condition x time x sex) demonstrated no significant interactions between sex and conditions or times for all the variables except for TMG. Therefore, sex (men, women) was used as a covariate to analyse changes in the TMG variables. For the rest of the variables, data were pooled between sexes and analysed together for the rest of the variables.

Eta squared ($\eta^2$) and Cohen's d values were reported to provide an estimate of the standardised effect size (small $\eta^2 = 0.01$, d = 0.2; moderate $\eta^2 = 0.06$, d = 0.5; and large $\eta^2 = 0.14$, d = 0.8). The significance level was set at 0.05. All results are reported as mean (standard deviation) unless stated otherwise. All statistics were performed using the Statistical Package for the Social Sciences (SPSS for Windows, version 26.0; SPSS, Inc., Chicago, IL, USA).

## 3.3. Results

The demographic characteristics of the sample are presented in Table 3.2.

**Table 3.2.** Demographic characteristics of the participants (described by sex).

| **Measures** | **Males (n=7) Mean ± SD (Range)** | **Females (n=7) Mean ± SD (Range)** |
|---|---|---|
| Age (yrs) | 49 ± 5<br>(45-58) | 49 ± 4<br>(45-55) |
| Height (cm) | 178 ± 5<br>(168-185) | 161 ± 3<br>(156-164) |
| Body mass (kg) | 85.8 ± 14<br>(67-107) | 71 ± 8<br>(58-82) |
| Fat-Free mass (kg) | 63.3 ± 5<br>(52–69) | 45.3 ± 5<br>(54-38) |
| Fat mass (kg) | 25.1 ± 10<br>(12-38) | 27.4 ± 9<br>(15-42) |
| Experience in RT (yrs) | 2.4 ± 1<br>(1-5) | 1.6 ± 1<br>(1-5) |
| Overhead Medicine ball Throw (m) | 6.1 ± 1.2<br>(4.5-7.9) | 4.6 ± 0.6<br>(3.6-5.4) |
| Countermovement jump (cm) | 26.4 ± 3.4<br>(21.4-30.7) | 17.1 ± 2.6<br>(12.9-20.4) |
| Maximal Isometric Mid-Thigh Pull (kg$_F$) | 179 ± 41<br>(114-240) | 108.4 ± 18.8<br>(90.50-145.5) |
| $\dot{V}O_2$peak (ml/kg/min) | 47 ± 11<br>(34-71) | 31 ± 5<br>(25-38) |
| Fat max intensity (Watts) | 103 ± 45<br>(66-196) | 55 ± 12<br>(41-74) |

No carryover effect was observed for the main performance (WVOL $p = 0.40$, SVOL $p = 0.51$) and metabolic (FAO $p = 0.39$, CHOox $p = 0.77$) variables, nor for the secondary variables (MBT $p = 0.43$, CMJ $p = 0.21$, MIF $p = 0.37$, Vc for VM $p = 0.36$, BFLH $p = 0.51$ and AD $p = 0.10$) and the exploratory variables (all $p > 0.05$).

### *3.3.1. Diet Analysis*

Table 3.3 shows the daily consumption of macronutrients (grams) and energy (kcal) including and not including the two analysed supplements. Overall, the ingestion of a 40 g daily dose of PREW increased protein and carbohydrate intake, while adding 27 g of maltodextrin increased daily carbohydrate ingestion alone. Both PREW and MALT significantly increased energy intake.

**Table 3.3** Descriptive analysis of participants' diet compositions, including and not including pre-workout supplementation.

| Macronutrients | No supplementation (n=14) | With Pre-Workout (n=14) | With Maltodextrin (n=14) |
|---|---|---|---|
| **Proteins** | | | |
| $g{\cdot}d^{-1}$ | 83.3 ± 31.4 | 92.9 ± 31.4 $^{*\ \phi}$ | 83.3 ± 31.4 $^{\phi}$ |
| $g{\cdot}kg^{-1}{\cdot}d^{-1}$ | 1.1 ± 0.3 | 1.2 ± 0.33 $^{*\ \phi}$ | 1.1 ± 0.3 $^{\phi}$ |
| % of total energy | 16.5 ± 4.4 | 17.5 ± 4.24 $^{*\ \phi}$ | 15.7 ± 4.2 $^{*\ \phi}$ |
| **Carbohydrate** | | | |
| $g{\cdot}d^{-1}$ | 242.0 ± 82.3 | 260.1 ± 83.1 $^{*\ \phi}$ | 267.4 ± 82.4$^{*\ \phi}$ |
| $g{\cdot}kg^{-1}{\cdot}d^{-1}$ | 3.2 ± 1.3 | 3.4 ± 1.3 $^{*\ \phi}$ | 3.5 ± 1.3 $^{*\ \phi}$ |
| % of total energy | 48.3 ± 13.3 | 49.0 ± 12.7 * | 50.8 ± 12.7$^{*\ \phi}$ |
| **Fats** | | | |
| $g{\cdot}d^{-1}$ | 77.4 ± 26.2 | 77.4 ± 26.2 | 77.4 ± 26.2 |
| $g{\cdot}kg^{-1}{\cdot}d^{-1}$ | 0.97 ± 0.2 | 0.97 ± 0.2 | 0.97 ± 0. |
| % of total energy | 35.2 ± 9.9 | 33.5 ± 9.4 | 33.4 ± 9.4 |
| **Energy** | | | |
| Total daily energy | 2053.2 ± 407.7 | 2167.4 ± 407.3 * | 2157.3 ± 407.6 * |
| $Kcal{\cdot}kg^{-1}{\cdot}d^{-1}$ | 26.4 ± 4.9 | 27.9 ± 5.0 * | 27.8 ± 5.0 * |

$^{*}$ $p < 0.01$ to baseline $^{\varphi}$ $p < 0.01$ Pre-workout vs. Maltodextrin. Notes: values are presented as mean ± standard deviation.

### *3.3.2. Primary Outcomes*

*Total Resistance Training Volume over the entire week (WVOL):* Under the PREW condition, the participants lifted more kg ($p = 0.001$, d = 1.26) than when they ingested only maltodextrin (Figure 3.2A).

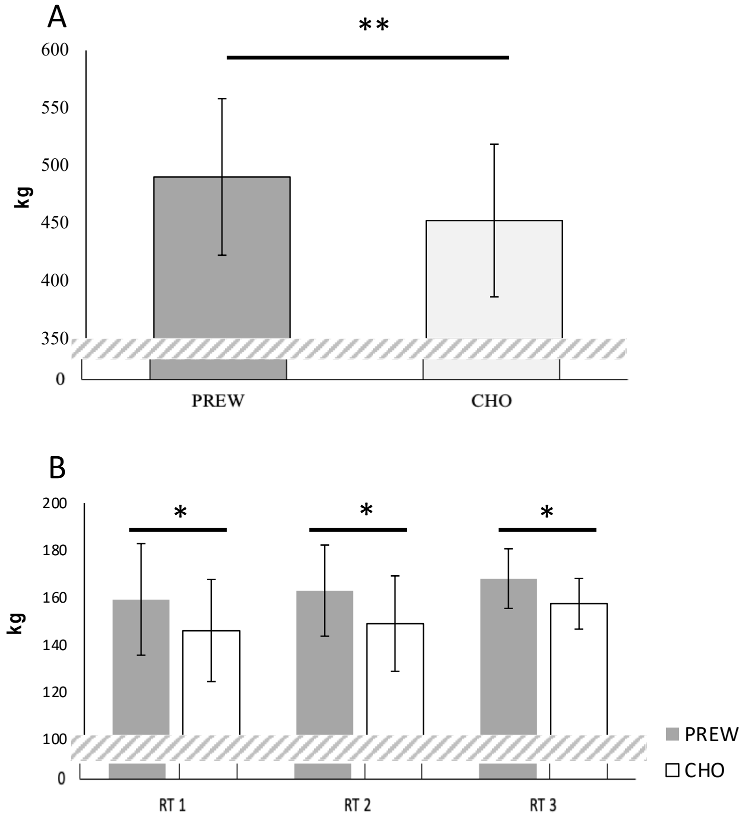


**Figure 3.2.** Estimated marginal mean values and 95% confidence intervals in resistance training volume (total kg lifted) per week (A) or per workout (B) under both treatment conditions: pre-workout (PREW) or carbohydrate (CHO). $^{**}p < 0.01$, $^{*}p < 0.05$ between conditions (PREW vs CHO).

#### *3.3.2.1. Total Resistance Training Volume Per Session (SVOL)*

Effects between workouts (F[2,26] = 15.082, $p = 0.001$, $\eta^2 = 0.010$) and supplements (F[2,26] = 22.295, $p = 0.001$, $\eta^2 = 0.023$) were observed but an interaction was not found (F[2,26] = 1.073, $p = 0.375$, $\eta^2 = 0.001$. Post hoc comparisons indicated that under PREW, the participants lifted more kilograms than under CHO ($p < 0.05$, d > 0.80) during the three RT sessions (Figure 2B). Additionally, under PREW, the training volume was significantly higher in RT3 vs. RT2 ($p = 0.026$) and RT1 ($p = 0.005$), as well as between RT2 vs. RT1 ($p = 0.016$). However, under CHO, no difference was observed between the

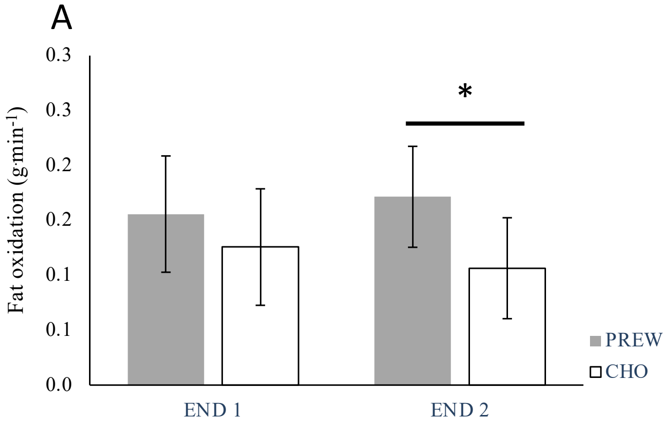
SVOL during the first two sessions (RT1 vs. RT2, $p$ = 0.471), while RT3 increased significantly compared to both RT1 ($p$ = 0.008) and RT2 ($p$ = 0.023).

### *3.3.2.1. Subtract Oxidation during Endurance Training Sessions*

Main effects between supplements (F[1,13] = 4.878, $p$ = 0.046, $\eta^2$ = 0.077), but not between workouts F[1,13] = 0.008, $p$ = 0.931, $\eta^2$ = 0.001) or interaction F[1,13] = 1.256, $p$ = 0.283, $\eta^2$ = 0.088) effects, were determined for FAO.

No main effects between workouts (F[1,13] = 0.169, $p$ = 0.663, $\eta^2$ = 0.001), supplements (F[1,13] =1.922, p = 0.189, $\eta^2$ = 0.007) or interaction (F[1,13] = 3.242, $p$ = 0.095, $\eta^2$ = 0.088) were determined for CHOox.

The post hoc analysis indicated a significantly higher FAO($p$ = 0.05, d = 0.53) and a non-significant ($p$ = 0.07, d = 0.51) lower CHOox under PREW compared to CHO during END 2 (Figure 3.3B).



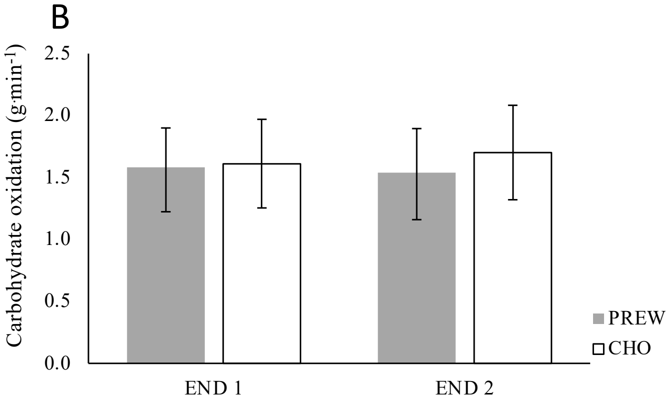


**Figure 3.3.** Estimated marginal mean values and 95% confidence intervals fat (A) and CHO (B), oxidation rate g·min−1 determined during the first (END 1) and second (END 2) endurance training session under both treatment conditions: pre-workout (PREW) or carbohydrate (CHO). $^* p < 0.05$ between conditions (PREW vs CHO).

### *3.3.3. Secondary and Exploratory Outcomes*

#### *3.3.3.1. Medicine Ball Throw (MBT)*

Compared to baseline, a significant decrease in performance was observed after the completion of RT2 and RT3 under the PREW condition, and after completion of the three RT workouts under the CHO condition. Additionally, the main between supplement effects were determined (F[1,13] = 12.65, p = 0.004, $\eta^2 = 0.065$), though not for workouts (F[2,26] = 1.920, p = 0.167, $\eta^2 = 0.019$) or interaction F[2,26] = 1.073, p = 0.357, $\eta^2 = 0.016$). A post hoc analysis revealed significantly lower performance reduction (p = 0.001, d = 1.46) under the PREW compared to the CHO condition after RT 2. No between supplement differences were observed after RT1 and RT3 (Figure 3.4A).

#### *3.3.3.2. Vertical Jump*

Compared to baseline, no significant reduction in VJ performance was measured under either condition (PREW and CHO) after the three RT workouts. No main effects were determined between workouts (F[2,26] = 1.994, p = 0.156, $\eta^2 = 0.011$), for supplements (F[1,13] = 0.876, p = 0.366, $\eta^2 = 0.006$), or interaction (F[2,26] = 0.651, p = 0.530, $\eta^2 = 0.005$) (Figure 3.4B).

#### *3.3.3.3. Maximal Isometric Force (MIF)*

Compared to baseline, significant strength decreases were observed after RT1 (*p* = 0.006, d = 0.88) and RT2 (*p* = 0.039, d = 0.61) only under CHO condition. No strength reduction was measured under the PREW condition (Figure 3.4C).

There were main effects between supplements (F[1,13] = 4.881, p = 0.046, $\eta^2 = 0.076$) but not for workouts (F[2,26] = 2.854, *p* = 0.076, $\eta^2 = 0.022$) or interaction F[2,26] = 0.858, *p* = 0.436, $\eta^2 = 0.005$).

The post hoc analysis revealed a significantly lower performance reduction (*p* = 0.007, d = 0.86) under the PREW compared to the CHO condition after RT 3. No between supplement differences were observed after RT1 and RT2 (Figure3.4C).

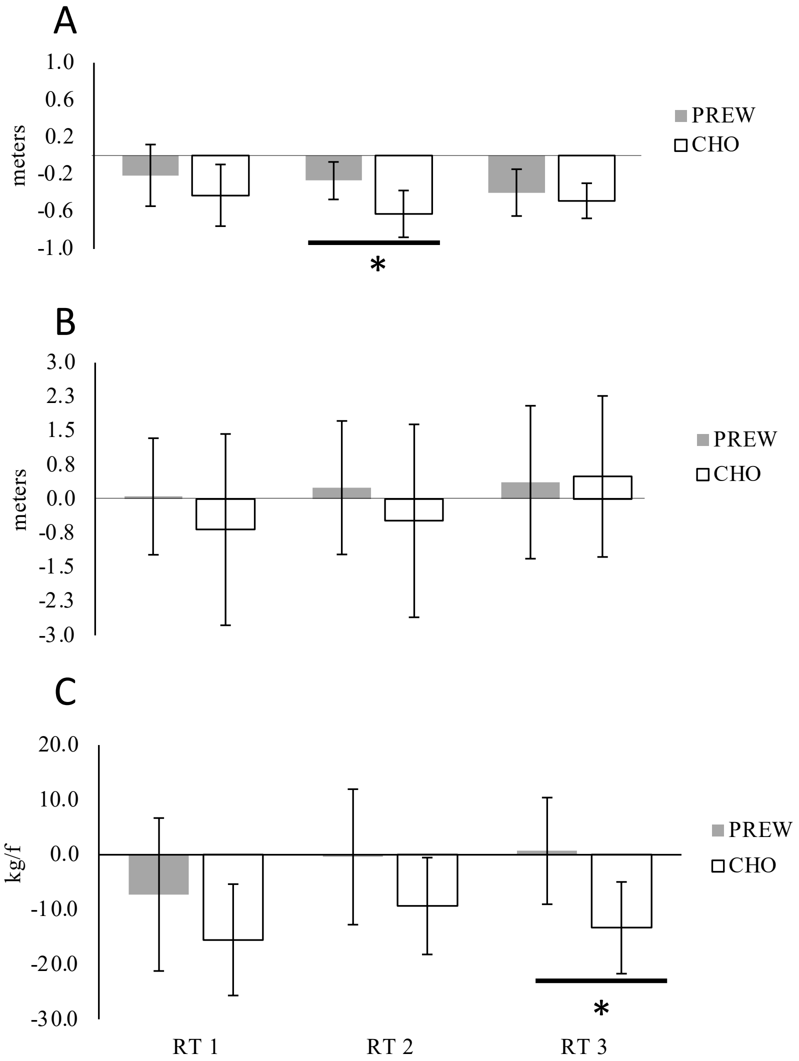


**Figure 3.4.** Estimated marginal mean values and 95% confidence intervals in delta changes in medicine ball throw (A), vertical jump (B), and maximal isometric force (C) measured after the completion of the three resistance training sessions (RT 1, RT 2 and RT 3) under both treatment conditions: pre-workout (PREW) or carbohydrate (CHO). * $p < 0.05$ between conditions (PREW vs CHO).

#### *3.3.3.4. Tensiomyography*

Compared to baseline, no significant changes (all $p > 0.05$) in any TMG variable were observed (Vc, Dm or Tc). No between workouts, supplements, or interaction effects were determined in the three analysed muscles (AD, BFLH and VM). Means and SD describing the changes measured after RT1, RT2, and RT3 of the three TMG analysed variables (Vc, Dm and Tc), including the 95% CI and the ANOVAs tests for the three muscles under the two assessed conditions (PREW and CHO), are presented as Supplementary Material (Table S2).

#### *3.3.3.5. Perception of Effort*

The GRPE rated by the OMNI-RES scale after completion of the three RT workouts were as follows: (i) RT1: PREW 7.9 ± 0.9; CHO 7.9 ± 1.2, (ii) RT2: PREW 8.0 ± 0.8; CHO 8 ± 0.8, (iii) RT3: PREW 8.1± 0.7; CHO 8.1 ± 0.8. Neither main effects between workouts (F[2,26] = 1.387, $p = 0.268$, $\eta^2 = 0.010$) and supplements (F[1,13] = 0.07, $p = 0.795$, $\eta^2 = 0.001$) nor an interaction F[2,26] = 0.038, $p = 0.963$, $\eta^2 = 0.001$) were determined.

The average RPE values rated by the Borg-scale (6–20 points) every 5 min during the endurance training were as follows: (i) END1: PREW 11.5 ± 1.1; CHO 12.0 ± 1.2, (ii) END2: PREW = 11.5 ± 1.2; CHO 11.7 ± 1.1. No main between workouts (F[1,13] = 1.230, $p = 0.287$, $\eta^2 = 0.003$), supplements (F[1,13] = 2.149, $p = 0.166$, $\eta^2 = 0.026$) or interaction F[1,13] = 2.997, $p = 0.107$, $\eta^2 = 0.006$) effects were determined.

#### *3.3.3.6. Subjective Feelings*

The responses to the subjective feelings questionnaire followed similar patterns for both the PREW and CHO supplementation protocols. No differences between conditions (all $p > 0.05$) were observed for the averaged energy levels values, fatigue level, feeling of alertness, or focus for the task before starting the workouts.

## 3.4. Discussion

The observed results suggest that PREW vs. CHO treatment increases the total volume lifted over three resistance training sessions performed across a five-day macrocycle (Figure 3.2). Additionally, ingesting the multi-ingredient admixture before low-intensity (Fatmax) endurance training sessions favoured fat oxidation (Figure 3.3A). However, even though participants experienced less performance decrease on the MBT and MIF under the PREW condition after completion of RT2 and RT3 respectively, the lack of interaction effect precludes any conclusion supporting the advantage of PREW over CHO to attenuate this decrease (Figure 3.4). No differences between supplements (PREW vs. CHO) were identified for the remaining variables, including the acute response of involuntary muscular function (TMG), the rating of perceived effort measured after both RT (by the OMNI-RES scale) and END (by the 6–20, Borg-scale), or the subjective feelings. Based on these findings, and within the confines of the study procedures, our research hypothesis was accepted asserting that compared to carbohydrates alone, a pre-workout multi-ingredient admixture may promote higher training volumes (kg lifted) during resistance workouts and may favour fatty acid oxidation during submaximal continuous endurance exercises. However, the hypothesis supporting the benefit of a pre-workout multi-ingredient admixture instead of carbohydrate alone to attenuate the post-workout decrease of muscular function, improving the perception of effort, and enhancing subjective feelings cannot be accepted.

Previous studies reported enhancement effects of ingesting pre-workout multi-ingredient admixtures on resistance training performance (Hoffman et al., 2009a; Gonzalez et al., 2011; Jagim et al., 2016; Bergstrom et al., 2018). Compared to the multi-ingredient admixture examined in our study, the formulations used in the aforementioned investigations provided less energy, containing lower amounts of carbohydrates and proteins, but included creatine, beta-alanine, or both. These two ingredients were not part of the admixture used in our study. However, all the formulations, including the one tested in the present study, contained alkaloids, mainly in the form of anhydrous caffeine, combined with amino-acids or derivatives (e.g., L-Leucine, Taurine, L-Citrulline-DL malate). The observed increased volume during the three RT workouts under the PREW condition could have been related to the role of caffeine as an adenosine receptor antagonist (Goldstein et al., 2010). Conversely, previous studies reported a positive effect of caffeine to decrease the perception of effort during and after high-intensity exercise sessions (Cooper et al., 2014) and improve task motivation (Lieberman et al., 2002), focus on the task, and energy feelings (Hoffman et al., 2009) compared to the ingestion of carbohydrate alone, even though such effects failed to be observed. However, it is worth noting that the higher RT volume accomplished under the PREW condition did not elicit a concomitant increase in the global post-workout perception of effort. Therefore, it is possible to speculate that the ingestion of the multi-ingredient admixture attenuated the concomitant rise of the perception of effort that could have been expected by the completion of a higher training volume. Additionally, the increased volume under the PREW condition should have been followed by a larger decrease in voluntary and involuntary muscular function. However, there was no difference between conditions. In practice, the ingestion of the multi-ingredient, in addition, to promote an increased RT volume, attenuated the concomitant loss of both voluntary and evoked muscular function that could have been expected after performing higher volumes during the resistance workout sessions (McLester et al., 2003; Bartolomei et al., 2017). On the other hand, the lack of clear differences between the two tested conditions in all the TMG variables can be explained by the expected blunted-fatigue effect of pre-workout supplementation (Kerksick & Leutholtz, 2005). Nutrients included in both PREW (e.g., isomaltulose and amino acids) and placebo (maltodextrin), to a certain extent, were effective at attenuating the drop in muscle function that is often expected with no nutrient ingestion (McLellan et al., 2013). In any case, the observed mitigation of usual performance decreases under the PREW condition on both MBT and MIF supports the efficacy of ingesting PREW vs. CHO. The mentioned benefit, however, still needs confirmation from further studies conducted over longer intervention periods (>4 weeks) and using larger samples.

It is worth mentioning that, up to a certain limit, e.g., ten sets per muscle group per week (Barbalho et al., 2019), substantially greater training volumes may be beneficial in enhancing muscle hypertrophy (Schoenfeld et al., 2019). Furthermore, appropriate lean and fat mass levels are two of the most relevant health-related factors to improve the quality of life and attenuate deleterious processes associated with typical ageing (Westcott, 2012). In those lines, multi-ingredient supplementation provides an additional nutritional stimulus to promote skeletal muscle anabolic responses to exercise with a moderate increase of caloric intake, which may actually assist older individuals in meeting their total daily energy intake requirements. Thus, for physically active, healthy, middle-aged, and older individuals, adding a pre-workout multi-ingredient admixture, as used in our study, could be an acceptable strategy to maximise training adaptations and counteract the progression of age-associated declines in muscle mass, strength, and physical function.

The multi-ingredient admixture used in the present study provided 400 mg of anhydrous caffeine (5.2 ± 0.8 $mg \cdot kg^{-1}$) and 300 mg of yerba mate extract (3.9 ± 0.6 $mg \pm kg^{-1}$), with an estimated per dose content of caffeine of about 1% (3 mg) (0.04 ± 0.01 $mg \cdot kg^{-1}$) (Bracesco et al., 2011). Thus, the resulting mean relative dose of caffeine per intake was 5.2 ± 0.8 $mg \cdot kg^{-1}$, which was within the range of recommended moderate doses (3 to 6 $mg \cdot kg^{-1}$) related to performance increase in resistance exercises (Pickering and Grgic, 2019). In addition, the dose used was also slightly higher than the relative amount of caffeine included in pre-workout supplements previously reported to be effective for improving resistance training volume (Gonzalez et al., 2011; Jagim et al., 2016; Bergstrom et al., 2018)

Regarding the impact of pre-workout supplementation on low-intensity endurance exercises, in line with the study of Bergstrom et al. (2018), those under the PREW treatment elicited higher fat oxidation. Nonetheless, different from the study of Bergstrom et al. (2018), who observed improvements in perceived response, our participants reported similar perception of global effort after both endurance and resistance training workouts. Discrepancies between studies may be due to differences between the supplements. Alkhatib and coworkers (2015) used a thermogenic-based multi-ingredient admixture with no protein, AA, or carbohydrates, instead containing a combination of different herbs, i.e., 210 g of green tea leaf and 300 mg of Guarana seed extract, as well as 150 mg anhydrous caffeine per intake. The effects of this admixture were compared to those of a noncaloric placebo. Our study compared an admixture including thermogenic compounds (caffeine and yerba matte) protein, amino-acids, and carbohydrates vs. an iso-energetic supplement containing nonprotein or derivatives, or any thermogenic compound ingested prior to a low-intensity, middle-length (30 min) endurance exercise. The ingestion of macronutrients (e.g., carbohydrates) with added caffeine has been shown to reduce the perception of effort at the end of long-duration or exhaustive endurance exercises (Cooper et al., 2014; Kumar et al., 2019). Our participants were following typical eating patterns, having the last pre-workout meal ~3-h before each exercise session. Therefore, it is unlikely that they were exercising with low muscle glycogen content or obtaining any advantage from the pre-workout supplementation in terms of attenuating the perception of effort after performing a 30 min, submaximal endurance training session.

Since it is not possible to identify the contribution of each individual ingredient to the observed effects, several previous isolated nutrient studies have offered insight into the potential effects of the various nutrients (Schwarz and McKinley-Barnard, 2020). In addition to the caffeine and yerba mate, the increased volume of weight lifted during RT may also be attributed to the improved vasodilatory response and higher muscular efficiency elicited by the ingestion of citrulline-DL-malate (Sureda et al., 2010) and L-Carnitine (Spiering et al., 2007). Furthermore, the inclusion of 2 g per dose of betaine hydrochloride could have acted as an osmolyte, thereby increasing the water retention of cells and attenuating fatigue as the workout sessions progressed (Hoffman et al., 2009b).

It has been claimed that the addition of theanine and tyrosine to caffeine favours cognitive function and focus on the task (Zaragoza et al., 2019). Although no effect was observed on the measured subjective feelings in this study, it is possible that these three ingredients worked synergistically with citrulline-malate, L-carnitine, amino-acids, and betaine to increase exercise volume by promoting cell hydration, blood flow, and the removal of metabolic by-products while attenuating the concomitant increase of the perception of effort due to the higher training volume. The dose of tyrosine used in the present investigation (1 g) was similar to the amount included in several multi-ingredient formulae claiming to enhance some aspects of exercise performance (Bergstrom et al., 2018; Figueiredo et al., 2020). Nonetheless, to the best of the authors' knowledge, there is still a lack of research on the synergistic effect of caffeine, thionine, and tyrosine to enhance muscular activation during exercise sessions.

The macronutrient admixture provided 14 g of isomaltulose (low glycaemic index ~32) and 1.9 g of maltodextrin (high glycaemic index > 90). Compared to ingesting maltodextrin only, the addition of isomaltulose to the protein and amino-acids admixture may have favoured a more stable glucose concentration (Stevenson et al., 2017), promoting a higher exercise volume during resistance training (Kraemer et al., 2015).

The present study had several limitations that must be considered when attempting to draw evidence-based inferences. Even though dietary records were kept, the participants' diets were not fully controlled outside of the supplement routine. Although this approach has been extensively used, providing a prepacked diet to participants before and during the intervention would have offered a more accurate reliable means of achieving dietary control (Jeacocke and Burke, 2010). The estimation of fat oxidation rates during the incremental exercise test was conducted under non-steady-state conditions. Although this method is widely used and allows for the determination of the Fatmax point, the continuous increase in workload may not allow sufficient time for substrate oxidation to reach equilibrium at each intensity stage. This can lead to transient imbalances in $VO_2$ and $VCO_2$ kinetics, especially at the onset of each new stage, potentially underestimating fat oxidation rates and overestimating carbohydrate reliance (Amaro-Gahete et al., 2019). Therefore, this marker should be cautiously interpreted and considered an estimate instead of a highly accurate physiological threshold (Jeukendrup & Wallis, 2005). As several previous investigations have demonstrated the effectiveness of pre-workout supplements on increasing performance outcomes vs. noncaloric conditions, the inclusion of another group receiving a nonenergy placebo supplement was not considered in the present investigation. Even though adjustment of the load during RT was allowed, variations of exercise intensity were not permitted during END. Thus, it was not possible to know whether the participants would have performed either a higher volume or maintained a higher exercise intensity with a similar rate of fat oxidation under the PREW condition compared to the CHO condition. Additionally, the supplementation protocol considered the absolute dose recommended by the manufacturer. Lastly, female participants were tested randomly over the menstrual cycle. The phase over which each participant was tested may influence individual strength performance (Knowles et al., 2019) and or willingness to train (Pallavi et al., 2017).

Future studies should consider both acute and long-term interventions using individualised doses based on the participants' body mass or fat-free mass. Regarding females, further studies analysing the impact of exercising in different phases of the menstrual cycle under diverse nutritional intervention are warranted.

### 3.5. Conclusions

The present investigation advocates for the ingestion of a pre-workout, protein-based, multi-ingredient admixture providing ~5.2 $mg \cdot kg^{-1}$ of caffeine, 16 g (~0.21 $g \cdot kg^{-1}$) of carbohydrate including a high proportion of isomaltulose (slow-release disaccharide), and 9 g (~0.12 $g \cdot kg^{-1}$) of protein including added amino-acids (~1.8 ratio of CHO/protein), instead of carbohydrates alone, to increase resistance training volume and possibly enhance fat oxidation during endurance training in middle-aged, physically active men and women.

# CHAPTER 4

# STUDY 3 – THE RECOVERY EFFECTS OF A VEGAN PROTEIN-BASED MULTI-INGREDIENT SUPPLEMENT ON MUSCULAR FUNCTION AND MUSCLE SORENESS IN MIDDLE-AGED PHYSICALLY ACTIVE INDIVIDUALS

## 4.1. Introduction

One of the key factors influencing recovery from resistance exercises is nutrition (Morton, McGlory & Phillips, 2015). Various nutritional strategies involving multi-ingredient preparations that provide micronutrients (e.g., minerals, vitamins, probiotics) and macronutrients (carbohydrates, proteins, or fats) have been examined for their potential to enhance recovery after intense workout sessions (Naclerio et al., 2020). Recent research has demonstrated the beneficial effects of post-workout mixtures containing high-quality, rapidly digestible proteins from animal sources (Trommelen, Betz & van Loon, 2019) such as whey (Lam et al., 2019) and beef (Valenzuela et al., 2019a) in stimulating muscle protein synthesis and improving recovery between training sessions in athletes (Cintineo et al., 2018). For example, in young resistance-trained males, consuming a complex milk-based protein beverage post-exercise was more effective than carbohydrate (CHO) alone in enhancing performance, as measured by agility, push-ups, and sprints (Lynch, 2013). More recently, Naclerio et al. (2020) indicated that a post-workout multi-ingredient supplement containing CHO, whey, and beef protein optimised strength and power performance recovery, as well as muscle function assessed by tensiomyography (TMG), following three consecutive resistance training sessions. Plant-based foods are low in one or more essential amino acids (EAA), termed 'limiting amino acids', and are thus considered 'incomplete protein sources' (Hoffman & Falvo, 2004). However, consuming animal-based protein sources (e.g., beef, pork, eggs) has been shown to result in a greater net protein balance compared to plant-based sources (e.g., kidney beans, nuts), likely due to enhanced protein synthesis and reduced protein breakdown in young adults (Park et al., 2021). However, although the proportion of EAA is lower in vegetable sources, when equivalent amounts of EAA (e.g. leucine) are ingested, whether by the ingestion of multiple plant-based protein sources (Trommelen et al. 2019) or by a blend admixture fortified with EAA, similar effects to optimise exercise-induced outcomes (e.g. increase of post-exercise muscle protein synthesis and promotion of post-workout tissue remodelling and recovery), are observed between plant and animal proteins (Joy et al., 2013). Nonetheless, some extracts of vegetable protein sources, such as pea protein (Babault et al., 2015), have exhibited scores close to or superior to 1.0 in the Digestible Indispensable Amino Acid Score (DIAAS), which is the most renowned method to analyse protein quality (Leser, 2013) by its specific true ileal digestibility and the content and profile of essential amino acids (EAA) concerning dietary requirements (Leser, 2013; Wolfe et al., 2017). For instance, Banaszek et al. (2019) compared the effects of providing pre- and post-workout pea protein vs whey protein, combined with an 8-week resistance training programme (4 training sessions per week). The authors concluded that whey and pea protein provided similar body composition, muscle thickness, force production, performance and strength adaptations. Moreover, a recent investigation evaluating the biochemical markers in under-20 years old soccer players 24 and 48 yr after a game highlighted the viability of considering plant protein as a potential alternative to animal protein without compromising athletic performance or recovery (Loureiro et al., 2023). Additionally, different studies have investigated the effects of post-workout supplementation on recovery using standard assessments of maximal strength, mechanical power, muscular endurance (Ratamess et al., 2003; Hoffman et al., 2010) and the time course of muscle soreness measured over 48-h (Rindom et al., 2016), and 72-h (Eddens et al., 2017) after a strenuous exercise bout. However, none of these investigations included pea protein, and therefore there is extremely limited research on the use of pea protein-based admixtures in this context. Hoffman et al. (2010) observed greater improvement in performance 24-h and 48-h after training in athletes ingesting a pre and post-workout blend protein including 2 g of carbohydrates vs. the ingestion of maltodextrin.

TMG is a sensitive non-invasive method for estimating in vivo, contractile and mechanical properties of individual muscles through the simple measurement of the muscle belly radial deformation in response to an electrical stimulus (Macgregor et al., 2018). Because no physical effort is required by participants being evaluated (Rey et al., 2012), TMG has been used to objectively estimate the fatigued muscle responses to different active or passive recovery strategies (Rey et al., 2012). TMG uses evoked muscular activity to estimate muscle function independently on voluntary drives, motivation, or the influence of technique when performing specific sports exercises involving multiple muscle groups (Macgregor et al., 2018). Nonetheless, in order to estimate the time course of recovery in athletic population within an applicable contextual scenario, the TMG parameters should be integrated with other measurements of in vivo human performance (maximal strength, mechanical power, etc.) and muscular disruption (Macgregor et al., 2018). Thus, well-controlled studies examining post-workout supplementation effects on recovery from intense training sessions, including changes in fatigued muscles' contractile properties, exercise performance, and muscle disruption indicators, are needed (Naclerio et al., 2021). The aim of the present study was, therefore, to investigate the effects of a multi-ingredient admixture containing CHO and high-quality protein (3:1 Vegan Recovery Crown® Sport Nutrition, Spain) on the recovery of muscular function following hard resistance training sessions. The primary outcomes included changes in upper and lower body impulsive and endurance strength performance. Secondary outcomes included changes in muscular contractile properties estimated by the evoked mean contraction velocity (Vc) using TMG, as well as changes in the perception of delayed onset muscle soreness (DOMS) due to its impact in limiting further exercise performance following hard workout sessions. Furthermore, the other TMG variables [muscle displacement (Dm), contraction time (Tc)] needed for determining the Vc were considered exploratory. It was hypothesised that compared to the ingestion of CHO alone, a vegan protein-based multi-ingredient will speed up recovery of voluntary and involuntary muscular function after hard resistance training sessions. As the most relevant factor to improve recovery after the completion of high-intensity training sessions is the energetic content of the supplement rather than the nutritional composition (McLellan, Pasiakos & Lieberman, 2014), the inclusion of another treatment receiving other protein sources (e.g. whey) or the comparison with a non-caloric condition was considered not necessary for the aim of the present investigation.

## 4.2. Material and Methods

### *4.2.1. Experimental Design*

A double-blinded, randomised, controlled, and counterbalanced, crossover design was performed. Following the inclusion criteria, familiarisation period, and baseline assessments, the participants were randomly allocated to receive either a vegan protein-based post-workout multi-ingredient (POSTW) or an isocaloric carbohydrates-only comparator (COMP). Thereupon, the participants completed two twin 5-day training and testing microcycle phases separated by a two-week washout period. (Figure 4.1)

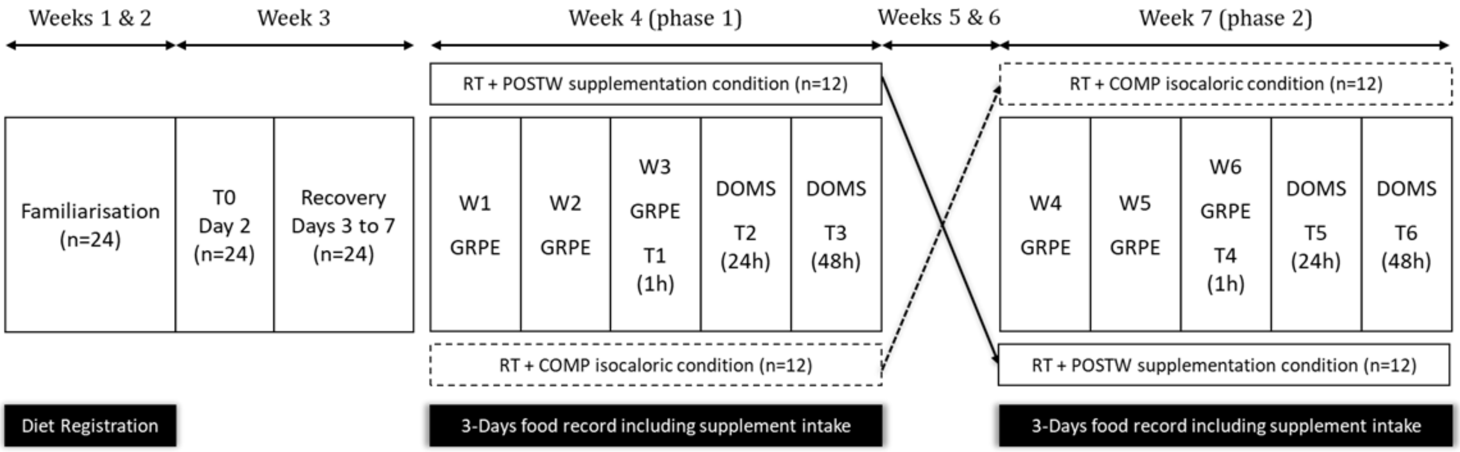


**Figure 4.1.** Schematic overview of the interventional design. The overall protocol involved a 7-consecutive-weeks period. First 2 weeks: Familiarisation; Third week: Baseline assessments (Tuesday) and a recovery period of 5 days (Wednesday-Sunday). Fourth week: First training and testing period, including 3 resistance training workouts on consecutive days (W1, W2, and W3); Fifth and Sixth weeks: Recovery and washout period; Seventh week: Second training and testing period, following the opposite supplementation condition.

### *4.2.2. Participants*

Twenty-four healthy, recreationally active, adults (50% females) 53 ± 5 y.o. were recruited and completed this interventional study. The inclusion criteria were to have a minimum regular resistance training history of 6 months before the study onset, to be 45 years or older and, for female participants, to present a minimum of two symptoms of menopause onset (such as hot flushes, menstrual cycle alterations, not menstruating for more than 1 year…) (NICE, 2019). Participants were not eligible if suffering from acute illness or chronic diseases (including advanced obesity, metabolic syndrome, long Covid-19, osteoporosis, or sarcopenia), following a medication prescription, or consuming supplements that could interfere with our research or affect exercise performance (i.e., creatine, protein amino-acids, NSAIDs, etc.). In accordance with the Declaration of Helsinki, all participants provided written informed consent. Procedures were approved by the University of Greenwich Research Ethics committee (FREC-EHHS-19-1-8.2.2) on 21 November 2021. The project was registered as a clinical trial at the U.S. National Institutes of Health. https://www.clinicaltrials.gov (NCT05769101).

According to preceding similar interventional studies from our research group (Naclerio 2020a; Naclerio 2020b; Puente-Fernandez et al, 2020), the sample size estimation was calculated based on the primary outcome assessment [the CMJ, jump height in centimetres]. Assuming an α-error of 0.05, for the resulting effect size of d = 0.95 calculated between two dependent means determined for the POSTW and COMP conditions, the required sample size of n= 12 was estimated to achieve >80% statistical power.

CONSORT
TRANSPARENT REPORTING of TRIALS

**CONSORT 2010 Flow Diagram**

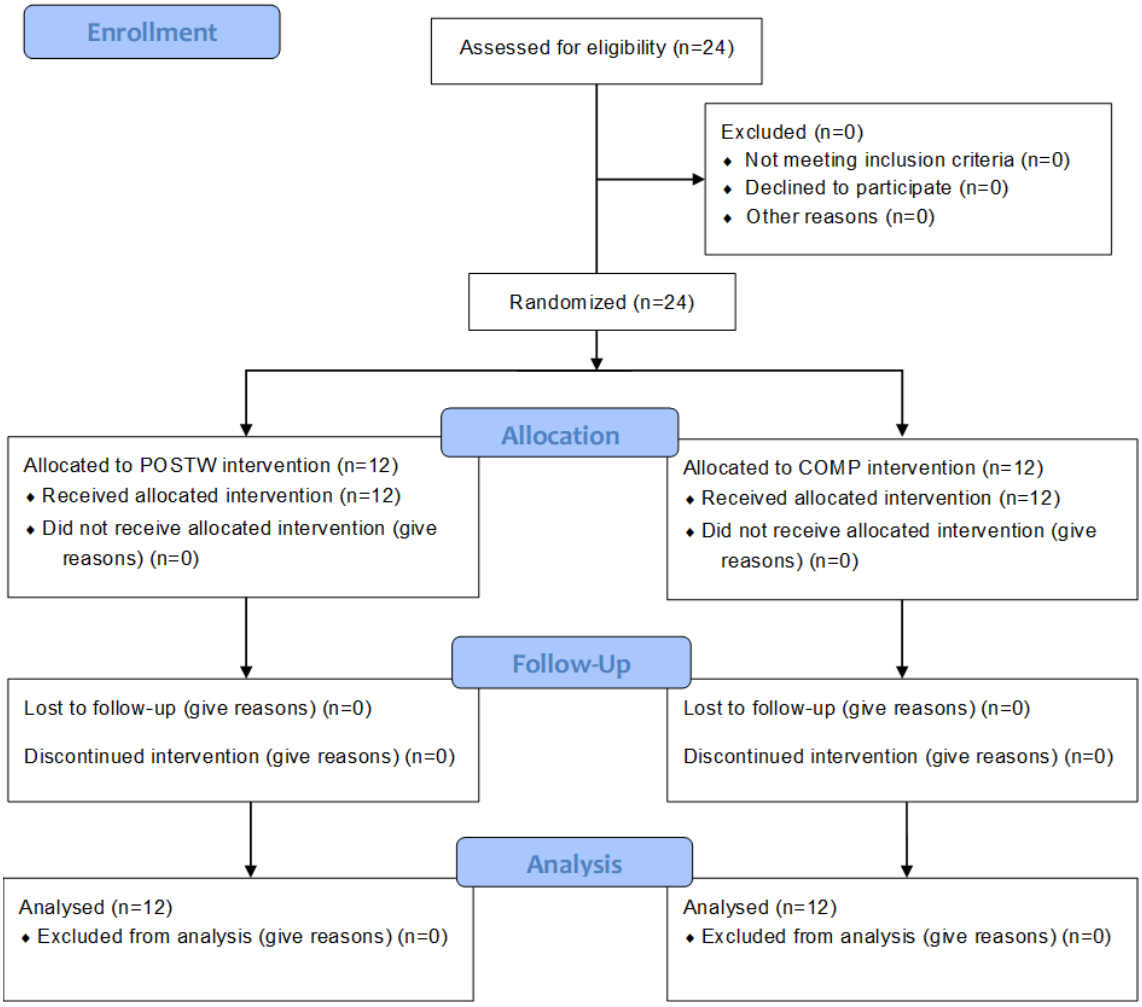


**Figure 4.2**. Schematic overview of CONSORT 2010 flow diagram of the recruited participants.

### *4.2.3. Assessments*

After being confirmed eligible and before the first baseline tests, the participants completed a familiarisation period of 2 weeks (6 sessions on alternate days) to ensure appropriate technique and estimate their individual self-perceived 16-RM (appropriate number of repetitions for muscular endurance development (Ratamess et al, 2009).

Baseline testing was conducted during weeks 3 and 6 (for the first and second training periods respectively) after 5 days of rest from the last familiarisation session (i.e., Friday to Wednesday). Participants refrained from moderate and vigorous physical activity or physical exercise for the previous 72 hours and before the training protocol. Additionally, no food or drink was allowed 2 hours before baseline assessments, training days, and testing sessions. The baseline evaluation was conducted on the same day and in the following order: (i) body composition, (ii) tensiomyography, (iii) maximal isometric force, (iv) handgrip strength, (v) countermovement jump, (vi) chest medicine ball throw, (vii) 30-seconds sit-to-stand, and (viii) 30-s bench press.

Environmental conditions were constant on every testing and training session; i.e., mean (SD): 19 (1.5) ◦C, 1020 (14) mBar, and 53.6 (5.7) % for air temperature, barometric pressure, and relative humidity, respectively. Body composition was assessed via air displacement plethysmography documented for descriptive purposes only and followed the methodology described by Dempster & Aitkens, (1995).

#### *4.2.2.1. Vertical Jump*

A countermovement jump (CMJ) was performed according to the methodology described by Brown & Weir (2001). A Kistler force platform (9287B, three-component force platform; Kistler, Hook, United Kingdom; dimensions: 900 × 600 × 100 mm) with a sampling rate of 2000 Hz was used to calculate the height from the difference, in meters (m), between maximum height of the centre of mass (apex) and the last contact of the toe on the ground during the take-off. Based on the height, the best of three jumps was chosen for the analysis.

#### *4.2.2.2. Chest Medicine Ball Throw (CMBT)*

Sitting on a chair placed against a wall, with the feet flat on the floor and separated at the shoulder widen, the participants threw the ball from their chest following the methodology defined by Harris et al (2011). Based on the distance, the best of three attempts was chosen for the analysis. Males used a five-kg (circumference of 0.30 m), and females a three-kg (circumference of 0.21 m) medicine ball. A range from 0.97-0.99 ICC has been observed for this test in recreationally trained adults (Beckham et al, 2019).

#### *4.2.2.3. 30-Seconds Sit-to-Stand (30STS)*

Participants were instructed to keep their hands on their opposite shoulders, with their arms crossed on their chest, and their feet flat on the floor at their individual shoulders wide during the entire test. The starting position was with the participant seated on a bench with her posterior thigh parallel to the floor. A repetition counted as accomplished when the participant stood with her knees extended and her hips aligned with her knees and shoulders and touched the bench with the thigh parallel to the floor. The total number of successfully completed repetitions in 30 seconds was collected.

#### *4.2.2.4. 30-Seconds Bench Press (30BP)*

A bench press with their subjective and self-determined ~16RM was performed. A repetition was successfully completed when the barbell touched the chest at the deepest position and the participant extended his arms completely again. The maximum number of completed repetitions was collected.

#### *4.2.2.5. Tensiomyography*

A TMG portable device (TMG Measurement System, 146 TMG-BMC Ltd., Ljubljana, Slovenia) with a maximal stimulation output of 110 mA·ms−1 was used to evaluate the contractile properties of the Anterior Deltoid (AD), Vastus Medialis (VM), and Biceps Femoris Long Head (BFLH) of the dominant limb (Rey, Lago-Penas & Lago-Ballesteros, 2012; Loturco et al, 2015). The same qualified and skilled researcher collected all the measurements, following the methodology described by Rey et al (2012), and applied in our previous investigations (Naclerio 2020a; Naclerio 2020b). The participants maintained relaxed and resting seated (AD), supine (VM), and prone (BFLH) positions. The electrodes and the sensor were placed in the same specific skin areas, which were accurately painted with a permanent marker on each participant's skin and kept for the whole week. At the end of the first week, the assessing points were copied in an A3 acetate paper and preserved from the first to the second training and testing week. Changes in the evoked muscular contractile properties were estimated by analysing the following variables (i) maximal radial displacement of the muscle belly in millimetres (Dm), (ii) contraction time between 10 and 90% Dm in milliseconds (Tc), and (iii) mean velocity of contraction in millimetres per millisecond (Vc), which was calculated by dividing the Dm by the sum of the Tc and the time delay (Td) (Rey, Lago-Penas & Lago-Ballesteros, 2012). High levels of accuracy, reliability, and sensitivity have been previously demonstrated for these markers with neuromuscular function changes analysed via TMG (Martin-Rodriguez et al, 2017). Furthermore, it is not uncommon for Tc and Dm to vary disproportionately relative to one another, and changes in Tc, independent from Dm, can be due to alterations in the rate of contraction, as measured by Vc (Mcgregor et al, 2018).

The intraclass correlation coefficients (ICC) at 95% confidence intervals (CI) for TMG variables ranged from 0.88 to 0.91, similar to those reported in previous investigations (Loturco et al, 2015).

#### *4.2.2.6. Training*

Resistance Training (RT): Three RT sessions were conducted on consecutive days (Monday, Tuesday, and Wednesday). All sessions took place late in the afternoon (4 to 6 pm). Each participant performed a supervised full-body resistance-training protocol involving a standardised warm-up followed by three circuits of one set of the following exercises: (i) box step-ups (ii) bench press, (iii) sit-and-stand from the box, (iv) bent-over row, (v) deadlift, (vi) alternate lunges, (vii) shoulder press, and (viii) leg extension. About 30 seconds rest between exercises and 3-min between circuits was allowed. As the workout aimed to create a high level of mechanical and metabolic stress, a muscle endurance training targeting 16 self-determined maximum repetitions (>40 to <60% 1RM) per set was designed (Ratamess et al, 2009). When participants were able to perform more than 16 repetitions per set, the load was increased between 2.5 and 5 kg. If fewer than 16 repetitions were completed, a rest period of ~10 s was allowed until the participants were able to reach the targeted number of repetitions per set. The time to complete the workouts was 49±8 min. After one hour from the completion of the last workout (W3), assessments of voluntary (CMJ, CMBT, 30STS, and 30BP) and evoked (TMG) muscular function were conducted, following the same protocol as used for the baseline assessment.

#### *4.2.2.7. Diet and supplementation*

Each participant completed a three-day food diary report (two weekdays and one weekend day) for three different time points: (i) first familiarisation week, (ii) first interventional week, and (iii) second interventional week. MyFitnessPal Inc.© (Version 2022, Texas, US) smartphone application, validated by Evenepoel et al (2020), was used to calculate the total (absolute and relative, in percentage) daily intake, and per-meal energy and macronutrient (proteins, carbohydrates, and fats) distribution. Participants were instructed to maintain their habitual diet throughout the study, including the washout period, and to avoid any caloric intake 2 hours after the training and assessment sessions. They were asked to report any minimal change regarding food composition and size, ingestion of supplements or compliance with the reported meals, including breakfast, lunch, pre- and post-workout food intake, and dinner. If any change had been detected (i.e., becoming vegetarian, restricting calories, fasting, taking additional nutritional supplements, etc.), that participant's data would have been excluded from the analysis.

During the 5-day training and testing periods (weeks 4 and 7), all participants consumed either one dose of a commercially available vegan protein-based multi-ingredient recovery supplement (3:1 RECOVERY + (PLUS) VEGAN, Crown Sport Nutrition, Spain) or an isoenergetic comparator (maltodextrin) (see Table 4.1).

**Table 4.1** Nutritional composition of supplements per intake mixed with ~400mL of plain water.

| **Description** | **Multi-Ingredient (POSTW) (100 g dose)** | **Comparator (COMP) (100 g dose)** |
|---|---|---|
| Energy value (kcal) | 365 | 364 |
| | | |
| **Macronutrients** | | |
| Total Fats (g) | 0.0 g | 0.0 g |
| - Saturated | 0.0 g | 0.0 g |
| Total carbohydrates (g) of which | 65.39 g | 90 g |
| - Maltodextrin | 32.99 g | 90 g |
| - Dextrose | 32.40 g | 0.0 g |
| Total Proteins (g) | 25.49 g | 1.1 g |
| - Pea protein | 17 g | 1.1 g |
| - Defatted cocoa powder | 8.49 g | 0.0 g |
| **COMPOSITION:** | | |
| Essential Amino Acids | 21.500 mg | 0.0 g |
| L-Leucine | 2.63 g | 0.0 g |
| L-glutamine | 1.43 g | 0.0 g |
| L-threonine | 0.4 g | 0.0 g |
| Clorhidrate L-lisine | 0.36 g | 0.0 g |
| L-isoleucine | 0.28 g | 0.0 g |
| L-methionine | 0.2 g | 0.0 g |
| Sodium | 0.2 g | 0.03 g |

The supplements under evaluation were presented in analogous white sachets of chocolate-flavoured powder to be dissolved in ~400 mL of room temperature plain water and administered within 15 minutes after each workout session and after post-24 hours assessments. Therefore, each participant ingested four doses per training-assessing week (8 in total). Even though the drinks were similar in appearance, texture, and taste, they were provided in exactly identical black and opaque bottle shakers to maximise the double-blinded procedure. The participants were instructed to ingest the next meal ~2 to 3 hours after supplement administration. A third investigator who was not involved in the data collection (AL) prepared and administered both supplements to all participants, increasing the double-blinding for the participants and the data collection researcher. No supplements were consumed on non-exercising days (e.g., weekends, and weeks 5 and 6) or after the post-48h assessments. Furthermore, to avoid possible confounding trial order effects, the conditions were tested following a balanced randomised order. Following the preintervention assessments, subjects were matched by body mass and maximal isometric force. Assignment of participants to treatments was performed by block randomisation using a block size of two and in a double-blind (POSTW or COMP) fashion.

### *4.2.3. Statistical Analysis*

A descriptive analysis was performed, and subsequently, the Kolmogorov-Smirnov and Shapiro-Francia tests were applied to assess normality. Raw changes in all outcome variables were calculated by subtracting pre-assessment from post-assessment values, without adjusting for pre-values, since the same subjects performed under both conditions acting as their own controls. To assess the magnitude of the differences from the baseline outcome, confidence intervals (CIs) of the differences were calculated and plotted. Those CIs not crossing zero were considered statistically significant from the baseline performance. Additionally, two-tailed one-sample Student's t-tests were used to test for a null effect hypothesis. Before testing the main hypothesis, the possible treatment order effect was checked using a 2 (order: POSTW-COMP vs. COMP-POSTW) x 2 (conditions: POSTW vs. COMP) analysis of variance (ANOVA). A 2 (conditions: POSTW vs. COMP) x 3 (times: after 1 hour, 24 hours, and 48 hours) repeated-measures ANOVA was used to compare differences between conditions and post-workout measurements in the raw change of lower body (countermovement jump, 30-seconds sit-to-stand) and upper body (chest medicine ball throw, 30-seconds bench press) mechanical power, and endurance. As TMG and DOMS were assessed at 1, 24 and 48 hours after completing the last training session, a 2 (conditions: POSTW vs. COMP) by 3 (times: 1, 24, and 48 hours) repeated-measures ANOVA was used. Differences over time were compared using Bonferroni-adjusted pairwise comparisons when appropriate. Generalized eta-squared (η2G) and Cohen's d values were reported to provide an estimate of standardized effect size (small $d$=0.2, $\eta^2_G = 0.01$; moderate $d$=0.5, $\eta^2_G = 0.06$; and large $d$=0.8, $\eta^2_G = 0.14$). The statistical significance level was set at 0.05. Results are reported as mean (SD) unless stated otherwise. All statistics were performed using the Statistical Package for the Social Sciences (SPSS for Windows, version 28.0.1.1 Windows; SPSS, Inc., Chicago, IL). An a priori power analysis was conducted using G*Power (version 3.1.9.7) to estimate the minimum sample size required to detect significant differences in the primary outcome variable, countermovement jump (CMJ) performance. Based on previous meta-analytic data, a large effect size (Cohen's d = 0.82) was anticipated following short-term neuromuscular interventions (Ramirez-Campillo et al., 2020). The analysis was set with an alpha level (α) of 0.05, a statistical power (1–β) of 0.80, and a two-tailed paired-sample t-test design, assuming a within-subjects comparison of pre- and post-intervention outcomes. Under these parameters, a minimum of 15 participants was required to detect significant changes in CMJ height. To ensure robustness against potential attrition or data loss, a total of 24 participants were recruited and included in the analysis.

## 4.3. Results

No participants were excluded or dropped out of the study after recruitment because of time constraints, personal affairs or other exceptional reasons. Therefore, twenty-four subjects successfully completed the entire intervention and testing sessions under both analysed conditions (Figure 4.2).

No order effect between treatments was observed ($p>0.05$) for any of the analysed variables: TMG markers: (Vc, Dm, and Tc), performance (CMJ, CMBT, 30STS, 30BP), and DOMS after 1-h, 24-h, or 48-h

### *Diet Analysis*

Daily absolute (in grams) and relative (percentage) macronutrients (carbohydrate, protein, and fat), and energy (kilocalories) consumption, without including and including both post-workout supplements are presented in Table 4.2.

**Table 4.2.** Descriptive analysis of the participants' diet composition in macronutrients

| Macronutrients | DIET pre (n = 24) | DIET + POSTW (n = 24) | DIET + COMP (n = 24) |
|---|---|---|---|
| **Proteins** | | | |
| **$g \cdot d^{-1}$** | 97.86 ± 28.1 | 112.99 ± 28.9$^{*\delta}$ | 98.96 ± 27.4$^{\delta}$ |
| **$g \cdot kg^{-1} \cdot d^{-1}$** | 1.24 ± 0.3 | 1.44 ± 0.4$^{*\delta}$ | 1.26 ± 0.3$^{\delta}$ |
| **% of total energy** | 16.7 ± 3.9 | 16.95 ± 3.6$^{\delta}$ | 14.6 ± 32.9$^{*}$ |
| **Carbohydrates** | | | |
| **$g \cdot d^{-1}$** | 251.39 ± 74.3 | 316.78 ± 73.1$^{*\delta}$ | 341.39 ± 72.2$^{*}$ |
| **$g \cdot kg^{-1} \cdot d^{-1}$** | 3.19 ± 1.3 | 4.03 ± 1.6$^{*}$ | 4.34 ± 1.2$^{*}$ |
| **% of total energy** | 44.3 ± 11.8 | 47.5 ± 12$^{*\delta}$ | 50.4 ± 10.1$^{*}$ |
| **Fats** | | | |
| **$g \cdot d^{-1}$** | 105.22 ± 27.6 | 105.22 ± 25.4 | 105.22 ± 25.3 |
| **$g \cdot kg^{-1} \cdot d^{-1}$** | 1.34 ± 0.4 | 1.34 ± 0.5 | 1.34 ± 0.6 |
| **% of total energy** | 39.8 ± 10.4 | 35.5 ± 10.1 | 35.0 ± 9.2 |
| **Energy** | | | |
| **Total daily energy** | 2344 ± 316 | 2708 ± 311$^{*}$ | 2708 ± 312$^{*}$ |
| **$Kcal \cdot kg^{-1} \cdot d^{-1}$** | 29.8 ± 4.8 | 34.4 ± 5.8$^{*}$ | 34.4 ± 5.9$^{*}$ |

Notes: Values are presented as mean ± standard deviation

$^{*}$p<0.01 respect to diet without post-workout supplementation.

$^{\delta}$p<0.01 from diet with REC supplementation compared to diet with CHO supplementation.

The ingestion of ~100g dose of the POSTW multi-ingredient determined significant increases in total daily protein and carbohydrate intake whereas, the ingestion of the COMP supplement significantly increased the total daily carbohydrate ingestion, with no changes in fat and protein daily intake.

### *4.3.1. Primary Outcomes (Performance)*

The mean ± standard deviation and 95% CI of the differences measured at 24 and 48 h for the performance variables (CMJ, CMBT, 30STS, and 30BP) assessed are presented as Supplementary Material (S4 – Table S2)

#### *4.3.1.1. Vertical Jump (cm)*

Compared to baseline assessment, a significant performance decline was observed for both POSTW and COMP after 1 hour (-1.33±1.5, *p*<0.05; and -1.33±1.3, *p*<0.05; respectively) from the last workout (Figure 3A). However, post-24 (+0.46±1.1; and

+0.17±1.2, $p>0.05$; respectively) and post-48 (+0.57±1.4; and +0.77±1.3, $p>0.05$; respectively) hours evaluations revealed no significant differences from baseline. or from post-24 to post-48 hours ($p>0.05$).

Compared to baseline assessment, a significant performance decline was observed for both POSTW and COMP after 1 hour (-1.33±1.5, $p<0.05$; and -1.33±1.3, $p<0.05$; respectively) from the last workout (Figure 3A). However, post-24 (+0.46±1.1; and +0.17±1.2, $p>0.05$; respectively) and post-48 (+0.57±1.4; and +0.77±1.3, $p>0.05$; respectively) hours evaluations revealed no significant differences from baseline or from post-24 to post-48 hours ($p>0.05$).

Significant main effects for time were observed (F[2,70] = 16.20, $p < 0.001$, $\eta^2 = 0.596$), indicating differences across the time points. However, no significant main effect for condition was detected (F[1,23] = 0.606, $p = 0.453$, $\eta^2 = 0.052$). The interaction between condition and time was also not significant (F[2,70] = 2.484, $p = 0.106$, $\eta^2 = 0.184$), suggesting that the changes over time were not different between conditions.

### *4.3.1.2. Chest Medicine Ball Throw (m)*

Compared to baseline, a significant decrease in performance was observed under both POSTW and COMP conditions after 1 hour (-0.10±0.2, $p<0.05$; and -0.10±0.2, $p<0.05$; respectively) from the third training session (Figure 3B). However, no significant differences were revealed from baseline to post-24 (+0.01±0.1; and -0.05±0.1, $p>0.05$; respectively) and to post-48 (+0.06±0.2; and +0.02±0.1, $p>0.05$; respectively) time points ($p>0.05$).

Significant main effects for time were observed (F[2,70] = 7.685, $p = 0.003$, $\eta^2 = 0.411$), indicating differences across the three time points. However, no significant main effect for conditions was detected (F[1,23] = 1.321, $p = 0.275$, $\eta^2 = 0.107$). The interaction between conditions and times was not significant (F[2,70] = 2.816, $p = 0.081$, $\eta^2 = 0.204$), suggesting that the changes over time did not significantly differ between conditions.

#### *4.3.1.3. 30-Second Sit-to-Stand*

Compared to baseline assessment, no significant performance decline was observed for either POSTW or COMP after 1 hour (-0.42±1.6, $p>0.05$; and -0.38±1.6, $p>0.05$; respectively) from the last workout (Figure 3C), or 24 hours (+0.46±1.1; and +0.17±1.2, $p>0.05$; respectively). However, post-48 hours assessment (+2±2, $p<0.05$; and +2±1.7, $p<0.05$; respectively) revealed a significant difference from baseline or from pre- to post-48 hours ($p<0.05$).

The analysis revealed a significant main effect of time ($F[2,70]= 27.40$, $p < 0.001$, $\eta^2 = 0.59$), indicating significant differences in performance across the different time points. However, there was no significant main effect of condition ($F[1,23] = 0.44$, $p = 0.52$), suggesting that both conditions did not significantly differ in their effects on the number of repetitions achieved. Furthermore, the interaction between condition and time was not significant ($F[2,70] = 0.89$, $p = 0.42$), indicating that the changes over time were similar under both conditions.

#### *4.3.1.4. 30-Seconds Bench Press*

Compared to baseline, a significant reduction of the number of completed repetitions was observed for both POSTW and COMP after 1 hour (-1.33±1.5, $p<0.05$; and -1.33±1.3, $p<0.05$; respectively) from the last workout (Figure 3A). However, at 24 hr (+0.46±1.1; and +0.17±1.2, $p>0.05$; respectively) and post-48 hr (+0.57±1.4; and +0.77±1.3, $p>0.05$; respectively) no significant differences ($p>0.05$). to baseline and between times (post-24 vs. post-48 hours) were determined.

The analysis revealed a significant main effect of time ($F[2,70] = 8.95$, $p = 0.001$, $\eta^2 = 0.45$), indicating differences across time points. However, no significant main effect of condition was observed, ($F[1,23] = 0.008$, $p = 0.932$, $\eta^2 = 0.001$), suggesting no difference between the POSTW and COMP conditions. Additionally, the interaction between condition and time was not significant, ($F[2,70] = 0.252$, $p = 0.779$, $\eta^2 = 0.022$), indicating that changes over time were similar between conditions.

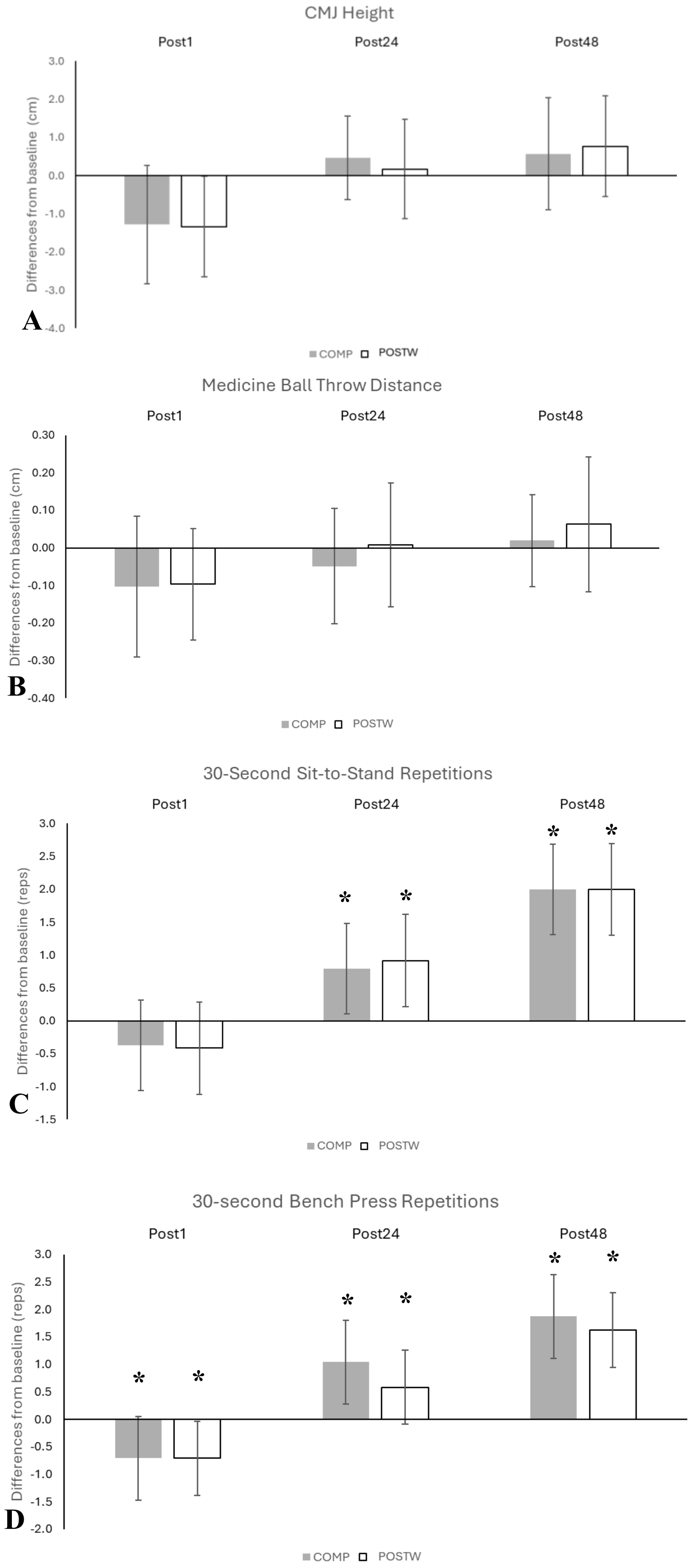


**Figure 4.3** Estimated marginal means and 95% confidence intervals of differences in vertical jump height (A), chest medicine ball throw distance (B), 30-second sit-to-stand (C), and 30-second bench press (D) exercises. *$p < 0.05$ from the baseline values. COMP: Isocaloric, carbohydrate-only condition; POSTW: Post-workout multi-ingredient condition.

### *4.3.2. Secondary Outcomes*

Table 4.3 summarises the changes measured at 1, 24 and 48 hours after performing the last resistance training workout of the TMG Vc for the three different muscles (AD, VM, and BFLH) analysed, including the 95% CI for both (POSTW and COMP) conditions.

#### *4.3.2.1. Tensiomyography*

Compared to baseline, a significant performance decline was observed in AD Vc for both POSTW and COMP after 1 hour (-0.006±0.04, $p<0.05$; and -0.006±0.04, $p<0.05$; respectively) from the last workout. However, post-24 (-0.015±0.03; and +0.03±0.03, $p>0.05$; respectively) and post-48 (+0.003±0.06; and +0.002±0.04, $p>0.05$; respectively) hours evaluations revealed no significant differences from baseline.

The results of the AD Vc repeated measures ANOVA revealed no significant main effect for condition ($F[1,23] = 0.000$, $p = 0.983$, $\eta^2 = 0.000$), indicating that there were no differences between the two treatment conditions across time. Additionally, no significant main effect for time was observed ($F[2,70] = 1.795$, $p = 0.190$, $\eta^2 = 0.140$), suggesting that performance did not significantly vary across the different time points (post-1h, post-24h, and post-48h).

Furthermore, the interaction effect between condition and time was not significant ($F[2,70] = 1.327$, $p = 0.286$, $\eta^2 = 0.108$), indicating that the changes over time were similar between the two conditions.

Compared to baseline, a significant performance decline was observed in VM Vc for both POSTW and COMP after 1 hour (-0.007±0.03, $p<0.05$; and -0.006±0.03, $p<0.05$; respectively) from the last workout. However, post-24 (+0.006±0.02; and +0.004±0.03, $p>0.05$; respectively) and post-48 (+0.002±0.02; and +0.003±0.03, $p>0.05$; respectively) hours evaluations revealed no significant differences from baseline.

The repeated measures ANOVA revealed no significant main effect for condition ($F[1,23] = 0.034$, $p = 0.856$, $\eta^2 = 0.003$), indicating no difference between the two conditions across time. Similarly, no significant main effect for time was observed ($F[2,70] = 1.749$, $p = 0.197$, $\eta^2 = 0.137$), suggesting no significant changes over the time points (post-1h, post-24h, post-48h).

Additionally, the interaction between condition and time was not significant (F[2,70] = 0.020, p = 0.980, $\eta^2 = 0.002$), indicating that the performance changes over time were similar under both conditions.

Compared to baseline, no significant performance decline was observed in BFLH Vc for both POSTW and COMP after 1 hour (-0.004±0.03; and -0.001±0.03, $p>0.05$; respectively) or post-24 (+0.003±0.02; and +0.001±0.02, $p>0.05$; respectively) from the last workout. However, post-48 hours (+0.007±0.02; and +0.006±0.02, $p<0.05$; respectively) evaluation revealed a significant difference from baseline assessment.

The results of the repeated measures ANOVA indicated a significant main effect of time (F[2,70] = 5.040, p = 0.016, $\eta^2 = 0.314$), suggesting differences in performance across the three time points. However, there was no significant main effect for condition (F[1,23] = 1.029, p = 0.332, $\eta^2 = 0.086$), indicating that the two treatment conditions did not differ overall.

No significant interaction between time and condition was found (F[2,70] = 0.002, p = 0.998, $\eta^2 = 0.000$), suggesting that the performance changes over time were similar between the two conditions.

#### *4.3.2.2. Delayed Onset Muscle Soreness*

Both conditions showed a significant DOMS increase before the third training session ($p<0.001$), and at both 24 hours ($p < 0.001$) and 48 hours ($p < 0.001$) after completing the last training session.

### ***4.3.3. Exploratory Outcomes***

#### *4.3.3.1. Tensiomyography Muscle Belly Radial Displacement and Contraction Time*

Both conditions exhibited a significant reduction in Dm and Tc after the third training session ($p < 0.05$), with no significant differences between groups ($p >0.05$). However, involuntary muscle properties returned to baseline values after 24 hours and remained stable through the 48-hour post-exercise period, with no differences between supplementation protocols or time points ($p > 0.05$). Thus, both supplementation strategies promoted comparable recovery patterns following the final training session. No between-sexes differences were identified for any of the outcomes assessed. Both sexes similarly responded under the two different conditions for the performance and tensiomyography values after 1h, 24h and 48h.

**Table 4.3.** Mean (M) ± standard deviation (SD) and 95% CI of the differences measured at 24 and 48 h for the tensiomyography variables in the two assessed conditions.

| **Conditions** | **POSTW (n=10)** | | | **COMP (n=10)** | | | **ANOVA Repeated Measures (3 times x 2 conditions)** | **Conditions Comparisons** | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Variables** | **Post-1h** | **Post-24h** | **Post-48h** | **Post-1h** | **Post-24h** | **Post-48h** | | **1h** | **24h** | **48h** |
| **Anterior Deltoids Vc (m.s$^{-1}$)** | -0.01±0.05<br>[-0.02, 0.01] | -0.01±0.06<br>[-0.03, 0.02] | 0.00±0.06<br>[-0.03, 0.03] | -0.01±0.04<br>[-0.03, 0.01] | 0.00±0.04<br>[-0.02, 0.02] | 0.00±0.04<br>[-0.02, 0.02] | Time: $F(2,22) = 1.92$; $p = 0.20$; $\eta^2_G=0.28$<br>Time x Condition: $F(2,70) = 1.31$; $p = 0.31$; $\eta^2_G=0.21$<br>Condition: $F(1,23) = 0.01$; $p = 0.98$; $\eta^2_G=0.001$ | $p = 0.80$<br>ES = 0.05 | $p = 0.66$<br>ES = 0.14 | $p = 0.93$<br>ES = 0.02 |
| **Vastus Medialis Vc (m.s$^{-1}$)** | -0.01±0.03<br>[-0.02, 0.00] | 0.01±0.02<br>[-0.01, 0.01] | 0.00±0.02<br>[-0.01, 0.01] | -0.01±0.03<br>[-0.02, 0.01] | 0.00±0.03<br>[-0.01, 0.02] | 0.00±0.03<br>[-0.01, 0.01] | Time: $F(2,22) = 1.17$; $p = 0.35$; $\eta^2_G=0.19$<br>Time x Condition: $F(2,70) = 0.16$; $p = 0.98$; $\eta^2_G=0.01$<br>Condition: $F(1,23) = 0.34$; $p = 0.86$; $\eta^2_G=0.01$ | $p = 0.69$<br>ES = 0.05 | $p = 0.79$<br>ES = 0.09 | $p = 0.94$<br>ES = 0.01 |
| **Biceps Femoris Long Head Vc (m.s$^{-1}$)** | 0.00±0.02*<br>[-0.01, 0.01] | 0.00±0.02<br>[-0.01, 0.01] | 0.01±0.02<br>[0.00, 0.01] | 0.00±0.03*<br>[-0.01, 0.01] | 0.00±0.02<br>[-0.01, 0.01] | 0.01±0.02<br>[0.00, 0.01] | Time: $F(2,22) = 20.17$; $p = 0.001$; $\eta^2_G=0.80$<br>Time x Condition: $F(2,70) = 0.01$; $p = 0.99$; $\eta^2_G=0.01$<br>Condition: $F(1,23) = 1.03$; $p = 0.33$; $\eta^2_G=0.09$ | $p = 0.65$<br>ES = 0.12 | $p = 0.54$<br>ES = 0.15 | $p = 0.96$<br>ES = 0.03 |

Notes: CI = Confidence Interval; ANOVA = Analysis of Variance.
ES is the Standardised Effect Size presented as Cohen's.
*p < 0.05 and $^{T}$p <0.10 respect to baseline values.

### 4.4. Discussion

Our findings indicated that when compared to the consumption of an isoenergetic carbohydrate supplement, a post-workout multi-ingredient formulation containing 17g of pea protein, ~8.5g of cocoa protein, and ~65.4g of carbohydrates (33g of maltodextrin and 32.4g of dextrose) did not confer any additional advantages in promoting the recovery of both voluntary and involuntary muscle function. Furthermore, no significant differences were observed in the perception of delayed onset muscle soreness (DOMS) at 24 and 48 hours following the completion of three consecutive high-intensity resistance training sessions.

One hour after completing the final training session, participants exhibited a generalised depletion of energy, as indicated by a decline in voluntary performance across all assessed tests. Both lower body (CMJ) and upper body (CMBT) mechanical power output, along with lower (30-second sit-to-stand) and upper body (30-second bench press) muscular endurance tests showed similar performance reductions at 1-hour post-exercise, with no differences between conditions. After 24 hours, performance in all tests returned to baseline levels, indicating a generalised voluntary contractile properties restoration, as no significant differences were observed between baseline and post-24-hour results ($p > 0.05$). Moreover, no differences in performance outcomes were detected between supplementation conditions at both the 24-hour and 48-hour post-training assessments. At 24 hours after having finished the last resistance training session, both conditions significantly suffered a decline in performance and muscle contractile properties (TMG) except for BFLH Vc. Nonetheless, no additional statistical differences were identified for any of the performance or the TMG outcomes at any time point. Moreover, when inter-individual differences from baseline results were analysed, no differential recovery effect was observed after POSTW or COMP intake in voluntary (Figures 4.3A-D) or involuntary contraction (Table 4.3). Therefore, as no recovery hastens under any condition was identified, our results suggest that regardless of the treatment (POSTW or COMP), all participants followed a similar pattern of recovery after performing three consecutive hard resistance training sessions.

These findings are consistent with previous research that has shown that while multi-ingredient supplements can accelerate recovery processes such as glycogen replenishment and muscle protein synthesis, their effects on immediate post-exercise performance restoration remain uncertain (Alghannam et al., 2018). Moreover, including intra-session or post-workout aiming at reducing muscle soreness is still a conflictive question, as inconsistent results have been observed in previous investigations (Pearson, Hind, Macnaughton, 2023).

However, previous investigations are opposite to our findings. An accelerated recovery process after consuming a combination of 38g (~0.45–0.60g·kg$^{-1}$) of CHO, and 18g (~0.20–0.30g·kg$^{-1}$) of high-quality protein from whey isolate and beef hydrolysate (Naclerio et al., 2020), or 31g (~0.30-0.50g·kg$^{-1}$) of CHO and 30g (~0.30-0.50g·kg$^{-1}$) of rice protein (Naclerio et al., 2021) were observed in highly trained young males. In the current study, the post-workout admixture provided ~65.4g (~0.6-0.93g·kg·BM$^{-1}$) of CHO and 25.49g (~0.24-0.42g·kg$^{-1}$) of protein, of which 17g were from pea. Additionally, the total energy provided was ~365kcal for both supplementation protocols. Compared to 222kcal (Naclerio et al., 2020), and 273kcal (Naclerio et al., 2021), the total energy difference and overall post-exercise CHO intake might have played a key role in the recovery process of muscle contractile properties by accelerating glycogen replenishment and additional physiological endocrine and musculoskeletal interactions (Burke et al., 2011; Fritzen et al., 2019). Additionally, similar outcomes on glycogen resynthesis have been observed after either CHO-only or a combination of CHO, fats and proteins ingested post-exercise, suggesting a potential contribution of other macronutrients as gluconeogenic precursors, thereby increasing substrate availability (Bird et al., 2024). However, the results of this current investigation align with the ones obtained by Nieman et al. (2020), who did not observe a similarity in whey and pea post-exercise training muscle damage markers, therefore suggesting that a higher dose of pea compared to whey, or a leucine-enriched pea protein supplement should be provided when aiming at maximising post-exercise recovery.

Herein, potential speculations might arise after finding controversial outcomes. Firstly, the possibility of protein quality (leucine content only 2.63g) not meeting the minimum recommended 3g for post-exercise muscle protein synthesis and recovery stimulation processes (Devries et al., 2018). Secondly, the likely stimulation impairment due to a reduced total protein quantity content of the blend, as even with a greater leucine content, dosages of ≥30 g have been suggested to be required by older adults to strongly increase MPS (Yang et al., 2012c) due to a slower recovery process in older adults after heavy-exercise compared to younger participants (Hayes, et al. 2023) due to anabolic resistance among other molecular bases (Li et al., 2024), which were included in the previously cited investigations. Thirdly, pea protein, even rich in amino acids and considered a high-quality protein, is low in methionine and cysteine and has shown a lower digestibility compared to animal proteins like whey or casein (Shanthakumar, 2022). Additionally, only four investigations have compared the effect of pea protein vs whey (Babault et al., 2015; Banaszek et al., 2019; Loureiro et al., 2023; Singh et al., 2024) on different markers. However, none of them have included pea protein as part of a multi-ingredient admixture, compared to isocaloric CHO-only contrast. On the one hand, Babault et al. (2015) evaluated elbow flexors strength and muscle thickness, after 12 weeks, including 2 daily doses containing ~25g of protein and a total of ~165kcal each dose, finding similar results under both conditions (Babault et al., 2015). Nevertheless, the participants recruited for this investigation were young males not engaged in resistance training or regular activity. On the other hand, Banaszek et al. (2019) evaluated body composition, muscle thickness, and strength in trained and experienced young adults after 8 weeks of 4 functional training sessions per week, providing 2 daily doses (pre- and post-exercise on training days) of ~24g of protein (either pea or whey). The results of this investigation were similar to Babault et al. (2015), as no differences were identified between groups for any of the parameters assessed. Contrarily, Loureiro et al. (2023) assessed biochemical and metabolic parameters (such as creatine kinase, lactate, urea or creatinine) in a crossover randomised controlled trial (RCT), using nuclear magnetic resonance spectroscopy after 10 days of daily supplementation (0.5 $g \cdot kg \cdot BM^{-1}$ of protein). However, these participants were very young adults and high-level footballers (~18 years old) with a training level of 2 hours per day, 6 days a week. Moreover, the test performed to compare between conditions was a football match. Finally, only one investigation (Singh et al., 2024) compared the effects of pea vs whey protein in middle-aged adults (30-59 years old). However, the participants recruited were sedentary, with no previous experience in resistance training. Thus, participants ingested one daily dose of ~21g of either pea or whey protein (post-exercise during training days: six 30-minute resistance training sessions per week) for 84 days of intervention. This study concluded that pea protein may be considered a viable alternative to whey protein without sacrifices in lean body mass improvements (Singh et al., 2024). Nevertheless, no investigations have compared the inclusion of pea protein as part of a multi-ingredient admixture or investigated the potential benefits of this protein type compared to carbohydrates on recovery markers.

As depicted by Table 4.2, at the beginning of the study, the participants of the present research were ingesting an average daily protein intake ~1.2g·kg$^{-1}$, which is around the minimum recommended amount to stimulate muscle function and preservation (Deutz et al., 2014), but in any case far below the optimal daily intake (~1.5 g·kg$^{-1}$) required for the older population to maintain bone musculoskeletal health and function (Wolfe, Miller & Miller, 2008). The ingestion of the POSTW still was slightly below the optimal daily protein requirements (1.4 vs. 1.5g·kg$^{-1}$) for active older populations engaged in regular resistance training looking for muscle and health gains (Deutz et al., 2014). Additionally, the added protein provided in the POSTW condition might not have been sufficient to stimulate further anabolic responses in middle-aged adults who are already consuming adequate protein (Ruijven et al., 2023). Moreover, our recent systematic review (Puente-Fernández et al., 2023) summarised the findings of 9 RCT in middle-aged and older adults consuming either a protein-based multi-ingredient supplement or an isocaloric CHO supplement on functional capacity, strength and body composition after a minimum interventional period of 6 weeks. The results concluded that current literature was still scarce in middle-aged adults and the evidence controversial when aiming at supporting post-workout multi-ingredient vs isoenergetic CHO supplementation in this population.

The present study is not without limitations that must be considered when interpreting the results. First, middle-aged men and peri-menopausal women between 45 and 55 years old were exclusively recruited. Given the unique hormonal and physiological changes occurring during this life stage, our findings may not be generalisable to younger women, post-menopausal women, or males. Second, dietary intake was assessed using self-reported food diaries. While widely used in nutritional research, this method may lack accuracy due to potential reporting biases and errors in portion size estimation, potentially affecting the reliability of our dietary data. These findings are primarily applicable to healthy, recreationally active middle-aged adults participating in resistance training who have an average daily protein intake of ~1.2 g·kg·BM$^{-1}$. Two plausible interpretations emerge from these results: either the vegan protein source (even one of high quality such as pea protein) may not elicit comparable outcomes to those observed with animal-based proteins unless consumed at higher doses (Church et al., 2024), or when protein intake falls below the recommended 1.6 g·kg·BM$^{-1}$, total energy availability may play a more critical role in optimising post-exercise recovery than the specific macronutrient composition (Alghannam et al., 2018). To elucidate this further, future research should explore the effects of higher protein dosages or adjust intake according to individualised nutritional needs to better assess the efficacy of plant-based proteins in enhancing recovery and adaptation.

## 4.5. Conclusions

In conclusion, our investigation does not support the notion that ingesting a post-workout pea-protein-based multi-ingredient provides additional benefits for the recovery of muscle contractile properties compared to carbohydrate supplementation alone when the energy intake is matched. Further research may be necessary to explore different nutrient combinations, dosages or supplementation strategies to enhance muscle recovery in this population.

# CHAPTER 5

# STUDY 4 – PRE-WORKOUT MULTI-INGREDIENTS OR CARBOHYDRATE ALONE PROMOTE SIMILAR RESISTANCE TRAINING OUTCOMES IN MIDDLE-AGED ADULTS: A DOUBLE-BLIND, RANDOMIZED CONTROLLED TRIAL.

## 5.1. Introduction

Multi-ingredient pre-workout formulations (PREW) represent a wide category of dietary supplements with purported efficacy in enhancing physical performance (Jagim et al., 2016). Previous interventions demonstrated valuable effects of PREW on exercise outcomes including strength (Beyer et al., 2024), power output (Jagim et al., 2016; Panayi & Galbraith, 2022), muscular endurance (Cameron et al., 2018) and gaining muscle mass (Smith et al., 2010; Harty et al., 2018; Cabre et al., 2022). In this context, caffeine has arisen as the predominant ingredient due to its thermogenic (Astrup et al., 1990) and ergogenic effect on both endurance (Jagim et al., 2016; Outlaw et al., 2016; Harty et al., 2020), and strength (Quesnele et al., 2014) performance. For instance, the co-administration of caffeine with yerba mate containing caffeoyl derivatives such as chlorogenic acid, phytosterols, and saponins, promoted fat metabolism (Alkhatib, 2014), increased fatty acid oxidation, and reduced the perception of effort during low-intensity endurance exercise (Alkhatib et al., 2015).

In addition to caffeinated substances, some commercially available PREW include high-quality proteins, such as whey fortified with amino acids (AA) (e.g., L-leucine, L-arginine, L-tyrosine, or L-taurine) or their derivatives (e.g., citrulline-malate, betaine, or L-carnitine) that may act synergistically with caffeine to enhance muscular efficiency and extend the onset of fatigue (Giannesini et al., 2011; Puente-Fernández et al., 2020). For example, L-carnitine is a conditionally essential AA derivative that plays a role in fatty acid metabolism. It may promote vasodilation and improve oxygen supply to the working muscles (Flanagan et al., 2010). L-arginine, a conditionally essential AA in adults, serves as a precursor for creatine, a component of the body's energy metabolism (Tapiero et al., 2002). L-citrulline is a non-essential and non-proteogenic AA which promotes vasodilation, rate of oxidative ATP production and phosphocreatine recovery after high-intensity exercise (Bendahan et al., 2002).

Recent studies have reported beneficial effects of L-citrulline or citrulline-malate supplementation in maximising strength, power output, and muscle endurance in both recreationally active and trained athletes (Trexler et al., 2019; Gonzalez & Trexler, 2020; Vårvik et al., 2021). Furthermore, combining L-arginine and L-citrulline has been proposed as an effective nutritional intervention to promote endogenous synthesis of nitric oxide (NO) which may optimise the removal of metabolic waste products, attenuate fatigue (Park et al., 2023), and enhance physical performance (Nyawose et al., 2022). The ingestion of L-tyrosine has been proposed to improve prolonged submaximal exercise in the heat, along with lower perceived exertion (Tumilty et al., 2011). L-taurine supplementation has also been associated with improved muscular endurance (De Luca et al., 2015). Betaine, a glycine aminoacidic derivative, favours muscle blood flow by elevating the levels of NO and promoting fluid and thermal homeostasis (Hoffman et al., 2009; Harty et al., 2018). Individually, or in combination, it is therefore feasible that such nutrients may have beneficial effects when consumed within a PREW formula.

Furthermore, carbohydrate (CHO) administration before workouts is widely accepted as a key dietary strategy to ensure the availability of circulating glucose to support limited muscle fuel stores during intense and prolonged steady or intermittent exercise (Karelis et al., 2010; Burke, 2021; Malone et al., 2021). Indeed, PREW including caffeine and CHO have been reported to promote faster intestinal absorption and increased exogenous CHO oxidation rates during exercise (Baur & Saunders, 2021). However, divergent conclusions concerning the advantages of PREW supplementation in healthy middle-aged and older adults have also been noted in the literature (Nabuco et al., 2018; Sugihara Junior et al., 2018; Schwarz & McKinley-Barnard, 2020; Beckner et al., 2022). Currently, there is a paucity of research regarding the use of PREW supplements to maximise exercise training adaptations in middle-aged and older physically active adults (Giráldez-Costas et al., 2023).

The aim of this study therefore was to compare the effectiveness of combining a 6-week resistance training (RT) programme with a commercially available PREW including caffeinated ingredients and plant-based protein extracts (Crown Sport Nutrition, Spain) vs. an isocaloric, carbohydrate-only supplement comparator (COMP) on body composition, muscle thickness, and physical performance in middle-aged, healthy, physically active individuals. Additionally, considering that females and males may show distinct benefits from physical exercise (Ji et al., 2024), exhibit differential molecular responses (O'Bryan et al., 2022), and training-induced adaptations to resistance exercise programmes (Jones et al., 2021), explored differences in the intervention-induced outcomes among female and male participants were also explored. Based on the literature, it was hypothesized that ingesting PREW over a 6-week RT programme would maximise fat loss, fat-free mass gain, and muscular hypertrophy, along with a more favourable performance enhancement effect than COMP alone.

## 5.2. Material and Methods

### *5.2.1. Experimental design*

The intervention followed a double-blinded, randomized, parallel-group controlled trial design. Ethical approval was granted by FREC-EHHS-21-2-23-03. Following the inclusion criteria, familiarization period, and baseline assessments, participants were randomly allocated to receive either a vegan protein-based multi-ingredient supplement (PREW) or an isocaloric, carbohydrate-only comparator (COMP). Primary outcomes were changes in body composition (fat mass, fat-free mass and waist circumference) and muscle thickness from vastus lateralis and elbow flexor. Changes in isometric strength, vertical jump, medicine ball throw, and 30-second continued repetition tests in sit-and-stand and bench press exercises were considered secondary outcomes. Additionally, the 15-to-20-minute post-workout global rating of perceived exertion (S-RPE) from the OMNI-RES (0-10) scale was considered an exploratory variable. All tests were performed at baseline and follow-up at 6 weeks.

### *5.2.2. Participants*

Forty-four healthy and recreationally active, middle-aged, and older adults (26 peri- and post-menopausal females and 17 males; age: 53 ± 5 years) were initially recruited. The inclusion criteria required participants to have a minimum regular resistance training history of 6 months before the beginning of the study and to be 45 years of age or older. Female participants were additionally required to be post- or peri-menopausal, exhibiting at least two symptoms of menopause onset, such as hot flushes, menstrual cycle alterations, and not menstruating for more than 1 year (NICE, 2019). Participants were not eligible if suffering from acute illness or chronic diseases (including obesity [BMI

≥30kg/m$^2$] (Haase et al., 2021), metabolic syndrome, long COVID-19, osteoporosis, or sarcopenia), following a medication prescription, or consuming supplements or medications that could interfere with our research or affecting exercise performance (i.e., creatine, protein amino-acids, NSAIDs, etc.).

All participants confirmed verbal compliance prior to providing written informed consent. All experimental procedures were conducted in accordance with the Declaration of Helsinki and registered with ClinicalTrials.gov, U.S. National Institutes of Health (Identifier: NCT05769088).

To assess the statistical power of the study, a sensitivity analysis of the final sample size (PREW, N = 22 and COMP, n = 19) was conducted to detect statistically significant differences between conditions in pre-post differences. Assuming a t-test model with two independent samples to compare adjusted means, 0.05 $\alpha$ error probability, and 0.80 power ($1 - \beta$), it was determined that differences between conditions could be detected with a Cohen's d above 0.89.

### *5.2.3. Procedures*

After inclusion and before the baseline assessment, participants performed eight sessions of familiarization on alternate days, aimed at minimizing any potential learning effects of the training procedures. Following the initial assessment, participants were matched by body mass and isometric strength. Assignment of participants to treatments was performed by block randomisation using a block size of two and in a double-blind (PREW or COMP) manner.

All pre- and post-intervention assessments were conducted in a single session conducted the week before and separately after the 6-week intervention period, at approximately the same time of the day and under the same conditions (i.e., the morning before the first training session, after an overnight fast). Participants were required to refrain from any hard exercise sessions 48 hours before the assessments. Furthermore, participants abstained from consuming food or beverages for 3 hours before the assessment sessions and from ingesting energy sources within 2 hours before the workouts. The pre-and post-intervention evaluation encompassed the following components in the specified order: (i) body composition, (ii) muscle thickness, and (iii) physical performance.

#### *5.2.3.1. Assessments*

##### *Body Composition*

Body mass (BM) and height were assessed according to the methods described by Ross & Marfell-Jones (1991). Height was measured in a stretched standing position to the nearest 0.01 m using a wall-mounted stadiometer (Seca GmbH, Hamburg, Germany), and BM was corrected to the nearest 0.01 kg using a digital scale (Seca GmbH, Hamburg, Germany). Whole body densitometry using air displacement via the Bod Pod (Life Measurements, Concord, CA) was used in accordance with the manufacturer's instructions for the assessment of body composition measures as detailed elsewhere (e.g., avoid strenuous exercise for at least 24 hours, no eating or drinking for at least 2 hours prior to the assessment) (Dempster & Aitkens, 1995).

##### *Waist and Hip Circumferences*

The circumferences of the waist and hip were assessed using a stretch-resistant measuring tape and following o the methodologies described by Stewart et al. (Stewart et al., 2011). Subsequently, the waist-to-hip ratio was calculated by dividing the waist circumference by the hip circumference. To minimize inter-rater variability and ensure measurement consistency, a single trained researcher performed all assessments.

##### *Muscle Thickness*

Muscular thickness, changes under relaxed and static conditions were evaluated using a real-time B-mode ultrasound imaging system (Philips Affiniti 70 Ultrasound, Philips Corporation, USA). At each marked site, an 18-5 MHz broadband linear-array transducer, along with water-soluble transmission gel (Aquasonic 100 Ultrasound Transmission gel), was positioned perpendicular to the skin surface and parallel to the longitudinal axis of the muscle, providing acoustic coupling during the test without depressing the dermal surface (Naclerio et al., 2019b). Following the methodology outlined by Bradley and O'Donnell (Bradley & O'Donnell, 2004), as described by Naclerio et al. (2019a), the same qualified and skilled researcher conducted all measurements using a standardized protocol. Muscle thickness of the elbow flexors (EF) and the vastus lateralis (VL) were assessed in the dominant limbs. Muscle thickness was determined as the distance between the superficial and deep muscle aponeuroses for the VL, or between the superficial aponeurosis of the muscle and the muscle-bone boundary for the EF. Figure 5.1. illustrates examples of ultrasonography images depicting measurement sites for muscle architecture in EF and VL.

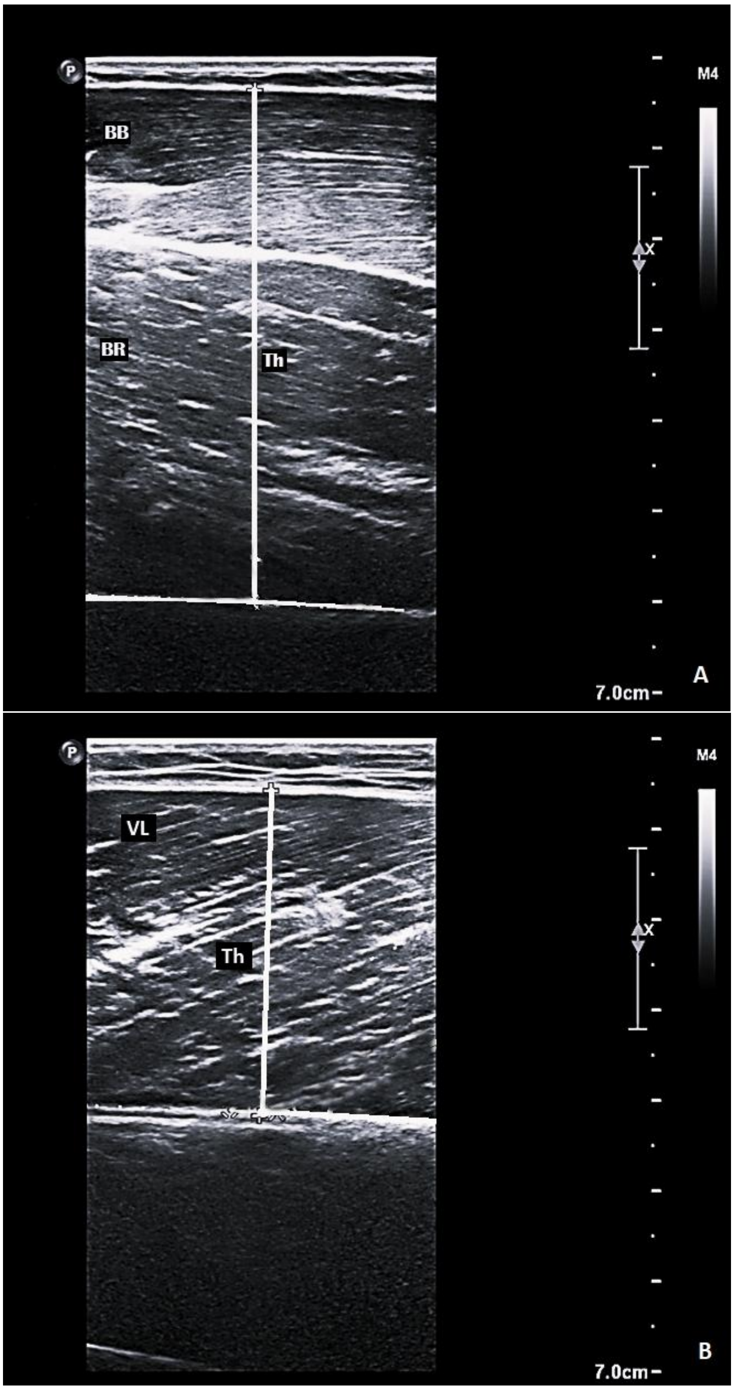


**Figure 5.1.** Ultrasonography images depicting measurement results for muscle thickness in EF (A) and VL (B) muscles.

To measure the thickness of the EF, participants were seated on a chair, with their torso straight and relaxed against the backrest. The assessed arm was maintained in a relaxed position at a 90° angle at the elbow joint on a bed, with the forearm in a relaxed pronated position. For VL thickness, participants were placed barefoot in a semi-recumbent and relaxed position on a bed set at 125°, with fully extended and relaxed knees and arms resting alongside the body. The EF assessment site was accurately located and marked at 80% of the distance between the coracoid process of the scapula and the medial epicondyle of the humerus. The VL assessment point was marked at 60% of the distance between the greater trochanter and the lateral condyle of the femur. To eliminate tissue distortion caused by excessive compression, the transducer was lightly rested on the skin surface, the ultrasound image on the screen was visually monitored, and participants were asked to provide verbal feedback on the pressure experienced on the skin. Three images were obtained at each location, and the median of the measurements was calculated and used for the analysis. To ensure accurate replication of the measurement location, the position of the probe was recorded on acetate paper, and pre- and post-intervention images were compared based on identifiable markings (such as moles and small angiomas) on the skin surface as reference points. This process increased the reliability of repeated measures. To prevent osmotic fluid shifts that could distort measurements of angle and thickness (Stasinaki et al., 2018), images were acquired at least 48 hours after the last training session and prior to the physical performance tests. Intra-rater reliability of muscle thickness measurements was assessed by a single trained researcher, who performed measurements on the same scan, demonstrating excellent interclass correlation coefficients (ICC) (>0.90).

### *Isometric Mid-Thigh Pull Test*

A T.K.K. 5402 dynamometer (Takei Scientific Instruments Co. Ltd., Niigata, Japan) with a base of 31.5 X 31.5 cm, equipped with a chain (51 cm), and a latissimus pulldown bar (120 cm; Perform Better, United Kingdom), was used to evaluate full-body maximal isometric force (MIF) (Till et al., 2018). Participants started in a standing position, on the foot grips, adjusting the chain length to position the bar slightly above their knees and gripped the bar without using straps. Before pulling, they were instructed to maintain tension on the chain to avoid jerking movements. Subsequently, participants exerted maximum force while pulling upwards (Haff et al., 2005). Three attempts of 5 seconds with a 30-second rest between each attempt were performed. The highest recorded value in kg of force (kgF) was selected for further analysis. Additionally, an excellent ICC (>0.90) was observed for this outcome.

### *Vertical Jump*

A countermovement jump (CMJ) was executed following the methodology described by Brown & Weir (Brown & Weir, 2001). To eliminate the influence of arm-swing on the final result, participants were required to maintain their hands on their hips throughout the jumping action (Harman et al., 1990). A Kistler force platform (9287B, three-component force platform; Kistler, Hook, United Kingdom; dimensions: 900 × 600 × 100 mm) with a sampling rate of 2000 Hz was used to calculate the jump height in centimetres (cm). The height was determined as the difference between the maximum height of the centre of mass (apex) and the last contact of the toe on the ground during the take-off. Based on the height, the best of three jumps was chosen for the analysis. The ICC was superior to 0.90, demonstrating excellent reliability.

### *Chest Medicine Ball Throw (CMBT)*

Participants were seated on a chair placed against a wall with the feet flat on the floor and positioned shoulder-width apart. Following the methodology defined by Harris et al. (Harris et al., 2011), participants performed a chest throw with a medicine ball (5 kg for males and 3 kg for females). Based on the distance achieved, the best of three attempts was chosen for the analysis. A range from 0.97-0.99 ICC has been observed for this test in recreationally trained adults (Beckham et al., 2019).

### *30-Seconds Bench Press (BP) and Squat (SQ)*

The BP exercise was performed using free weights with an individually tailored load to approximate sixteen maximum repetitions. Participants were instructed to perform all possible repetitions in 30 seconds. They commenced the exercise in a supine position on a flat bench, with their elbows fully extended, and were instructed to lower the barbell towards the chest before engaging in the concentric phase.

The squat (SQ) exercise was performed with no external overload. Participants began seated on an individually adjusted bench, so that the posterior thighs were parallel to the ground, back straight, feet positioned parallel at shoulder width apart, and toes angled slightly outwards. Arms were crossed at the wrists and held against the chest. The participants were instructed to stand up and sit as many times as possible in 30 seconds.

For both exercises, the total number of completed repetitions was considered for the analysis. One qualified instructor monitored the appropriate execution of both BP (arms' range of motion and bar path from the chest to the end position with elbows completely extended) and SQ (ensuring the back was straight, avoiding additional impulse from the arms and thighs, and returning to parallel position at the end of the descent phase). The ICC was higher than 0.90 for both tests, indicating excellent reliability.

### *5.2.4. Training and control of the intervention compliance*

Training sessions were conducted on alternate days (i.e., Monday, Wednesday, and Friday). Each participant engaged in a supervised full-body resistance-training protocol, which included a standardized warm-up of about 12 minutes followed by three circuits of one set of the following exercises: (i) box step-ups (ii) bench press, (iii) sit-and-stand from the box, (iv) bent-over row, (v) deadlift, (vi) alternate lunges, (vii) shoulder press, and (viii) leg extension. Approximately 30-sec rest between exercises and 3 minutes between circuits were allowed. The aim of the workout was to induce a high level of mechanical and metabolic stress, focusing on muscle endurance training with a target of 16 self-determined (Steele et al., 2017) maximum repetitions per set (American College of Sports Medicine, 2009). When participants were able to perform more than 16 repetitions per set, the load was slightly increased between 2.5 to 5 kg. If fewer than 16 repetitions were completed, a rest period of approximately 10 seconds was allowed until the participants were able to reach the targeted number of repetitions per set. The duration of the workouts was 49±8 minutes. Furthermore, the S-RPE was measured after 15 to 20 min of having completed each workout session. The participants rated their global perception of effort on the OMNI-RES scale (Robertson et al., 2003) by answering the question "How hard was your entire workout?" (Lodo et al., 2012).

### *5.2.5. Supplementation Protocol*

The two products were presented in analogous white sachets of citric-flavoured powder to be dissolved in ~400 mL of room-temperature plain water and dispensed in identical 500-mL black and opaque bottle shakers. The diluted isoenergetic drinks were similar in appearance, texture, and taste. Both supplements were ingested on training days, within 15 minutes before each workout session. No supplement was consumed during non-exercising days. The nutritional composition of each product is presented in Table 5.1.

**Table 5.1.** Nutritional composition of supplements per intake mixed with ~400mL of plain water.

| Description | Multi-Ingredient (30 g dose) | Comparator (30 g dose) |
|---|---|---|
| Energy value (kcal) | 60 | 60 |
| **Macronutrients** | | |
| Total carbohydrates from maltodextrin (g) | 5 g | 15 g |
| Fats | 0 g | - |
| Total proteins included added amino acids (g) | 9 g | - |
| **Amino acids and other ingredients** | | |
| L-Leucine (g) | 3 | - |
| L-Isoleucine (g) | 1.5 | - |
| L-Valine (g) | 1.5 | - |
| L-Lysine (g) | 2.7 | - |
| L-Arginine Base (g) | 2.5 | - |
| L-Methionine (g) | 0.7 | - |
| L-Phenylalanine (g) | 1.1 | - |
| Taurine (g) | 1 | - |
| L-Threonine (g) | 1.2 | - |
| L-Tryptophan (g) | 0.3 | - |
| Tyrosine (g) | 1 | - |
| Citrulline Malate | 2.5 | - |
| Betaine (HCl) | 2 | - |
| Acetyl-L-Carnitine | 1.3 | - |
| Caffeine (mg) | 406 | - |
| Yerba Mate (2% in Caffeine) | 300 | - |

### *5.2.6. Dietary Monitoring*

Each participant's baseline diet (3 days, 2 weekdays, and 1 weekend day) was analysed using MyFitnessPal Inc.© (Version 2022, Texas, US) smartphone application (Evenepoel et al., 2020). Participants were instructed to maintain their normal diet throughout the intervention. They were asked to report any minimal change regarding food composition and size, ingestion of supplements or compliance with the reported meals, including breakfast, lunch, pre- and post-workout food intake, and dinner. If any change had been detected (i.e., becoming vegetarian, restricting calories, fasting, taking additional nutritional supplements, etc.), that participant's data would have been excluded from the analysis. To evaluate differences caused by the supplementation protocol, the diet was analysed again during the last week of the intervention.

### *5.2.7. Statistical Analysis*

A descriptive analysis was performed and subsequently the Shapiro–Francia tests were applied to assess normality. Sample characteristics at baseline were compared between groups using an independent means Student's t-test. All pre- and post-intervention data were summarized and reported as mean ± standard deviation unless stated otherwise. Raw changes in all outcome variables were calculated by subtracting pre from post assessment values. Under the assumption that both treatment groups would promote changes from baseline values due to the common training intervention and that the amount of change would also be dependent on each participant's baseline performance levels, one-way analysis of covariance (ANCOVA) models were used to compare differences in raw change between groups, using the pre-assessment values as covariates. A one-sample t-test of the pre-to-post differences in each outcome variable was performed for each treatment condition. To assess the magnitude of the differences from the baseline outcome, confidence intervals (CIs) of the differences were calculated and plotted. Confidence intervals not crossing zero were considered statistically significant. Eta squared (η2) and Cohen's d standardized effect sizes of the adjusted differences between intervention groups were calculated from the ANCOVA F tests and compared to common benchmarks (Cohen, 1988) (small η2 = 0.01, d = 0.2; moderate η2 = 0.06, d = 0.5; and large η2 = 0.14, d = 0.8). The significance level was set to $p \leq 0.05$. A power analysis was previously conducted using G*Power (version 3.1.9.7) to determine the minimum sample size required to detect statistically meaningful changes in body composition (FM and FFM), which were the primary outcome variables of the study. The meta-analysis by Benito et al. (2020) examined the effects of resistance training on whole-body muscle hypertrophy in healthy adult males and reported a moderate to large overall effect size for changes in FFM, with a pooled Hedges' $g = 0.55$ (95% CI: 0.38–0.71, $p < 0.001$). This approximately corresponds to Cohen's $d = 0.55$, and was considered an appropriate and conservative estimate. Using a two-tailed, within-subjects (pre–post) design, an α level of 0.05, and a desired statistical power of 0.80, a minimum of 24 participants would be necessary to detect significant changes in FFM or FM. To ensure statistical robustness and accommodate potential dropouts or missing data, a total of 41 participants were recruited and included in the final analysis. Results are reported as mean ± standard deviation unless stated otherwise. All statistics were performed using the Statistical Package for the Social Sciences (SPSS for Windows, version 28.0.1.1 Inc., Chicago, IL, USA).

## 5.3. Results

As summarised in Figure 5.2, forty-one of the initially recruited 44 participants completed all aspects of the study (Figure 5.2). Groups characteristics were equivalent at baseline: PREW [n= 22, 13 females (59%) and 9 males]: Age 54±4 years, height 1.72±0.10 m, body mass 77.6±16.0 kg, isometric strength 128±49 kg. COMP (n= 19, 11 females (58%) and 8 males): age 52±4 years, height 1.72±0.10 m, body mass 80.6±16.0 kg, isometric strength 126±33 kg.

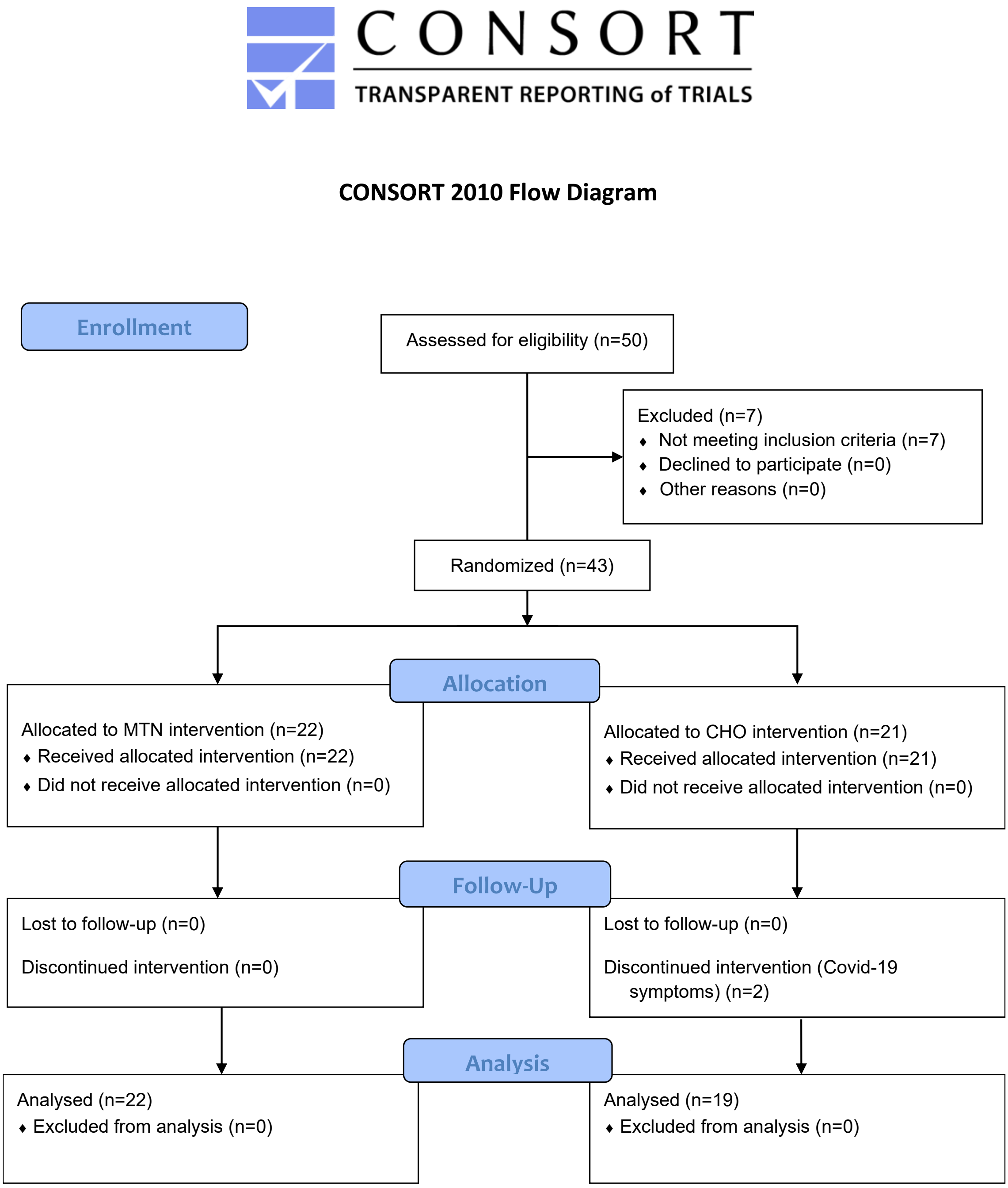


**Figure 5.2**. Flow diagram of the participants throughout the course of the study. MTN: Multi-Ingredient supplementation group; CHO: Comparator group.

Table 5.2 shows the dietary monitoring results, determined before and after the intervention.

**Table 5.2.** Descriptive Analysis of the Participants' Diet Composition

| **Macronutrients** | **MTN pre (n = 22)** | **MTN post (n = 22)** | **COMP pre (n = 19)** | **COMP post (n = 19)** |
|---|---|---|---|---|
| **Proteins** | | | | |
| $g \cdot d^{-1}$ | 88.5 ± 29.3 | 87.5 ± 28.9 | 88.7 ± 28.3 | 89.5 ± 29 |
| $g \cdot kg^{-1} \cdot d^{-1}$ | 1.14 ± 0.3 | 1.13 ± 0.3 | 1.1 ± 0.3 | 1.11 ± 0.3 |
| % of total energy | 15.9 ± 3.9 | 15.6 ± 3.6 | 14.5 ± 3.2 | 14.8 ± 3.3 |
| **Carbohydrates** | | | | |
| $g \cdot d^{-1}$ | 247 ± 78.6 | 255.42 ± 79 | 249.9 ± 78.1 | 244.4 ± 77.8 |
| $g \cdot kg^{-1} \cdot d^{-1}$ | 3.2 ± 1.4 | 3.3 ± 1.6 | 3.1 ± 1.3 | 3 ± 1.2 |
| % of total energy | 44.3 ± 11.8 | 45.5 ± 12 | 40.7 ± 11.6 | 40.3 ± 11.5 |
| **Fats** | | | | |
| $g \cdot d^{-1}$ | 98.7 ± 29.7 | 97.1 ± 29.4 | 122.1 ± 32.3 | 120.9 ± 31.8 |
| $g \cdot kg^{-1} \cdot d^{-1}$ | 1.3 ± 0.4 | 1.3 ± 0.5 | 1.5 ± 0.7 | 1.5 ± 0.7 |
| % of total energy | 39.8 ± 10.4 | 38.9 ± 10.1 | 44.8 ± 11.1 | 44.9 ± 11 |
| **Energy** | | | | |
| Total daily energy | 2231 ± 479 | 2246 ± 467 | 2453 ± 489 | 2426 ± 477 |
| $Kcal \cdot kg^{-1} \cdot d^{-1}$ | 28.8 ± 5.9 | 29 ± 5.7 | 30.4 ± 6.2 | 30.1 ± 6 |

Notes: Values are presented as mean ± standard deviation

No differences between groups were either found at baseline or as a result of the nutritional intervention for energy, carbohydrates, protein and fat intake. No complaints about any negative symptoms (i.e., hypoglycaemic reaction) or gastric discomfort due to the ingestion of supplements were reported.

Table 5.3 describes the mean and standard deviation values along with the observed absolute changes [95% CI] in body composition (BM, waist circumference, hip circumference, waist-to-hip ratio, fat mass and fat-free mass), muscle thickness (EF and VL), and performance (CMJ, seated chest medicine ball throw, midthigh pull isometric strength and 30-s sit to stand and bench press muscular endurance tests) for each of the intervention groups. No significant differences were observed at pre-intervention in any of the analysed dependent variables.

**Table 5.3.** Mean (M) ± standard deviation (SD) of the pre- and post-values and changes M±SD [95% CI] of the analysed variables for the two intervention groups.

| **Variable** | Multi-Ingredient (n=24) | | | Comparator (n=19) | | | Between-Groups Comparisons | |
|---|---|---|---|---|---|---|---|---|
| | **Pre** | **Post** | **Changes [95% CI]** | **Pre** | **Post** | **Changes [95% CI]** | ***p* value** | **ES** |
| Body Mass (kg) | 77.6 ± 16 | 77.4 ± 15 | -0.25±1.6 [-0.98,0.49] | 80.6 ± 16 | 80.6 ± 16 | 0.08±1.8 [-0.78,0.90] | 0.38 | -0.285 |
| Waist Circumference (cm) | 85 ± 10.9 | 83.2 ± 10.5 | -1.8±1.8 [-2.65,-0.94]** | 88 ± 13.7 | 87.3 ± 12.8 | -1.16±2.5 [-1.68,0.27] | 0.10 | -0.546 |
| Hip Circumference (cm) | 101.2 ± 6.7 | 100.3 ± 6.9 | -0.81±2.4 [-1.94,0.31] | 105 ± 8.9 | 104.1 ± 9.2 | -0.61±2.9 [-2.19,0.37] | 0.70 | -0.130 |
| Waist-to-hip ratio | 0.84 ± 0.08 | 0.83 ± 0.08 | -0.01±0.02 [-0.02, 0.01]** | 0.84 ± 0.12 | 0.84 ± 0.11 | -0.005±0.03 [-0.01,0.01] | 0.38 | -0.278 |
| Fat Mass (%) | 31.5 ± 9.3 | 30.1 ± 9 | -1.41±1.6 [-2.09,-0.74]** | 34.5 ± 9.2 | 33.5 ± 9 | -1.01±1.5 [-1.78,-0.25]* | 0.20 | -0.419 |
| Fat Mass (kg) | 24.4 ± 8.2 | 23.2 ± 7.7 | -1.15±1.5 [-1.82,-0.47]** | 28.4 ± 11.2 | 27.5 ± 11 | -0.88±1.6 [-1.66,-0.12]* | 0.30 | -0.343 |
| Fat-Free Mass (%) | 68.5 ± 9.3 | 69.9 ± 9 | 1.41±1.6 [0.74,2.09]** | 65.5 ± 9.2 | 66.5 ± 9 | 1.01±1.5 [0.25,1.78]* | 0.20 | 0.419 |
| Fat-Free Mass (kg) | 53.2 ± 13.5 | 54.1 ± 13.2 | 0.91±1.1 [0.48,1.34]** | 53.5 ± 9.6 | 54.2 ± 9.5 | 0.76±0.9 [0.25,1.23]** | 0.67 | 0.113 |
| Vastus Lateralis Muscle Thickness (cm) | 2.64 ± 0.5 | 2.8 ± 0.5 | 0.16±0.2 [0.05,0.27]** | 2.65 ± 0.3 | 2.78 ± 0.4 | 0.13±0.6 [0.04,0.21]* | 0.75 | 0.099 |
| Elbow Flexors Muscle Thickness (cm) | 3.6 ± 0.8 | 4.1 ± 0.8 | 0.51±0.3 [0.39,0.63]** | 3.6 ± 0.7 | 4.1 ± 0.7 | 0.42±0.2 [0.2,0.56]** | 0.58 | 0.176 |
| CMJ (cm) | 25.5 ± 6 | 26.8 ± 6 | 1.33±1.7 [0.70,1.95]** | 24.3 ± 5 | 25.3 ± 5 | 0.98±1.8 [0.14,1.29]* | 0.29 | 0.342 |
| Seated Chest Medicine Ball Throw (m) | 3.49 ± 0.6 | 3.67 ± 0.6 | 0.18±0.16 [0.11,0.26]** | 3.61 ± 0.4 | 3.71 ± 0.4 | 0.1±0.2 [0.02,0.19]* | 0.21 | 0.401 |
| Maximal Isometric Mid-Thigh Pull (kg) | 128.2 ± 49 | 137.8 ± 46 | 55±24 [-14.83,124.83] | 126 ± 33 | 129.9 ± 30 | 2.47±12 [-76.97,81.91] | 0.33 | 0.313 |
| 30-s Sit-to-Stand Repetitions (n) | 24.3 ± 5 | 27.3 ± 5 | 3±2.1 [1.8,4.2]** | 24.7 ± 6 | 27.8 ± 5 | 3.1±2.3 [1.8,4.4]** | 0.79 | 0.084 |
| 30-s Bench Press Repetitions (n) | 23.2 ± 5.1 | 27.8 ± 5.1 | 4.6±2.1 [3.61,5.58]** | 25.7 ± 4.2 | 29.8 ± 3.5 | 4.1±2.4 [3.05,5.16]** | 0.98 | 0.008 |
| Total Volume Lifted (kg) | 9222 ± 2733 | 13303 ± 3784 | 4082±1928 [3273,4890]** | 9677 ± 2280 | 13496 ± 3849 | 3669±2079 [2749,4589]** | 0.42 | 0.173 |

$^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{t}p \leq 0.10$ respect to baseline levels; ES is the standardised effect size presented as Cohen's *d*.

Even though both groups showed a significant reduction of body fat (percentage and absolute [kg])) along with a concomitant increase of fat-free mass (percentage and absolute [kg]) a significant reduction of the waist circumference and waist-to-hip ratio was observed only for the PREW group. However, no further difference between treatments was identified when the adjusted values were analysed post-intervention (Figure 5.3).

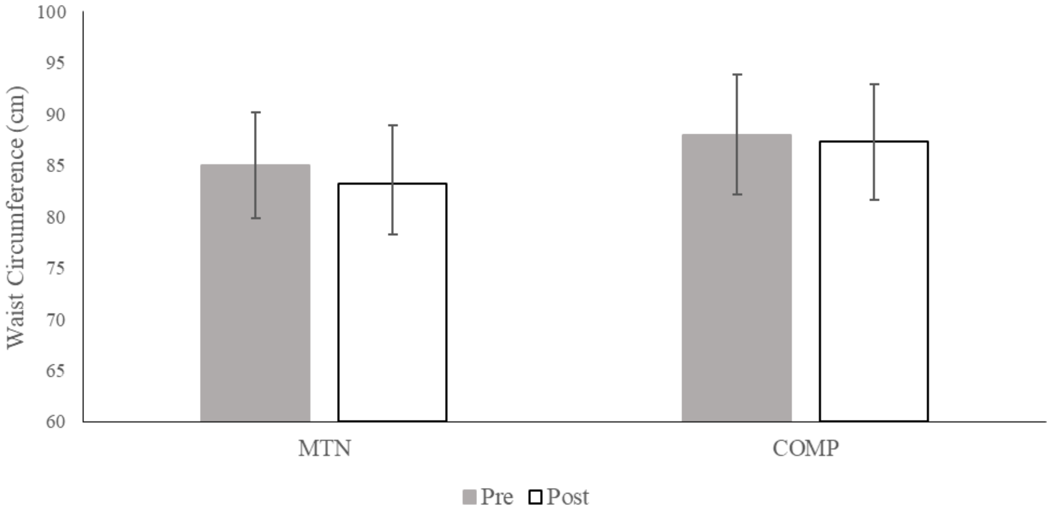


**Figure 5.3.** Waist circumference changes from baseline to post-intervention.

A non-significant trend effect was observed for FM in kg (F(1,39) = 2.79, p = 0.104, $\eta^2$ = 0.065) and percentage (F(1,39) = 3.723, p = 0.061, $\eta^2$ = 0.086) values. Additionally, no significant between-groups difference emerged upon adjustment for pre-intervention values for FM in kg (p = 0.302, d = -0.343) and percentage (p = 0.201, d = -0.42) values.

While no significant absolute FFM change was evident when adjusted from baseline values (p = 0.48), a significant increase from pre- to post-intervention in VL thickness was observed (F (1,39) = 6.52, p = 0.015, $\eta^2$ = 0.132). Remarkably, both supplement protocols yielded similar hypertrophy outcomes (p = 0.754). Similarly, a significant difference of larger magnitude was observed from pre- to post-values for EF muscle thickness (F (1,39) = 21.25, p < 0.001, $\eta^2$ = 0.288). However, no significant differences were noted between conditions (p = 0.578).

Both the PREW and COMP groups demonstrated significant absolute and adjusted increases in CMJ (F (1,39) = 6.48, p < 0.015, $\eta^2$ = 0.128). However, no significant difference emerged between conditions when values were adjusted for baseline results ($p = 0.287$), despite the PREW group showing larger CMJ increases (1.55 vs 0.99 cm). Similarly, CMBT exhibited a trend towards a significant increase from pre- to post-values ($F(1,39) = 3.07$, $p < 0.088$, $\eta^2 = 0.066$). Nonetheless, no significant difference between conditions was observed ($p = 0.212$, $d = 0.4$), although PREW yielded a larger increase than COMP (18.9 vs. 12.0 cm, respectively).

Both the 30-second sit-to-stand and 30-second bench press tests significantly improved under both conditions from pre- to post-intervention results ($F(1,39) = 8.92$, $p = 0.005$, $\eta^2 = 0.194$, and $F(1,39) = 6.33$, $p = 0.016$, $\eta^2 = 0.143$, respectively). However, no significant difference was observed between groups when the average mean difference adjusted by pre-values was considered.

The total volume (kg) lifted during workouts increased significantly under both conditions ($p<0.001$) with no differences ($p>0.05$) between groups.

### *5.3.1. Exploratory variables*

The average S-RPE scores did not reveal any statistically significant disparities between groups (PREW: 7.79 ± 0.50 vs. COMP: 7.94 ± 0.60). Additionally, no effect of sex (all $p>0.05$) was observed for any of the analysed variables.

## 5.4. Discussion

Results of the present study suggest that ingesting a pre-workout caffeinated vegan protein-based multi-ingredient providing 12 g of EAA, and 406 mg of caffeine promoted similar body composition and performance outcomes to an isoenergetic carbohydrate-only supplement in middle-aged physically active female and male adults. The waist circumference was the only variable showing a significant reduction in the PREW group (Figure 5.3). Based on these findings, and within the limitations of our study procedures, except for the observed reduction of waist circumference, our hypothesis asserting that compared to a carbohydrate-only isocaloric comparator, a caffeinated protein-based PREW might further stimulate fat loss and optimise more favourable body composition and performance outcomes must be rejected.

No significant energy or macronutrient consumption changes were observed from pre- to post-intervention for any group (Table 5.3). Both groups ingested an acceptable amount and distribution range of macronutrients across the study (Thomas, Erdman & Burke, 2016; Seidelmann et al., 2018). Therefore, there were no limitations due to insufficient energy, protein, carbohydrate and fat for any of the groups.

The multi-ingredient admixture used in our study included 406 mg of anhydrous caffeine (4.9±2 $mg \cdot kg^{-1}$) and 300 mg of yerba mate extract with 2% of caffeine (~6 mg of caffeine) (~0.01$mg \cdot kg^{-1}$). Thus, the resulting mean relative dose of caffeine per intake was ~4.9 $mg \cdot kg^{-1}$, which was within the range of recommended moderate doses (3 to 6 $mg \cdot kg^{-1}$) related to ergogenic effects for resistance training (Pickering & Grgic, 2019). Nonetheless, it is worth noticing that the non-interaction effects between the PREW and COMP at post-intervention agree with previous investigations reporting no performance benefits from the pre-workout ingestion of 3 $mg \cdot kg^{-1}$ of caffeine in habitual caffeine consumers (Filip-Stachnik et al., 2023).

Previous acute studies conducted in middle-aged adults reported beneficial effects of caffeinated PREW containing protein and fortified with amino acids to maximise resistance training outcomes (Puente-Fernández et al., 2020). Similar to the current study, the participants ingested a PREW providing ~5.2 $mg \cdot kg^{-1}$ of caffeine, 0.21 $g \cdot kg^{-1}$ of carbohydrates with a high proportion of isomaltulose (a slow-release disaccharide), and 0.12 $g \cdot kg^{-1}$ of protein along with citrulline-malate, L-leucine, L-tyrosine, L-taurine, and betaine. The proposed attenuation of effects due to habitual caffeine intake could have been the cause of discrepancies between the current 6-week intervention study and acute trial designs. Even though regular caffeine consumption does not diminish acute performance benefits on muscular function (Carvalho et al., 2022), the scientific consensus on its ergogenic effects remains divided, particularly concerning long-term adaptations, and individual and genetic variability in caffeine metabolism (Virgili et al., 2023). In our study, both groups significantly improved CMJ, CMBT, and both lower and upper body muscle endurance tests with no significant difference between them (Table 3). These results are supported by the meta-analysis by Grgic & Varovic (2022), suggesting that the long-term ergogenic effects of caffeine supplementation, particularly for upper body (e.g., medicine ball throw), may not significantly differ from placebo.

The variability in responses to caffeine supplementation across different studies highlights the complexity of its ergogenic properties. While acute caffeine intake has been shown to enhance performance in specific contexts, the translation of these benefits to long-term training adaptations remains uncertain (Valenzuela et al., 2019b). Our research supports the notion that, over a 6-week RT regimen, PREW supplementation including caffeine does not confer additional advantages to enhance muscle function and promote body composition outcomes beyond those achieved through regular training alone. Indeed, for middle-aged recreationally trained adults, performing a regular resistance exercise programme seems to be the most important aspect impacting the observed training adaptations (Choi et al., 2021). Along those lines, unlike previous studies reporting a significant effect of pre-workout supplementation to reduce the perceptual response to endurance (Alkhatib et al., 2015), and strength (Grgic & Mikulic, 2017) exercise bouts, differences in the global perceptual response between groups were not identified, even during the initial workout sessions.

Our study is not without limitations. Firstly, although diet composition was registered with a self-reported food diary and analysed with a validated application (MyFitnessPal Inc.©), providing a prepared and prepacked diet to participants during the intervention would have offered an ideal scenario to standardize and control the influence of diet on the observed results. Secondly, most of the participants had a daily caffeine intake of 3-5 coffees or teas (100-300 mg of caffeine), which could have impacted the observed results. However, because our study was designed as a highly ecological intervention, avoiding diet modifications, the participants were asked to maintain their usual caffeine intake (avoiding caffeine or energetic intake 3 hours pre- and 2 hours post-workout during training days) so that the impact of integrating PREW supplement to the habitual nutritional habits could be assessed. Although concerns regarding the variability of the individual responses to different protocols of caffeine ingestion, including the caffeine format (e.g., coffee, capsules, etc) or the timing (Wickham & Spriet, 2018), it is worth highlighting that the participants of our study ingested the PREW supplement 15 minutes before performing a ~65-minute workout (including the 12-minute warm-up). Considering that caffeine is rapidly absorbed by the body, when consumed in coffee, powders or capsules, appearing in the blood within 5–15 min and peaking between 40 and 80 min (Guest et al., 2021), the window for observing caffeine ergogenic effects covered from the beginning of the workout and likely peaking during the latter half of the session, when physical fatigue is more pronounced, and performance begins to decline. Thirdly, considering that the most prominent resistance training adaptations and nutritional support benefits are likely to occur in the most heavily utilised muscle groups (Bernárdez-Vázquez et al., 2022), the low training volume, only one exercise, directly impacting the elbow flexors may have limited the observed adaptations on this muscular group. Lastly, we aimed to include peri- and post-menopausal women but no additional blood tests were performed aside from the symptoms questionnaire (Heinemann et al., 2003), which might potentially influence the results between early peri-menopausal and post-menopausal women. Future longer investigations using larger sample sizes are needed to comprehensively understand the long-term effects of combining pre-workout supplementation with resistance training in middle-aged female and male individuals.

## 5.5. Conclusions

Despite observing a significant reduction in waist circumference for the PREW treatment group, compared to the ingestion of only carbohydrates a pre-workout vegan protein-based caffeinated admixture did not maximise body composition and performance outcomes in middle-aged individuals engaged in a resistance training programme over 6 weeks.

# CHAPTER 6

# STUDY 5 – POST-RESISTANCE TRAINING SUPPLEMENTATION USING A MULTI-INGREDIENT OR CARBOHYDRATES ALONE OVER SIX WEEKS IN MIDDLE-AGED INDIVIDUALS.

## 6.1. Introduction

Nutrition is universally acknowledged as one of the primary determinants impacting post-exercise recovery (Morton, McGlory & Phillips, 2015). Post-workout muti-ingredients are designed with the primary objective of engaging in the "3R" paradigm (rehydration, refuelling, and muscle repair) (Morton, McGlory & Phillips, 2015). Furthermore, recent scientific literature asserts that some amino-acid derivatives, such as creatine (Kerksick et al., 2018; Barnes, 2023), and β-hydroxy-β-methylbutyrate (β-HMB) (Kerksick et al., 2018; Barnes, 2023), have the potential to expedite post-exercise recovery and support optimal muscle function in athletes and physically active individuals via synergistic mechanisms. The proposed beneficial effects of post-workout admixtures rely on mitigating declines in physical performance after hard training sessions and promoting skeletal muscle mass accretion and remodelling (Nabuco et al., 2018; Naclerio et al., 2020). The claimed superior effects of multi-ingredients compared to the intake of each ingredient in isolation are based on their synergistic interaction or amplification effect once the included ingredients are ingested as a component of a special formulation (Liao et al., 2017). Nevertheless, the expected outcomes are based on the proven effects of each singular ingredient examined separately. For instance, whey protein is considered the highest quality protein due to its complete nutritional profile and rapid digestion kinetics (Boirie et al., 1997). Furthermore, its high protein quality is attributed to the rich array (>40%) of essential amino acids (EAA), particularly the branched-chain amino acids (BCAAs), including leucine (Phillips & van Loon, 2011).

In this regard, high-quality protein extracts have proven to maximise muscle protein synthesis and attenuate muscle protein breakdown during exercise and resting conditions (Naclerio & Larumbe-Zabala, 2016). Additionally, adding high-quality protein to the habitual diet favours metabolic health in older adults (Nabuco et al., 2019) and maximises muscle mass and strength gains over long-term resistance training interventions (Huecker et al., 2019) in physically active middle-aged and older adults (Camargo et al., 2020).

Moreover, *β*-HMB, a leucine metabolite, may also attenuate catabolism and exercise-induced muscle damage (Arazi et al., 2021; Kim & Kim, 2022), favouring growth hormone production, tissue repair (Arazi et al., 2021) and muscular protein synthesis (Kraemer et al., 2015). Furthermore, creatine has been proven to promote muscle mass accretion and maximise strength and endurance outcomes in athletes and recreationally active individuals (Mills et al., 2020). Vitamin D is a cofactor associated with optimal muscular function and growth (Chiang et al., 2017), predominantly lower body strength (Beaudart et al., 2014) particularly in older individuals or those with vitamin D insufficiency. Indeed, adding Vitamin D3 to whey protein isolate enhanced skeletal mass index, strength, and anabolic markers in sarcopenic older adults (Bo et al., 2019). This may be attributed to the influence of Vitamin D receptors on human skeletal muscle (Zhang et al., 2019) although the specific critical link between vitamin D and muscle growth and remodelling remains unclear (Bouillon et al., 2022).

To the best of the authors' knowledge, there is still a paucity of research analysing the convenience of regularly ingesting post-workout muti-ingredients to maximise exercise adaptation outcomes in physically active middle-aged and older adults. The aim of this study, therefore, was to compare the effects of ingesting either a commercially available post-workout multi-ingredient including, carbohydrates, Leucine-enriched whey protein, β-HMB, creatine monohydrate, and Vitamin D3 (Crown Sports Nutrition, Spain) or an isoenergetic carbohydrate-only comparator (COMP) on body composition (including muscular hypertrophy) and physical performance over a 6-week resistance training intervention. The primary outcomes were changes in fat mass, free mass, waist, and hip circumference along with vastus lateralis and elbow flexors muscular thickness. Secondary outcomes included changes in strength, power output, and muscular endurance. Furthermore, the post-workout global perceptual response, the total volume (kg lifted per session), and changes in endurance performance were considered exploratory outcomes. Based on previous literature it was hypothesised that ingesting the above-described post-workout multi-ingredient instead of an isocaloric, only carbohydrate supplement will elicit higher training outcomes in physically active middle-aged and older (females and males) adults.

## 6.2. Material and Methods

### *6.2.1. Experimental design*

The intervention followed a double-blinded, randomised, controlled, and counterbalanced, parallel group design. Following the inclusion criteria, familiarisation, and baseline assessments, the participants were randomly allocated to receive either a post-workout multi-ingredient (POST-MTN) or isoenergetic comparator (COMP) composed of carbohydrates alone. Subsequently, the participants completed a standardised 6-week circuit-shaped resistance training programme including 3 workouts per week (18 total sessions).

### *6.2.2. Participants*

Twenty-two recreationally active, middle-aged and older adults (12 peri- and post-menopausal females and 10 males; mean age: 54 ± 6 years) were initially recruited. The inclusion criteria were a minimum resistance training history of 6 months before the study onset and 45 years or older. Additionally, post- or peri-menopausal female participants, presenting a minimum of two symptoms of menopause onset (such as hot flushes, menstrual cycle alterations, and not menstruating for more than 1 year) (NICE, 2019) were included. Participants were not eligible if suffering from acute or chronic diseases (including advanced obesity, metabolic syndrome, long Covid-19, osteoporosis, or sarcopenia), following a medication prescription, or consuming supplements that could interfere with our research or affect exercise performance (i.e., creatine, protein amino-acids, NSAIDs, etc.). In accordance with the Declaration of Helsinki, all participants provided written informed consent. Procedures were approved by the University Research Ethics Committee. The study was registered as a clinical trial at the U.S. National Institutes of Health. https://www.clinicaltrials.gov (NCT05769088).

To assess statistical power, a sensitivity analysis of the final sample size (POST-MTN. n = 10 and COMP, n = 10) was conducted to detect statistically significant differences between conditions in pre-post differences. Assuming a t-test model with two independent samples to compare adjusted means, 0.05 $\alpha$ error probability, and 0.80 power ($1 - \beta$), it was determined that differences between groups could be detected with a Cohen's d above 1. As summarised in Figure 6.1, twenty-two participants were randomly allocated into one of the two intervention groups (POST-MTN or COMP). However, Due to non-intervention-related reasons, two participants (one per group) dropped out of the study and eventually, twenty participants completed all aspects of the intervention protocol and were considered for the analysis. The final composition of the groups was equivalent at baseline: POST-MTN (n=10, 50% females) age 52.0 ± 5 years, 1.76 ± 0.1m, 82.0 ± 18 kg; COMP (n=10, 50% females): age 51.0 ± 3 years, height 1.74 ± 0.1 m, body mass 85.0 ± 17 kg.

CONSORT
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**CONSORT 2010 Flow Diagram**

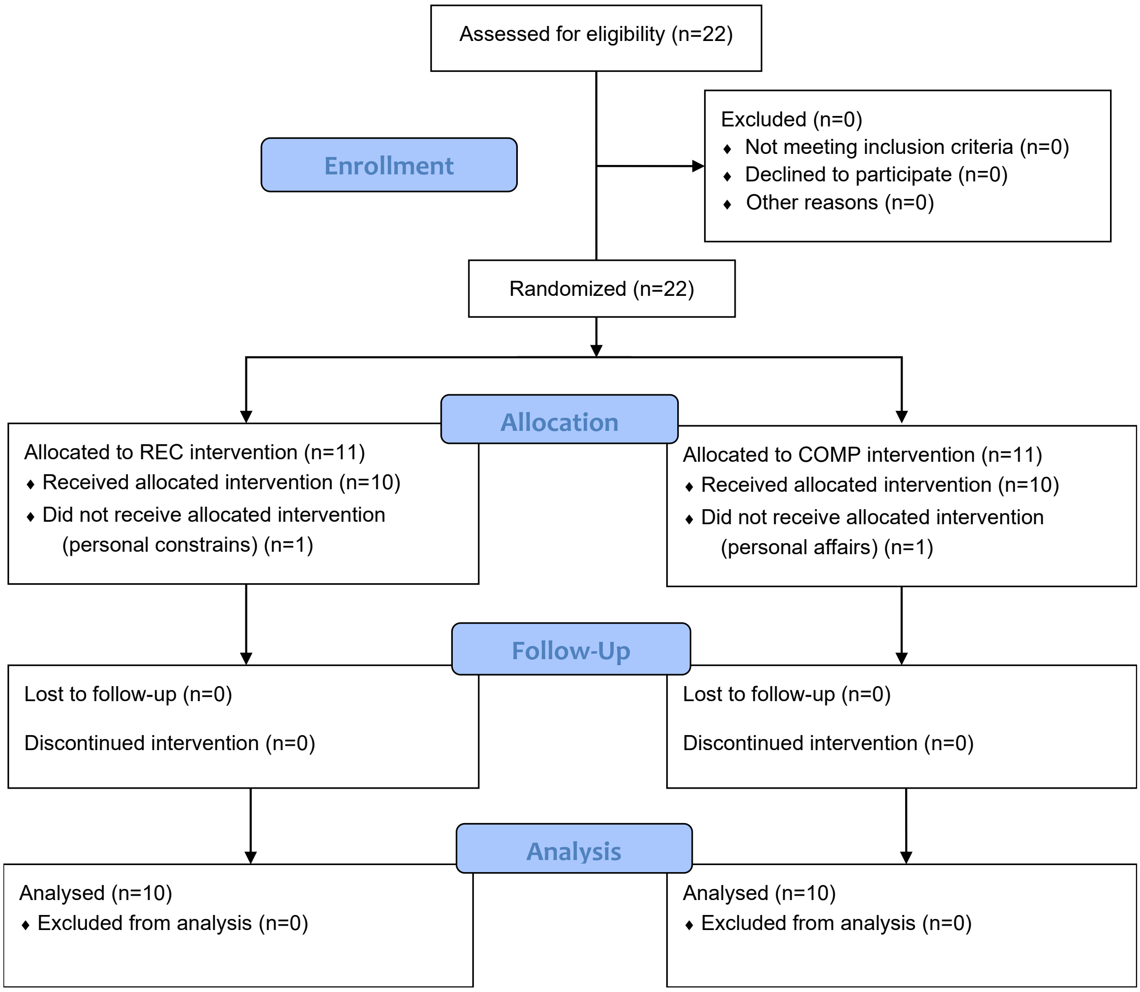

**Figure 6.1**. Flow diagram of the participants throughout the course of the study. REC: Multi-Ingredient supplementation group; COMP: Isoenergetic, carbohydrate-only, comparator group.

### *6.2.3. Procedures*

Following confirmation of eligibility, enrolment, and before the baseline assessment, participants performed a 2-weekly familiarisation programme over 4 weeks (8 sessions) aimed at minimizing any potential learning effects with the assessment and training procedures. After the preintervention assessments, participants were matched by body mass and maximal isometric force. Assignment of participants to treatments was performed by block randomisation using a block size of two and in a double-blind (POST-MTN or COMP) manner.

### *6.2.4. Assessments*

Measurements were determined over two sessions. Day 1 included (i) muscle thickness using ultrasonography, (ii) waist and hip circumferences, and body composition via plethysmography (iii) vertical jump and (iv) medicine ball chest throw. Day 2 included the progressive cycling to exhaustion test. Before any testing session, participants were instructed to refrain from any vigorous activity. Environmental conditions at the sports science laboratory were controlled and maintained at a constant level during both testing and training sessions. Specifically, the mean±SD air temperature, barometric pressure, and relative humidity were 19±1.5ºC, 1020±14 mBar, and 53.6±5.7 %, respectively.

#### *6.2.4.1. Body Composition*

Absolute (kg) and relative (percentage) fat mass and fat-free mass were assessed via air displacement plethysmography (BodPod®, Life Measurements, Concord, CA, USA) following the methodology described elsewhere (MacDougall, Wenger & Green, 1993; Dempster & Aitkens, 1995). Height was assessed in a stretched standing position to the nearest 0.01 m using a wall-mounted stadiometer (Seca GmbH, Hamburg, Germany) and body mass (BM) was corrected to the nearest 0.01 kg using a digital scale (Seca GmbH, Hamburg, Germany). Additionally, waist and hip circumferences were measured with a standard measuring tape, and the waist-to-hip ratio was calculated by dividing the waist by hip circumference. One trained researcher assessed all participants to eliminate inter-rater variability and ensure measurement consistency.

### *6.2.4.2. Muscle Structure*

Muscular architecture changes under relaxed and static conditions were evaluated using a real-time B-mode ultrasound imaging system (Philips Affiniti 70 Ultrasound, Philips Corporation, USA). At each marked site, an 18-5 MHz broadband linear-array transducer, along with water-soluble transmission gel (Aquasonic 100 Ultrasound Transmission gel), was positioned perpendicular to the skin surface and parallel to the longitudinal axis of the muscle, providing acoustic coupling during the test without depressing the dermal surface (Naclerio et al., 2019b). Following the methodology outlined by (Bradley & O'Donnell, 2004), as described by Naclerio et al. (2019), the same qualified and skilled researcher conducted all measurements using a standardised protocol. Muscle thickness (Th) of the elbow flexors (EF) at 80% of the humeral length, and the vastus lateralis (VL) at 60% of the femoral length were assessed in the dominant limbs. Muscle thickness was determined as the distance between the superficial and deep muscle aponeuroses for the VL, or between the superficial aponeurosis of the muscle and the muscle-bone boundary for the EF. Figure 6.2 illustrates examples of ultrasonography images depicting measurement sites for muscle architecture in EF and VL.

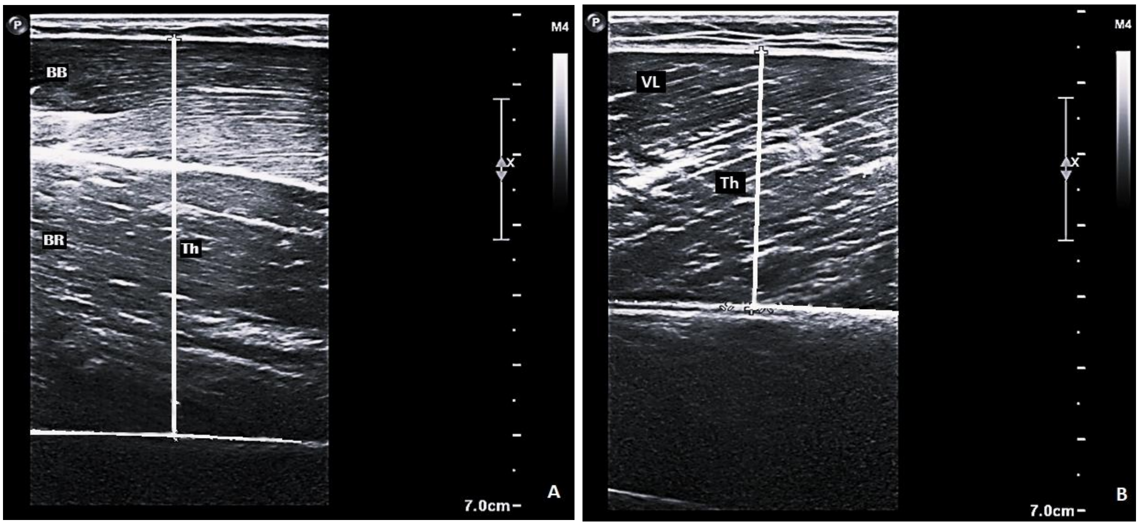

**Figure 6.2.** Ultrasonography images depicting measurement results for muscle thickness in EF (A) and VL (B) muscles.

To measure EF thickness, participants were seated on a chair, with their torso straight and relaxed against the backrest. The assessed arm was maintained in a relaxed position at a 90° angle at the elbow joint on a bed, with the forearm in a relaxed pronated position. For

VL thickness, participants were placed barefoot in a semi-recumbent and relaxed position on a bed set at 125°, with fully extended and relaxed knees and arms resting alongside the body. The EF assessment site was accurately located and marked at 80% of the distance between the coracoid process of the scapula and the medial epicondyle of the humerus. The VL assessment point was marked at 60% of the distance between the greater trochanter and the lateral condyle of the femur. To eliminate tissue distortion caused by excessive compression, the transducer was lightly rested on the skin surface, the ultrasound image on the screen was visually monitored, and participants were asked to provide verbal feedback on the pressure experienced on the skin.

Three images were obtained at each location, and the median of the measurements was calculated and used for the analysis. To ensure accurate replication of the measurement location, the position of the probe was recorded on acetate paper, and pre- and post-intervention images were compared based on identifiable markings (such as moles and small angiomas) on the skin surface as reference points. This process increased the reliability of repeated measures.

To prevent osmotic fluid shifts that could distort measurements of angle and thickness (Stasinaki et al., 2018), images were acquired at least 48 hours after the last training session and prior to the physical performance tests. Intra-rater reliability of muscle thickness measurements was assessed by a single trained researcher, who performed measurements on the same scan, demonstrating excellent interclass correlation coefficients (ICC) (> 0.90).

#### *6.2.4.3. Vertical Jump*

A countermovement jump (CMJ) was performed according to the methodology described by Brown & Weir (Brown & Weir, 2001). To eliminate the impact of arm-swing, the participants were required to maintain their hands on their hips for the entire action (Harman et al., 1990). A Kistler force platform (9287B, three-component force platform; Kistler, Hook, United Kingdom; dimensions: 900 × 600 × 100 mm) with a sampling rate of 2000 Hz was used to calculate the height from the difference, in meters (m), between maximum height of the centre of mass (apex) and the last contact of the toe on the ground during the take-off. Based on the height, the best of three jumps was chosen for the analysis.

#### *6.2.4.4. Seated Medicine Ball Chest Throw (SMBT)*

Sitting on a chair placed against a wall, with the feet flat on the floor and separated at shoulder width, the participants threw the ball from their chest following the methodology defined by (Harris et al., 2011). Based on the distance, the best of three attempts was chosen for the analysis. Males used a five-kg (circumference of 0.30 m), and females a three-kg (circumference of 0.21 m) medicine ball. A range from 0.97-0.99 ICC has been observed for this test in recreationally trained adults (Beckham et al., 2019).

#### *6.2.4.5. Progressive Cycling to Exhaustion Test*

Following a standardised warm-up, participants completed a maximal incremental laboratory exercise test to exhaustion on a cyclo-ergometer (Lode Corival®). The test commenced at a work rate of 30 or 50 W (females and males, respectively) and increased 15 W every minute. The participants were encouraged to keep a constant cycling rate between 60 and 90 rpm while remaining in a sitting position. When cadence dropped by more than 10 rev·min$^{-1}$ for more than 10 s despite strong verbal encouragement, tests were terminated. The test was designed to avoid long-term muscular fatigue, and every trial lasted <18 min. Expired gases were collected continuously during the test using a Cortex MetaLyzer 3B gas analyser (Cortex Biophysik, Leipzig, Germany). The maximum heart rate (HRmax) and $\dot{V}$O2peak (calculated as the highest mean oxygen consumption over a 30-s period (Karsten et al., 2015)) were determined and used for the analysis. Additionally, the oxidation and relative contributions of carbohydrates and fat across the test were estimated following the methodology described by (Alkhatib et al., 2015). Fat oxidation (FAO) was estimated using specific equations (Alkhatib et al., 2015). Assuming minimal protein contribution during exercise the maximal fatty acid oxidation (Fatmax) (g·min$^{-1}$) was determined from the progressive test as the highest amount of FAO averaged over 30s. Fatmax corresponding intensity was determined in watts (W) (Alkhatib, 2010).

### ***6.2.5. Resistance Training***

Training sessions were conducted on alternate days (i.e. Monday, Wednesday, and Friday). Each participant performed a supervised full-body resistance-training protocol involving a standardised warm-up followed by three circuits of one set of the following exercises: (i) box step-ups (ii) bench press, (iii) back squat with barbell, (iv) upright row, (v) deadlift, (vi) alternate lunges, (vii) shoulder press, and (viii) biceps curl. About 30-s rest between exercises and 2-min between circuits was allowed. As the workout aimed to create a high level of mechanical and muscular stress, a muscle hypertrophy training targeting 10-12 self-determined maximum repetitions (>70 to <75% 1RM) per set was designed (American College of Sports Medicine, 2009). When participants were able to perform more than 12 repetitions per set, the load was increased between 2.5 and 5 kg (Steele et al., 2017). If fewer than 10 repetitions were completed, a rest period of ~10 s was allowed until the participants were able to reach the targeted number of repetitions per set. The time to complete the workouts was 43±8 min. Additionally, the OMNI-RES scale (Robertson et al., 2003) was used to quantify the session rating of perceived exertion. To avoid easy or difficult elements toward the end of the sessions from skewing the overall rating of the exertion, the participants were asked to rate their session RPE by answering the question “How hard was your entire workout?” 15 min after the completion of each resistance training workout (Lodo et al., 2012).

### *6.2.6. Diet and supplementation protocol*

Each participant completed a three-day food diary report (two weekdays and one weekend day) for three different time points: (i) first familiarisation week, (ii) first interventional week, and (iii) last training week. The MyFitnessPal Inc.© (Version 2022, Texas, US) smartphone application was used to calculate the total (absolute and relative, in percentage) daily intake, and per-meal energy and macronutrient (proteins, carbohydrates, and fats) distribution (Evenepoel et al., 2020). Participants were instructed to maintain their normal diet throughout the intervention. They were asked to report any minimal change in food composition and size, ingestion of supplements, or compliance with the reported meals, including breakfast, lunch, pre- and post-workout food intake, and dinner. If any change had been detected (i.e., becoming vegetarian, restricting calories, fasting, taking additional nutritional supplements, etc.), that participant’s data would have been excluded from the analysis.

During the 6-week training period (weeks 6 to 11), all participants consumed either one dose of the multi-ingredient (Crown Sport Nutrition, Spain) or an isoenergetic providing only maltodextrin beverage (Table 6.1).

**Table 6.1.** Nutritional composition of supplements per intake mixed with ~400mL of plain water.

| **Description** | **Post-workout multi-ingredient (60 g dose)** | **Comparator (60 g dose)** |
|---|---|---|
| Energy value (kcal) | 204 | 203 |
| **Macronutrients** | | |
| Total Fats (g) | 0.8g | 0.6 g |
| - Saturated | 0.5 g | 0 |
| Total carbohydrates (g) of which | 23 g | 48 g |
| - Saccharides | 0.8 g | |
| Protein (from whey) | 26 *(21.5)* g | 1.3 g |
| **COMPOSITION:** | | |
| Aspartic Acid | 2.07 g | 0 |
| Glutamic Acid | 3.29 g | 0 |
| Alanine | 0.95 g | 0 |
| Arginine | 0.35 g | 0 |
| Cysteine + Cystine | 0.41 g | 0 |
| Phenylalanine | 0.52 g | 0 |
| Glycine | 0.30 g | 0 |
| Histidine | 0.27 g | 0 |
| Isoleucine | 1.2 g | 0 |
| Leucine | 1.87 g | 0 |
| Cystine + Methionine | 0.81 g | 0 |
| Lysine | 1.79 g | 0 |
| Methionine | 0.40 g | 0 |
| Proline | 1.18 g | 0 |
| Serine | 0.91 g | 0 |
| Tyrosine | 0.50 g | 0 |
| Threonine | 1.31 g | 0 |
| Tryptophan | 0.33 g | 0 |
| Valine | 0.99 g | 0 |
| Phenylalanine + Tyrosine | 1.02 g | 0 |
| Creatine | 5 g | 0 |
| HMB calcium | 1.8 g | 0 |
| Vitamin D3 | 1000 IU | 0 |
| Sodium | 0.2 g | 0.03 g |

The supplements were presented in analogous white sachets of chocolate-flavoured powder to be dissolved in ~400 mL of room-temperature plain water and administered immediately after each workout session. No supplement was consumed during non-exercising days. Therefore, each participant ingested a total of eighteen doses. Even though the drinks were similar in appearance, texture, and taste, they were provided in identical black and opaque bottle shakers to maximise the double-blinded procedure. In order to avoid any interference with digestion, absorption or effects of the assigned supplement, the participants were instructed to avoid caffeine-containing substances or food intake 3 hours pre- and 2 hours post-workout during training days.

### *6.2.7. Statistical Analysis*

A descriptive analysis was conducted, and subsequently, the Shapiro-Wilk test was utilized to evaluate the normality of the data. Baseline sample characteristics were compared between conditions using Analysis of Covariance (ANCOVA), where sex served as the covariate. Changes from pre-to-post-treatment were assessed using a 2 (times) × 2 (treatments) repeated-measures ANCOVA, again employing sex as the covariate. One-sample t-tests of the pre-to-post differences in each outcome variable were conducted for each treatment condition. To assess the magnitude of the differences from the baseline outcome, confidence intervals (CIs) of the differences were calculated and plotted, with statistically significant intervals being those that did not cross zero. Furthermore, mean differences between treatment conditions were evaluated with a one-way ANCOVA and Bonferroni-adjusted post hoc analysis was performed for pairwise comparisons in all ANCOVA models. Eta squared ($\eta^2$) and Cohen´s d values were reported to provide estimates of the standardised effect size (small d = 0.2, $\eta^2 = 0.01$; moderate d = 0.5, $\eta^2 = 0.06$; and large d = 0.8, $\eta^2 = 0.14$). The significance level was set to $p < 0.05$. Results are presented as mean ± standard deviation unless otherwise specified. The Statistical Package for the Social Sciences (SPSS for Windows, version 28.0.1.1 Windows; SPSS, Inc., Chicago, IL, USA) was employed for all statistical analyses.

## 6.3. Results

No differences between groups were observed at baseline and as a result of the nutritional intervention regarding energy, carbohydrate, and protein intake (Table 6.2). Additionally, no participants reported negative symptoms related to supplement ingestion, such as hypoglycaemic reactions or gastric discomfort.

**Table 6.2.** Descriptive Analysis of the Participants' Diet Composition.

| Macronutrients | POST-MTN pre (n = 10) | POST-MTN post (n = 10) | COMP pre (n = 10) | COMP post (n = 10) |
|---|---|---|---|---|
| **Proteins** | | | | |
| $g \cdot d^{-1}$ | 89.1 ± 27 | 88.6 ± 27 | 88.7 ± 26 | 89.1 ± 26 |
| $g \cdot kg^{-1} \cdot d^{-1}$ | 1.09 ± 0.3 | 1.07 ± 0.2 | 1.04 ± 0.3 | 1.04 ± 0.3 |
| % of total energy | 15.9 ± 3.9 | 15.7 ± 3.6 | 15.6 ± 3.2 | 15.6 ± 3.3 |
| **Carbohydrates** | | | | |
| $g \cdot d^{-1}$ | 249.3 ± 75 | 253.2 ± 79 | 248.8 ± 78 | 249.4 ± 78 |
| $g \cdot kg^{-1} \cdot d^{-1}$ | 3.04 ± 1.3 | 3.06 ± 1.4 | 2.93 ± 1.2 | 2.92 ± 1.2 |
| % of total energy | 44.4 ± 11.8 | 44.9 ± 12 | 43.9 ± 11.6 | 43.7 ± 11.5 |
| **Fats** | | | | |
| $g \cdot d^{-1}$ | 99.1 ± 29 | 98.6 ± 29 | 102 ± 33 | 103 ± 32 |
| $g \cdot kg^{-1} \cdot d^{-1}$ | 1.21 ± 0.4 | 1.19 ± 0.3 | 1.2 ± 0.5 | 1.21 ± 0.4 |
| % of total energy | 39.7 ± 10.4 | 39.4 ± 10.1 | 40.5 ± 11.1 | 40.6 ± 11 |
| **Energy** | | | | |
| Total daily energy | 2245 ± 467 | 2254 ± 461 | 2268 ± 479 | 2281 ± 472 |
| $Kcal \cdot kg^{-1} \cdot d^{-1}$ | 27.4 ± 5.6 | 27.3 ± 5.7 | 26.7 ± 6.1 | 26.7 ± 6 |

Notes: Values are presented as mean ± standard deviation

Table 6.3 describes the mean and standard deviation values along with the absolute changes [95% CI] in body composition (body mass, fat mass, fat-free mass, waist circumference and waist-to-hip ratio), muscle thickness (EF and VL), and performance (CMJ, seated chest medicine ball throw and total volume lifted during workouts, $\dot{V}O2peak$ and Fatmax intensity) for the two analysed groups. No significant differences between groups were observed at pre-intervention for any of the analysed variables at baseline (all $p > 0.05$).

**Table 6.3.** Mean (M) ± standard deviation (SD) of the pre- and post-values and the changes M ± SD [95% CI] of the analysed variables for the two intervention groups.

| | POST-WORKOUT MULTI-INGREDIENT (n=10) | | | COMPARATOR (n=10) | | | BETWEEN-GROUPS COMPARISONS | |
|---|---|---|---|---|---|---|---|---|
| Variables | Pre | Post | Changes [95% CI] | Pre | Post | Changes [95% CI] | *p* value | ES |
| Body Mass (kg) | 82 ± 17.9 | 82.6 ± 18.5 | 0.59 ± 1.3 [-1.48, 0.29] | 84.9 ± 17.6 | 85.4 ± 17.6 | 0.45 ± 1.4 [-1.34, 0.44] | 0.81 | 0.11 |
| Fat Mass (%) | 32.1 ± 6.4 | 30.7 ± 6.6 | -1.38 ± 0.7 [-1.94, -0.82]** | 31.9 ± 6.6 | 31.3 ± 6.7 | -0.52 ± 1 [-1.08, 0.04]$^{t}$ | 0.07 | 0.90 |
| Fat Mass (kg) | 26.7 ± 8.6 | 25.6 ± 8.7 | -1.09 ± 0.7 [-1.64, -0.54]** | 27.3 ± 8.9 | 26.9 ± 8.6 | -0.34 ± 1 [-0.89, 0.21] | 0.10 | 0.80 |
| Fat-Free Mass (%) | 67.9 ± 6.4 | 69.3 ± 6.6 | 1.38 ± 0.7 [0.82, 1.94]** | 68.2 ± 6.6 | 68.7 ± 6.7 | 0.52 ± 1 [-0.04, 1.08]$^{t}$ | 0.07 | 0.90 |
| Fat-Free Mass (kg) | 55.4 ± 11.6 | 56.8 ± 12.4 | 1.34 ± 1.2 [0.54, 2.15]** | 57.7 ± 11.8 | 58.5 ± 12.2 | 0.79 ± 1.2 [-0.02, 1.60]$^{t}$ | 0.25 | 0.56 |
| Waist Circumference (cm) | 88.2 ± 12.9 | 85.7 ± 12.5 | -2.5 ± 1.8 [-1.41, -3.59]** | 89.2 ± 12.3 | 88.8 ± 12.8 | -0.40 ± 1.5 [-1.49, 0.69] | 0.02 | 1.19 |
| Waist-to-hip ratio | 0.85 ± 0.08 | 0.82 ± 0.08 | -0.03 ± 0.03 [-0.04, -0.01]** | 0.85 ± 0.11 | 0.84 ± 0.11 | -0.01 ± 0.02 [-0.03, 0.01] | 0.20 | 0.62 |
| Vastus Lateralis Muscle Thickness (cm) | 2.54 ± 0.5 | 2.79 ± 0.5 | 0.24 ± 0.1 [0.17, 0.32]** | 2.61 ± 0.4 | 2.82 ± 0.5 | 0.21 ± 0.1 [0.14, 0.39]** | 0.40 | 0.40 |
| Elbow Flexors Muscle Thickness (cm) | 3.93 ± 0.7 | 4.3 ± 0.8 | 0.36 ± 0.2 [0.24, 0.49]** | 4.2 ± 0.7 | 4.36 ± 0.7 | 0.16 ± 0.2 [0.03, 0.28]* | 0.16 | 0.75 |
| CMJ (cm) | 27.6 ± 6.4 | 30.5 ± 6.4 | 2.87 ± 1.1 [2.12, 3.62]** | 27.2 ± 5.3 | 28.3 ± 5.6 | 1.12 ± 1.2 [0.38, 1.87]** | 0.01 | 1.47 |
| Seated Chest Medicine Ball Throw (m) | 3.54 ± 0.4 | 3.82 ± 0.3 | 0.27 ± 0.2 [0.15, 0.40]** | 3.82 ± 0.4 | 4.02 ± 0.4 | 0.2 ± 0.2 [0.07, 0.33]** | 0.99 | 0.01 |
| Total Volume Lifted (kg) | 10022 ± 2904 | 13687 ± 3791 | 3664 ± 1693 [2693, 4637]** | 10973 ± 2678 | 14412 ± 3411 | 3439 ± 1189 [2468, 4411]** | 0.48 | 0.34 |
| $\dot{V}O_2$peak (mL·kg$^{-1}$·min$^{-1}$) | 36.8 ± 8.2 | 40.1 ± 8.3 | 3.3 ± 2.5 [1.61, 4.99]** | 40.2 ± 6.4 | 42.6 ± 5.7 | 2.4 ± 2.6 [0.71, 4.09]** | 0.73 | 0.16 |
| Fatmax intensity (Watts) | 70 ± 31 | 80 ± 25 | 10.1 ± 9.2 [3.41, 16.79]** | 63 ± 18 | 73 ± 15 | 9.6 ± 10.9 [2.91, 16.29]* | 0.55 | 0.28 |

*$p \le 0.05$, **$p \le 0.01$, $^{t}p \le 0.10$ respect to baseline levels; ES is the standardized effect size presented as Cohen's *d*.

### *6.3.1. Changes in Body Composition*

Significant reductions of absolute (kg) and percentage of fat mass along with increases of fat-free mass were only observed for the POST-MTN group. Moreover, waist circumference and waist-to-hip ratio significantly diminished in the POST-MTN (-2.5 ± 1.8, d = 0.9 and -0.03 ± 0.03 d = 0.6 respectively) but not for the COMP group. Indeed, when adjusted values were considered, a significantly higher waist circumference reduction (p = 0.02) favouring the POST-MTN treatment with an effect size (d = 1.19) large enough to identify differences between groups was observed.

Both groups significantly enhanced VL (POST-MTN: +2.4±0.5mm; COMP: 2.1±0.5mm) and EF (POST-MTN: +3.6 ± 0.6 mm; COMP: +1.6±0.6 mm) muscle thickness, with no differences between groups.

### *6.3.2. Changes in Performance*

Compared to baseline, both POST-MTN and COMP groups significantly increased CMJ height (POST-MTN: 2.87 ± 1.1 d = 1.1; COMP: 1.12 ± 1.2, d = 0.8) and SMBT (POST-MTN: 0.27 ± 0.2; d = 1.2 COMP: 0.2 ± 0.2, d = 0.9). When adjusted values were considered, a more pronounced significant improvement (p = 0.005) and substantial effect size (d = 1.47) favouring the POST-MTN group. Nonetheless, no differences between groups were determined for SMBT at post-intervention. The total lifted workout volume significantly increased for both groups, with no differences between them when adjusted values were considered.

### *6.3.3. Exploratory Variables*

Both groups significantly improved $\dot{V}O2$peak and Fatmax intensity with no significant differences between them when adjusted values were considered.

The average scores on the Global Rating of Perceived Effort (RPE), assessed by the OMNI-RES (0-10) scale did not reveal any statistically significant disparities between the POST-MTN (8.3±0.8) and COMP (8.1±0.9) conditions. Additionally, no between-sexes differences were identified for any of the variables analysed, both groups similarly responded to treatment-induced changes in body composition and performance.

## 6.4. Discussion

Results of the present study suggest that ingesting a post-workout supplement, including carbohydrates, whey protein, creatine monohydrate, β-HMB and vitamin D, promoted noticeable body composition outcomes, not observed when consuming an isocaloric-only carbohydrate supplement. Nonetheless, except for the lower body impulsive force, where the POST-MTN group reached a higher post-intervention performance than the COMP, no differences between groups, were determined in supporting hypertrophy, upper body impulsive force, and endurance performance enhancements. Furthermore, no impact of the type of supplement was observed on workout volume (kg lifted) or the experienced global rating of perceived effort.

No significant change in energy or macronutrient intake was detected for either group (Table 6.2). Consequently, the primary distinction between treatments relied on the composition of the supplements. The analysis of dietary patterns indicated that all participants regardless of the group consumed ~3 $g \cdot Kg^{-1}$ of BM of carbohydrate (~44% of calories) slightly higher than 1 $g \cdot kg^{-1}$ of BM of protein (~15% of calories) and ~1.2 $g \cdot kg^{-1}$ of BM of fat (~40% of calories). These figures are below the recommended daily intake for carbohydrates (4-7 $g \cdot kg^{-1}$ of BM) and proteins (~1.6 $g \cdot kg^{-1}$ of BM) (Morton et al., 2018) while slightly above the recommended fat intake (30% of the calories) (Thomas, Erdman & Burke, 2016) for physically active individuals looking for hypertrophy (Thomas, Erdman & Burke, 2016).

Our results align with previous studies indicating the significance of specialised nutrition on body composition (Bonilla et al., 2021). The observed fat mass reduction experienced by the POST-MTN group underscores the impact of protein-containing post-workout meals on fat metabolism. The ingestion of whey protein has been associated with suppressed appetite, increased satiety and favours protein synthesis, which in turn would increase post-exercise thermogenesis (Acheson et al., 2011). Therefore, a hypothetically increased use of fat as the predominant fuel to support muscle remodelling and recovery could be the cause of the significant fat mass reduction observed in POST-MTN but not in the COMP group. Moreover, it has been suggested that lactalbumin and lactoferrin found in whey protein may increase postprandial lipolysis markers, improve energy balance, and decrease adiposity (Naclerio et al., 2019a). On the other hand, an increased post-workout protein intake could have been particularly impactful in stimulating post-exercise muscle protein synthesis in our participants who consumed lower daily protein intake than the recommended to support muscle mass accretion (Stokes et al., 2018; van Vliet et al., 2018). Although both groups similarly enhanced VL and EF thickness, a remarkably significant increase in FFM was observed only for the POST-MTN group ($p = 0.007$, $d = 1.10$), while only a trend towards significance ($p = 0.064$, $d = 0.65$) was noted for the COMP group. Furthermore, it is worth noticing, that although no statistical differences between groups was identified at post-intervention, a large effect size ($d = 0.90$), favouring the POST-MTN treatment was determined.

The relative amount of carbohydrate (~0.29 $g \cdot kg^{-1}$), protein (0.33 $g \cdot kg^{-1}$) and creatine monohydrate (0.06 $g \cdot kg^{-1}$) included in the post-workout multi-ingredient, administered to our participants was within the recommended effective doses to maximise muscle mass accretion (Cooper et al., 2012; Naclerio et al., 2020). Nonetheless, it seems that for middle-aged physically active participants ingesting a multi-ingredient supplement three times per week was not enough to induce significantly superior outcomes compared to the ingestion of only carbohydrates, at least over a 6-week training period. In summary, our results seem to support the notion that while specific nutrient compositions (reduced carbohydrate in favour of protein content while maintaining a similar energy consumption) may promote fat loss, body mass gain may also need an appropriate energy intake combined with resistance training to facilitate quality weight gain characterised primarily by FFM increase (Slater et al., 2019).

On the other hand, when considering performance outcomes, it is worth highlighting that the POST-MTN group showed greater CMJ performance increases than the COMP. However, such a difference between treatments was not seen for the medicine ball throw. In this regard, the applied training routine imposed a higher training volume on lower body muscles involved in knee and hip extension and therefore contributed to vertical jump-like action during four exercises (parallel squat, hang clean; and alternate lunges) whilst the upper body muscle action during shoulder flexion and elbow extension was mainly activated in only one exercise (bench press). These differences in the training overload may have impacted the observed results, particularly when considering that the most prominent resistance training adaptations and nutritional support benefits are likely to occur in the most heavily utilised muscle groups (Bernárdez-Vázquez et al., 2022). Additionally, it seems that for middle-aged physically active individuals, a 6-week resistance training programme, whether combined with the ingestion of a multi-ingredient or carbohydrates alone (Table 6.3), could be similarly effective in promoting endurance capacity with no differentiated effects on the global perception of effort. These results suggest that while targeted muscle hypertrophy and strength gains are influenced by specific training loads and muscle utilisation, broader endurance improvements can be achieved through consistent circuit-based resistance training and nutritional support, regardless of the specific supplement used.

Our investigation may be predisposed to several limitations. Firstly, despite the employment of a validated application (MyFitnessPal Inc.©) for recording nutritional intake, the self-reporting method employed might introduce measurement inaccuracies. A more standardised methodology would have entailed participants weighing their food prior to consumption. Secondly, the non-individualisation of the supplement dosage resulted in varying relative amounts of proteins (0.24 to 0.43 $g{\cdot}kg^{-1}$ of BM), carbohydrates (0.21 to 0.38 $g{\cdot}kg^{-1}$ of BM) and creatine (0.05 to 0.08 $g{\cdot}kg^{-1}$ of BM) among participants, potentially affording broader benefits to those with lighter body mass. However, subsequent statistical analysis examining individual variances in muscle mass accrual did not identify any notable differences between participants. Additionally, because our study was designed as a highly ecological intervention, avoiding diet modifications, the participants were asked to maintain their usual caffeine intake (avoiding caffeine or energetic intake 3 hours pre- and 2 hours post-workout during training days) so that the impact of integrating post-workout supplementation to the habitual diet could be assessed. Lastly, the included peri- and post-menopausal women were not complemented by any additional blood tests beyond a symptomatology questionnaire, which might have nuanced the results across different stages of menopause. Despite this, statistical assessments to discern variations across menopausal statuses did not demonstrate disparities for any investigated parameter. The potential for observing more pronounced differences between supplements could likely be enhanced with longer interventions using larger sample sizes. Nevertheless, given the study's ecological validity, we adhered to the dosages typically recommended by manufacturers.

Future research should endeavour to delineate the specific contributions of each constituent within multi-ingredient supplements to elucidate their singular and synergistic impacts on performance and body composition. Further exploration into the timing, dosage, and formulation of these supplements, in the context of diverse exercise modalities, promises to deepen our understanding of their effectiveness and mechanisms of action, particularly considering the widespread commercial/consumer use of such supplementation.

## 6.5. Conclusions

Compared to ingesting a post-workout-only carbohydrate beverage, a multi-ingredient providing carbohydrates, whey protein, and creatine enriched with L-leucine, β-HMB, and Vitamin D3, promoted fat mass decrease and fat-free mass increase, along with noticeable differences in waist circumference reduction and higher vertical jump performance enhancements. Nonetheless, no differences between supplements were observed in muscle thickness, upper body impulsive force, endurance performance, workout volume, or the experienced global rating of perceived exertion.

# CHAPTER 7

# SUMMARY AND CONCLUSIONS

The research conducted throughout the course of this PhD, culminating in the current thesis, has primarily focused on the effects of multi-ingredient supplementation on body composition, including changes in the muscular structure, and functional capacity in middle-aged and older adults. Eventually, 99 adults between 45-65 years old were assessed. Several research groups have investigated the specific nutritional needs of older populations (Liao et al., 2017; Morton et al., 2018; O'Bryan et al., 2019), with a focus on developing individualised dietary strategies (Boushey et al., 2020; Hinojosa-Nogueira et al., 2024; Hu, 2024), supplementation protocols (McGee et al., 2024; Rivero-Segura et al., 2024), and daily nutritional management (Chedraui & Pérez-López, 2013; Mazza et al., 2021; Bojang & Manchana, 2023) that address the unique requirements of this age group and enhance their functional capacity (Caçador et al., 2021), body composition (Verlaan et al., 2017; Dowling et al., 2024), strength (Cruz-Jentoft et al., 2020), and overall well-being and quality of life (Govindaraju et al., 2018; Gouela et al., 2024). Studies have consistently demonstrated that nutritional interventions, particularly in combination with tailored exercise programmes, can positively impact these outcomes (Cruz-Jentoft et al., 2020; Gielen et al., 2021). Despite the growing body of research on the potential benefits of combining proper nutritional strategies to maximise exercise adaptation in young athletic populations, a scientific gap regarding interventional investigations in healthy middle-aged and older (45-65 years old) physically active individuals, especially those who regularly engage in resistance training or moderate-to-vigorous exercise (Etnier et al., 2017; Erickson et al., 2023; Li, 2023) was identified. Most existing studies have also focused predominantly on sedentary populations (Martínez-Rodríguez et al., 2020), leaving a significant portion of active, healthy individuals underrepresented in current research. Furthermore, a gender disparity exists in the physical exercise and sports nutrition scientific literature (Devries & Jakobi, 2021; Kelly, 2023; Sims et al., 2023), with the majority of studies recruiting male participants only, thereby neglecting female populations. This gap was particularly evident in research exploring the effects of dietary interventions during the menopausal transition, a critical phase that influences various health outcomes in middle-aged women (Grigolon et al., 2023; Yelland et al., 2023).

The majority of existing research has concentrated on the effects of single macronutrient (Hettiarachchi et al., 2024), micronutrient (Giustina et al., 2023; De la Cruz-Góngora et al., 2024), or ergogenic aid supplementation such as proteins (Hettiarachchi et al., 2024), branched-chain amino acids (BCAAs) (Caldo-Silva et al., 2023), omega-3 fatty acids (Loong et al., 2023), vitamin D (Giustina et al., 2023), caffeine (Torres-Collado et al., 2018), and creatine (Candow et al., 2019). Furthermore, most studies investigated the effects of a single supplement (Cuyul-Vásquez, 2023). However, they did not address the interactions and synergistic effects of combining multiple nutrients, nor did they explore the optimal dosages or timing of multi-ingredient supplementation around physical exercise (Candow et al., 2006, 2008; Arnarson et al., 2013; Leenders et al., 2013; Bell et al., 2017; Holwerda et al., 2018; Nabuco et al., 2018, 2019; Krause et al., 2019). This background and the current state of the problem represent a significant gap in the literature, particularly regarding healthy, active, middle-aged populations who may benefit from the combined effects of pre- and post-workout supplementation strategies. Addressing this knowledge gap is essential for developing effective dietary protocols that can enhance performance, health, and well-being in this demographic (Puente-Fernández et al., 2023).

A systematic review with meta-analysis was conducted to evaluate the effects of multi-ingredient nutritional supplementation (MTN) combined with exercise on body composition, muscle strength, and functional capacity in healthy, recreationally active middle-aged and older adults (≥45 years). The key findings of this review can be summarised in different aspects. Firstly, the analysis of fat-free mass (FFM) and lean body mass (LBM) across ten treatment groups showed no significant improvements in MTN groups compared to the comparator. The overall effect size was very small, indicating no significant effect of adding MTN supplementation instead of an isocaloric carbohydrate supplement to resistance exercise programmes on body composition outcomes. Similarly, differences between groups consuming MTN vs. those ingesting the comparator in upper and lower body strength, as measured by the 1-repetition maximum (1-RM) test for bench press, leg press, and leg extension, were negligible. Furthermore, various functional tests (e.g., timed up-and-go, 5-times sit-to-stand…) revealed no significant differences between the MTN and comparator groups in terms of improvements in movement efficiency and lower limb function.

It is worth mentioning that the average daily protein intake in both MTN and comparator groups did not reach 1.6g·kg$^{-1}$, the recommended optimal intake for physically active individuals (Morton et al., 2018). The lack of significant differences in outcomes suggests that protein intake below this threshold may be insufficient to enhance exercise-induced benefits. Nonetheless, these findings contrast with prior studies that reported benefits of protein supplementation in older adults with chronic conditions, such as obesity or sarcopenia (Liao et al., 2017; O'Bryan et al., 2019). However, this review focused on healthy, non-obese populations, further highlighting the differential response to supplementation based on health status. The results from this review highlighted the importance of conducting further research focusing on the response of healthy and physically active middle-aged individuals, to elucidate the dose-response effects on those outcomes related to health and well-being, namely, changes in body composition (increase in fat-free mass and muscle thickness, and reduction in fat mass, waist circumference, and waist-to-hip ratio), functional capacity, including strength, mechanical power output and muscular endurance.

According to the results of the systematic review and meta-analysis, crossover studies aimed to analyse the acute and short-term impact of pre-workout (Study 2) and post-workout (Study 3) multi-ingredient supplementation were designed and subsequently conducted. These two studies were followed by two 6-week parallel pre- to post-interventional studies, comparing the effect of using a pre-workout (Study 4) or a post-workout (Study 5) protein-based multi-ingredient supplement vs. an isocaloric comparator.

The first interventional study (Study 2) aimed to analyse the acute impact of a caffeine-based multi-ingredient pre-workout supplement including amino acids compared to an isocaloric carbohydrate-only comparator on resistance and endurance training performance, substrate oxidation and muscle function in middle-aged, recreationally active men and women. Additionally, the subjective feelings of perceived effort were explored. Collectively, it was observed that when the participants consumed the MTN pre-workout supplement lifted more external load throughout the week, in total, counting the three resistance training workouts, and during each single resistance training session compared to the ingestion of only carbohydrates. Additionally, a significant increase in fatty acid oxidation under the pre-workout condition compared to the ingestion of only carbohydrates was also observed. This finding suggests that, at least during short-term interventions (1 week), pre-workout supplementation may promote higher fat metabolism during submaximal endurance exercise. This finding could suggest that the increased fat mobilisation observed under the pre-workout condition may also influence RT adaptations during longer interventions lasting for several weeks. However, in the 6-week intervention (Study 4) the participants allocated to ingest a similar pre-workout supplement failed to demonstrate a significant cumulative benefit to increase fat oxidation. Nonetheless, it is worth highlighting that the pre-workout group, showed a significant reduction in fat mass, along with a decrease in waist circumference, which likely suggests a reduction in visceral fat (Gadekar et al., 2020). Even though no differences between groups were observed at the end of the intervention period, the observed results would suggest that ingesting pre-workout supplements providing 406 mg of caffeine and 12g of essential amino acids, instead of only carbohydrates, may favour exercise performance and metabolic efficiency in middle-aged physically active individuals.

The third study compared the acute effects of a post-workout vegan protein-based multi-ingredient supplement (including 17g of pea protein, 8.49g of defatted cocoa protein powder, 32.99g of maltodextrin and 32.40g of dextrose) to the ingestion of carbohydrates alone on muscle voluntary (upper-and lower-body muscular mechanical power and endurance performance) and involuntary (tensiomyography) contractile properties. Furthermore, the perception of muscle soreness and weekly training volume (total external load lifted along the three resistance training circuits) was also explored. Overall, regardless of the conditions (pre-workout or carbohydrate only), a similar pattern of response was observed, with no significant difference across the 48h recovery time identified between conditions, for any of the analysed variables. Additionally, no sex-based differences were observed for any of the outcomes evaluated.

Finally, the last investigation (Study 5) aimed to compare the effectiveness of post-workout supplementation using a multi-ingredient mixture versus an isoenergetic carbohydrate-only comparator. The multi-ingredient supplement included carbohydrates, whey protein, creatine monohydrate, β-HMB, and Vitamin D3. The main findings were that the group consuming the post-workout experienced significant reductions in fat mass and waist circumference, along with increases in fat-free mass, and led to a significant reduction in waist-to-hip ratio. However, even though these changes were not determined for the comparator group, an interaction effect between groups was only observed for waist circumference. Both treatment groups exhibited significant improvements in muscle thickness and performance outcomes. However, for the vertical jump height, a greater increase was determined for post-workout treatment compared to the comparator group. Thus, it was concluded that over a 6-week intervention, compared to ingesting carbohydrates only in the post-workout period, a multi-ingredient providing whey protein, carbohydrates, creatine, β-HMB, and Vitamin D3, fortified with leucine promoted noticeable body composition outcomes and better vertical jump improvements with no further effects on hypertrophy, upper body and endurance performance.

In summary, this three-year research project successfully evaluated the impact of commercially available protein-based multi-ingredient formulations ingested pre- or post-workout on exercise-induced adaptations in middle-aged and older adults engaged in resistance training. Although the results did not consistently demonstrate a clear advantage of multi-ingredient supplements over isocaloric carbohydrate-only comparators across all measured outcomes, notable improvements in specific parameters such as fat mass reduction, waist circumference, and vertical jump performance were observed in certain conditions. These findings underscore the potential of multi-ingredient supplementation to influence body composition and specific performance metrics in a time-efficient manner. However, the overall lack of significant benefits in muscle hypertrophy and functional capacity calls for further research to determine the optimal composition, dosage, and timing of such formulations.

Future studies should focus on longer intervention periods and explore individualised strategies to enhance the synergistic effects of combined nutrients, thereby optimising exercise-induced outcomes in active middle-aged and older populations.

## CONCLUSIONS

When compared to an isoenergetic carbohydrate-only supplement, a caffeinated protein-based multi-ingredient formulation (Study 2) appears to enhance fatty acid oxidation during moderate-intensity endurance exercise, while concurrently offering ergogenic effects that facilitate increased total load lifted during resistance training sessions.

Ingesting a post-workout pea-protein-based multi-ingredient supplement (containing cocoa protein and combined with carbohydrates) vs. an isoenergetic carbohydrate-only comparator (Study 3) immediately after hard resistance training sessions does not seem to confer any acute or short-term benefits on muscle strength, muscular endurance, muscle contractility (evaluated using tensiomyography), or perceived muscle soreness.

While the multi-ingredient pre-workout supplement containing vegan protein and caffeine did not yield significant improvements over the carbohydrate-only supplement in most outcomes (Study 4), it exhibited a modest advantage in reducing waist circumference. However, the overall results indicate that regular pre-workout supplementation, administered three times per week, does not confer additional benefits in terms of body composition, muscle hypertrophy, or performance beyond those observed with an isoenergetic carbohydrate-only supplement in middle-aged, recreationally active adults undergoing resistance training. Thus, acutely consuming a caffeine-containing pre-workout supplement might provide benefits at specific resistance training sessions when aiming at lifting greater training loads or boosting fatty acid contribution during endurance training. However, this effect might only be present at specific single bouts of exercise, not leading to cumulative effects in the long term.

Post-workout supplementation with a multi-ingredient blend (whey protein, leucine, creatine monohydrate, β-HMB, and Vitamin D3) led to greater reductions in fat mass and waist circumference, as well as increased fat-free mass and improved waist-to-hip ratio, compared to an isoenergetic carbohydrate-only supplement (Study 5). Additionally, while both conditions improved muscle thickness and performance, the multi-ingredient supplement elicited a superior increase in vertical jump height, although no interaction between conditions was observed. These findings suggest that incorporating a high-leucine, multi-ingredient post-workout supplement may enhance muscle accretion and fat metabolism more effectively, making it a preferable choice for sustained adaptations in muscle mass development and fat loss.

# CHAPTER 8 – FUTURE WORK AND LINES OF RESEARCH

The research presented in this thesis has advanced our understanding of the impact of protein-based multi-ingredient formulations on exercise performance, training outcomes, and functional capacity in physically active adults. Despite the valuable insights gained, several gaps remain that warrant further investigation. This chapter outlines potential future research directions aimed at addressing expanding the applicability of our findings as well as translational applications, considered key applications applicable to the population of interest that has been the scope of this research.

## 8.1. Future lines of research suggested

### *8.1.1. Extending the Research to Older Populations*

Our studies primarily recruited middle-aged individuals between 45 and 65 years old. While this age group is critical for understanding age-related physiological changes, it is imperative to investigate older populations beyond 65 years of age. Ageing is associated with sarcopenia, reduced anabolic responses, and altered nutrient metabolism (Morley et al., 2010). Future research should focus on whether protein-based multi-ingredient supplementation can mitigate muscle loss, enhance functional capacity, and improve quality of life in older adults. Longitudinal studies involving this demographic could provide valuable insights into the long-term benefits and optimal dosing strategies for elderly populations.

### *8.1.2. Inclusion of Diverse Female Populations*

Our studies focused on peri-menopausal and recently post-menopausal women. Hormonal fluctuations during menopause can influence muscle mass, fat distribution, and metabolic rates (Lovejoy et al., 2008; Ambikairajah et al., 2019). Future research should include young and normally menstruating women, women undergoing hormonal replacement therapy (HRT), and those who have been post-menopausal for several years. This would allow for a comprehensive understanding of how hormonal status modulates the effectiveness of protein-based supplementation. Comparative studies across these subgroups could elucidate potential interactions between hormonal levels and nutrient metabolism.

### *8.1.3. Individualising Nutrient Quantities Relative to Body Mass*

The effects of specific, commercially available quantities of nutrients (such as 25 g of protein or 400 mg of caffeine) without adjusting for individual differences in body mass were assessed. Given that nutrient metabolism and physiological responses are often dose-dependent and influenced by body size (Thomas, Erdman & Burke, 2016), future studies should consider individualizing nutrient dosages relative to each participant's body mass or lean body mass. This approach could optimise the efficacy of supplementation and provide more personalised recommendations.

### *8.1.4. Evaluating Psychological Status and Wellbeing*

Physical performance and well-being are intrinsically linked to psychological factors such as motivation, mood, and perceived stress (Craft & Perna, 2004). Our research did not evaluate psychological status, which could moderate the effects of supplementation and training outcomes. Incorporating psychometric assessments in future studies would provide a holistic view of participant well-being and could identify potential psychosocial benefits of supplementation. Tools such as the Profile of Mood States (POMS) or the Perceived Stress Scale (PSS) could be employed for this purpose.

### *8.1.5. Extending Intervention Duration*

Our interventions spanned six weeks, encompassing 18 training sessions. Physiological adaptations to resistance training and nutritional interventions may require longer periods to manifest significantly (Wernbom, Augustsson & Thomeé, 2007). Extending the duration of future studies to several months or even up to a year would allow for the observation of chronic adaptations in muscle hypertrophy, strength gains, and metabolic changes. Long-term studies would also enable the assessment of sustainability and adherence to supplementation protocols.

### *8.1.6. Increasing Supplementation Frequency*

Participants in our studies consumed supplements three times per week, coinciding with training sessions. Daily supplementation may yield more pronounced effects by maintaining consistent nutrient availability and supporting recovery on non-training days (Areta et al., 2013). Future research should investigate the impact of increased supplementation frequency on muscle protein synthesis rates, recovery kinetics, and overall training adaptations.

### *8.1.7. Investigate other supplementation protocols*

Considering the acute ergogenic benefits observed with the caffeinated, protein-based pre-workout supplementation and the cumulative effects associated with the animal protein-based post-workout supplement, future research should explore a combined supplementation strategy. Specifically, regular post-workout supplementation could be implemented alongside pre-workout supplementation on days when participants experience reduced energy levels or increased fatigue, thereby potentially optimising performance and recovery outcomes.

## 8.2. Translational applications:

This last section integrates key findings and learnings from the present research programme, and translational applications to recreationally active middle-aged populations (>40-65 years old) who practice resistance training on a weekly basis. Therefore, after this 5-year process of research, several recommendations can be designed regarding pre- and post-workout multi-ingredient supplementation for this population.

Firstly, regarding overall supplementation protocols, three potential messages arise: (I) One standard dose does not fit everyone; therefore, specific calculations relative to body mass must be performed before initiating a supplementation protocol; (II) Generally speaking, subjects respond slightly differently to supplementation intake, so it is important to observe potential secondary (stomach bloating, gut disorders, etc.) or no effects to avoid unnecessary supplementation in certain populations; (III) Overall daily ingestion and nutritional habits are more relevant than taking a single supplement dose before or after training in order to maximise training outcomes (body composition or performance).

Secondly, specifically related to pre-workout supplementation, 3-6 mg/kg of caffeine intake (either combined with stimulant AA or CHO) consumed 30-45 minutes before endurance or resistance training has been observed to be effective to acutely maximise fatty acid mobilisation during light-to-moderate continuous cyclic and cardiovascular exercise, as well as increase to promote more visceral fat reduction in the long term, when consumed regularly. Therefore, this type of supplements would be interesting for those overweight populations (or high visceral adipose tissue accumulation) aiming at increasing fatty acids contribution to oxidative metabolism during training sessions. Additionally, although the caffeinated pre-workout supplement group lifted a greater total volume (measured in kilograms per session) during one training week, these pre-workout supplements failed to demonstrate similar or cumulative benefits in the long term (6 or more weeks). Moreover, a reduction in RPE or an increase in perception of energy levels or feeling of alertness were not observed either in the acute or 6-weeks interventional protocol. Therefore, the greatest interests of these supplements rely on the stimulant capacity and potential benefits of fatty acid mobilisation.

Thirdly, regarding post-workout supplementation aiming at speeding up recovery and promoting further gains in body composition, muscle strength and endurance capacity in the long term, a combination of ~25g of whey (but not pea) protein, with ~25g ~5g of creatine, ~1.5g of HMB, 1000 IU of Vitamin D, and enriched with leucine (~3g) might be beneficial compared to placebo. However, dosage would play a key role and thus, these dosages might be improved by individualising them with a thorough calculation relative to body mass (i.e.: $0.1g{\cdot}kg{\cdot}BM{\cdot}day^{-1}$ of creatine), fat-free mass, and/or to specific deficiencies such as Vitamin D or protein intake, as healthy subjects with a high dietary daily relative intake of high quality protein ($>1.2g{\cdot}kg{\cdot}BM^{-1}$) might not be benefited from these recommendations.

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# APPENDICES

## 1. Supplementary Material 1. Table 1 - Risk of Bias of the studies included for the Systematic Review and Meta-Analysis.

| Study | Sequence generation | Allocation concealment | Blinding of the participants and personnel | Blinding of outcome assessment | Incomplete outcome data | Selective outcome reporting | Similarity in baseline characteristics | TOTAL (-7 to 7) |
|---|---|---|---|---|---|---|---|---|
| **Candow et al. (2006)** | 1 | 1 | 1 | 1 | 0 | -1 | 0 | **3** |
| **Candow et al. (2008)** | 1 | 1 | 1 | 1 | 0 | -1 | 0 | **3** |
| **Arnarson et al. (2013)** | 1 | 1 | 1 | 1 | 0 | 0 | 0 | **4** |
| **Leenders et al. (2013)** | 1 | 1 | 1 | 1 | 0 | -1 | 0 | **5** |
| **Bell et al. (2017)** | 1 | 1 | 1 | 1 | 1 | -1 | 0 | **4** |
| **Holwerda et al. (2018)** | 1 | 1 | 1 | 1 | 1 | 1 | 0 | **6** |
| **Nabuco et al. (2018)** | 1 | 1 | 1 | 1 | 0 | 1 | -1 | **4** |
| **Krause et al. (2019)** | 1 | 1 | 1 | 1 | 0 | 0 | -1 | **3** |
| **Nabuco et al. (2019)** | 1 | 1 | 1 | 1 | 0 | 1 | -1 | **4** |

## 2. Supplementary Material 2. Figures S1-5. Funnel Plots of the Systematic Review and Meta-Analysis for the included studies and variables evaluated.

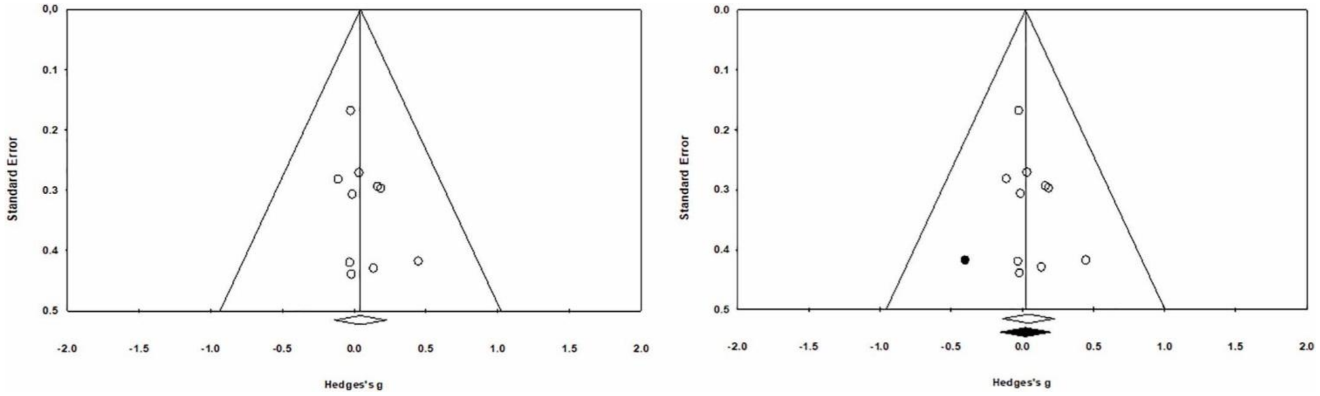

**Figure S1.** Upper-body strength calculated.

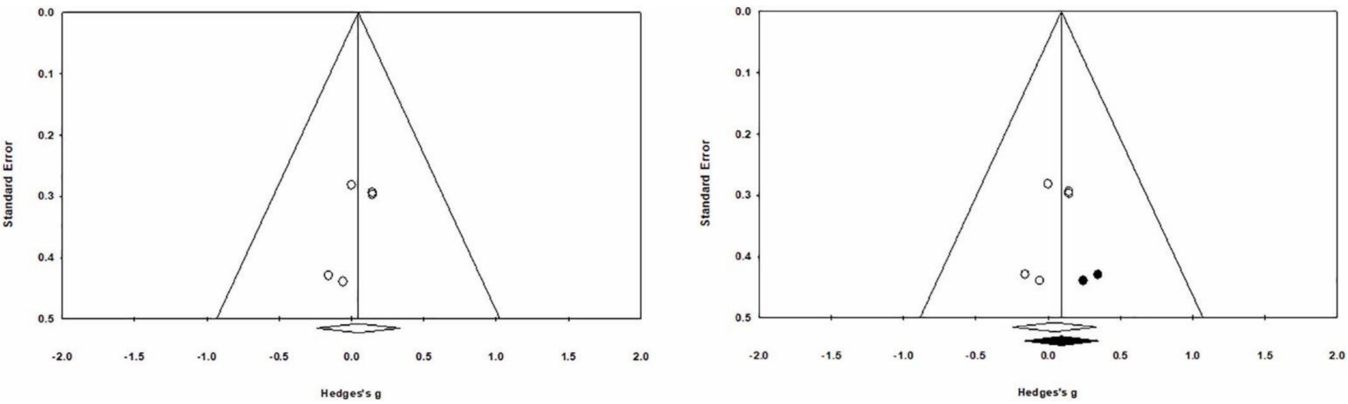

**Figure S2.** Fat-free mass calculated.

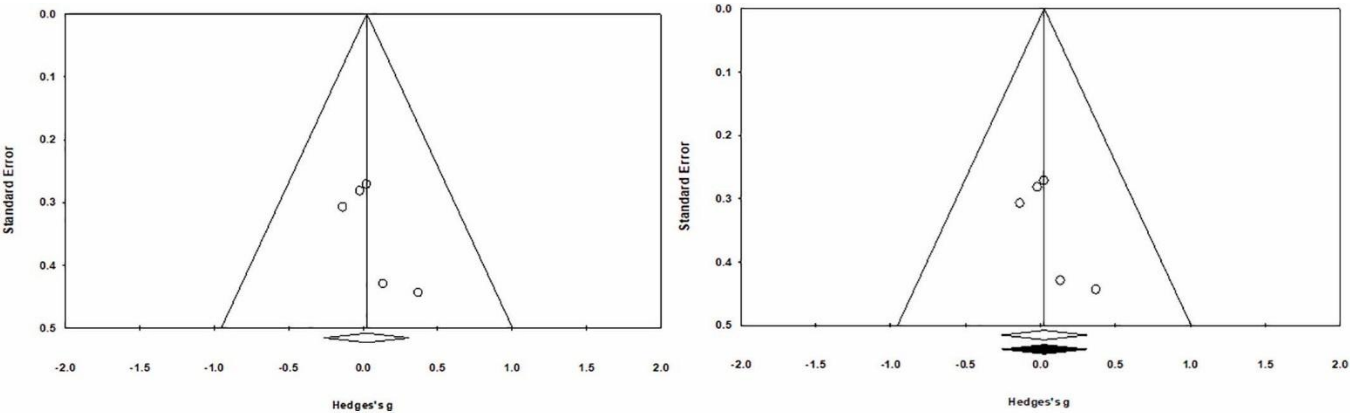

**Figure S3.** Lower-body (leg press) strength calculated.

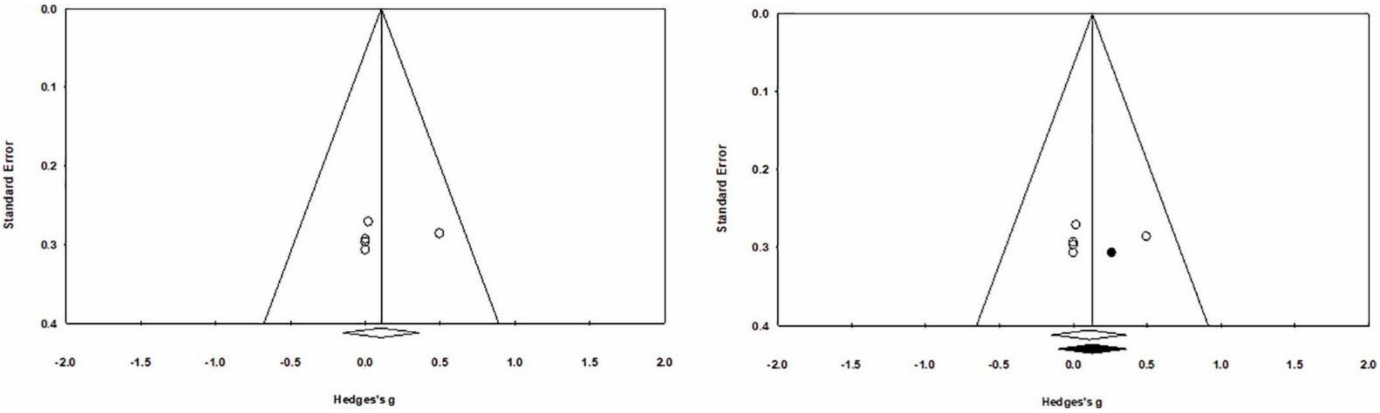

**Figure S4.** Lower-body (leg extension) strength calculated.

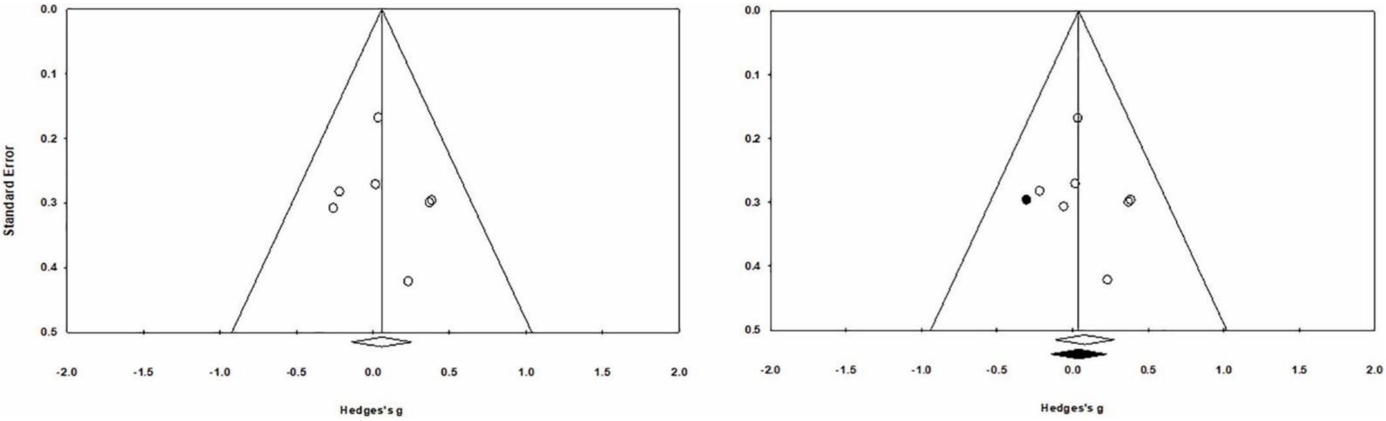

**Figure S5.** Functional capacity (TUG & 5-STS) calculated.

3. **Supplementary Material 3. Table S1 presents the results of the tensiomyography analysis for the three muscles evaluated at the 3 different time points in Study 2.**

| **Muscles** | **Conditions** | **PREW (n=14)** | | | **CHO (n=14)** | | | **ANOVA Repeated Measures (3 workouts x 2 supplements)** |
|---|---|---|---|---|---|---|---|---|
| | **Variables** | **RT 1** | **RT 2** | **RT 3** | **RT 1** | **RT 2** | **RT 3** | |
| Anterior Deltoids | Vc ($m \cdot s^{-1}$) | 0.002 ± 0.04<br>[-0.02, 0.03] | 0.01 ± 0.1<br>[-0.02, 0.04] | 0.02 ± 0.1<br>[-0.02, 0.05] | -0.001 ± 0.1<br>[-0.03, 0.03] | 0.01 ± 0.1<br>[-0.03, 0.05] | 0.00 ± 0.1<br>[-0.04, 0.05] | Workout: F(2,24)=0.970; p=0.392; $\eta^2 = 0.009$<br>Supplement: F(1,13)=0.393; p=0.542; $\eta^2 = 0.003$<br>Workout x Supplement: F(2,24)=0.797; p=0.461; $\eta^2 = 0.004$ |
| | Dm (mm) | -0.08 ± 1.7<br>[-1.11, 0.95] | 0.53 ± 2.1<br>[-0.75, 1.82] | 0.58 ± 2.2<br>[-0.72, 1.89] | -0.18 ± 2.1<br>[-1.47, 1.10] | 0.53 ± 2.6<br>[-1.05, 2.11] | -0.02 ± 2.8<br>[-1.72, 1.68] | Workout: F(2,24)=1.325; p=0.283; $\eta^2 = 0.014$<br>Supplement: F(1,13)=0.447; p=0.516; $\eta^2 = 0.003$<br>Workout x Supplement: F(2,24)=0.457; p=0.638; $\eta^2 = 0.003$ |
| | Tc (ms) | 0.04 ± 2.4<br>[-1.42,1.49] | 0.52 ± 2.0<br>[-0.69, 1.73] | 0.46 ± 2.4<br>[-0.96, 1.87] | -0.03 ± 2.1<br>[-1.30, 1.24] | 0.31 ± 2.3<br>[-1.09, 1.71] | -0.08 ± 1.8<br>[-1.18, 1.02] | Workout: F(2,26)=0.490; p=0.618; $\eta^2 = 0.006$<br>Supplement: F(1,13)=0.623; p=0.444; $\eta^2 = 0.004$<br>Workout x Supplement: F(2,26)=0.199; p=0.821; $\eta^2 = 0.002$ |
| Biceps Femoris Long Head | Vc ($m \cdot s^{-1}$) | 0.001 ± 0.02<br>[-0.01, 0.02] | 0.01 ± 0.02<br>[-0.01, 0.02] | 0.02 ± 0.02<br>[0, 0.03] | -0.004 ± 0.02<br>[-0.02, 0.01] | 0.001 ± 0.03<br>[-0.02, 0.02] | 0.002 ± 0.03<br>[-0.02, 0.02] | Workout: F(2,24)=1.141; p=0.336; $\eta^2 = 0.008$<br>Supplement: F(1,12)=0.001; p=0.976; $\eta^2 = 0.001$<br>Workout x Supplement: F(2,24)=0.503; p=0.611; $\eta^2 = 0.001$ |
| | Dm (mm) | -0.12 ± 1.3<br>[-0.94, 0.7] | 0.23 ± 1.43<br>[-0.67, 1.13] | 0.32 ± 1.46<br>[-0.60, 1.24] | -0.28 ± 1.20<br>[-1.03, 0.47] | -0.28 ± 1.70<br>[-1.35, 0.79] | -0.20 ± 1.75<br>[-1.29, 0.91] | Workout: F(2,24)=0.272; p=0.765; $\eta^2 = 0.001$<br>Supplement: F(1,12)=0.026; p=0.874; $\eta^2 = 0.000$<br>Workout x Supplement: F(2,24)=0.040; p=0.961; $\eta^2 = 0.000$ |
| | Tc (ms) | -1.20 ± 10.78<br>[-7.81, 5.42] | -1.46 ± 8.94<br>[-6.77, 3.85] | -3.05 ± 9.49<br>[-8.56, 2.47] | -0.20 ± 8.4<br>[-5.16, 4.76] | -3.6 ± 8.85<br>[-8.43, 1.23] | -2.83 ± 7.98<br>[-7.33, 1.67] | Workout: F(2,24)=0.397; p=0.677; $\eta^2 = 0.003$<br>Supplement: F(1,12)=0.266; p=0.615; $\eta^2 = 0.001$<br>Workout x Supplement: F(2,24)= 0.474; p=0.628; $\eta^2 = 0.038$ |
| Vastus Medialis | Vc ($m \cdot s^{-1}$) | -0.004 ± 0.02<br>[-0.02, 0.01] | 0.001 ± 0.03<br>[-0.02, 0.02] | -0.02 ± 0.02<br>[-0.02, 0.01] | -0.006 ± 0.02<br>[-0.02, 0.01] | -0.002 ± 0.02<br>[-0.02, 0.01] | -0.01 ± 0.02<br>[-0.02, 0.00] | Workout: F(1,12)=1.561; p=0.231; $\eta^2 = 0.019$<br>Supplement: F(1,12)=0.992; p=0.339; $\eta^2 = 0.018$<br>Workout x Supplement: F(2,24)=1.052; p=0.365; $\eta^2 = 0.009$ |
| | Dm (mm) | -7.14 ± 1.5<br>[-1.64, 0.21] | -0.36 ± 1.8<br>[-1.46, 0.74] | -0.63 ± 1.4<br>[-1.44, 0.18] | -0.82 ± 1.2<br>[-1.54, -0.1] | -0.59 ± 1.16<br>[-1.3, 0.12] | -0.93 ± 1.23<br>[-1.68, -0.17] | Workout: F(2,24)=1.779; p=0.190; $\eta^2 = 0.013$<br>Supplement: F(1,12)=1.364; p=0.265; $\eta^2 = 0.013$<br>Workout x Supplement: F(2,24)=0.542; p=0.589; $\eta^2 = 0.004$ |
| | Tc (ms) | -3.37 ± 12.5<br>[-11.14, 4.4] | -2.57 ± 13.3<br>[-10.88, 5.74] | -3.9 ± 12.2<br>[-11.28, 3.48] | -3.62 ± 12.2<br>[-11.19, 3.95] | -3.38 ± 12.4<br>[-11.17, 4.42] | -3.37 ± 11.3<br>[-10.44, 3.7] | Workout: F(2,24)=0.355; p=0.705; $\eta^2 = 0.001$<br>Supplement: F(1,12)=1.247; p=0.286; $\eta^2 = 0.002$<br>Workout x Supplement: F(2,24)=1.176; p=0.326; $\eta^2 = 0.002$ |

Notes: All values are adjusted using sex as a covariate. All p>0.05

### 4. Supplementary Material 4. Table S2 presents the results of the analysis for the four exercises evaluated at the 3 different time points in Study 3.

| **Conditions** | **POSTW (n=12)** | | | **COMP (n=12)** | | | **ANCOVA Repeated Measures (3 times x 2 conditions)** | **Conditions Comparisons** | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Variables** | **Post-1h** | **Post-24h** | **Post-48h** | **Post-1h** | **Post-24h** | **Post-48h** | | **Post-1h** | **Post-24h** | **Post-48h** |
| **CMJ (cm)** | -1.33±1.32$^{*\S}$<br>[-1.90, -0.76] | 0.17±1.30<br>[-0.39, 0.73] | 0.77±1.31<br>[0.20, 1.34] | -1.28±1.55$^{*\S}$<br>[-1.95, -0.61] | 0.46±1.10<br>[-0.01, 0.94] | 0.57±1.46<br>[-0.06, 1.20] | Time: *F*(2,22) = 13.01; *p* = 0.002; $\eta^2_G$=0.72<br>Time x Condition: *F*(2,70) = 1.41; *p* = 0.29; $\eta^2_G$=0.22<br>Condition: *F*(1,23) = 0.61; *p* = 0.45; $\eta^2_G$=0.05 | *p* = 0.84<br>ES = 0.04 | *p* = 0.42<br>ES = 0.24 | *p* = 0.65<br>ES = 0.14 |
| **CMBT (m)** | -0.10±0.15$^{*\S}$<br>[-0.16, -0.03] | 0.01±0.16<br>[-0.06, 0.08] | 0.06±0.18<br>[-0.02, 0.14] | -0.10±0.19$^{*\S}$<br>[-0.18, -0.02] | -0.05±0.15<br>[-0.12, 0.02] | 0.02±0.12<br>[-0.03, 0.07] | Time: *F*(2,22) = 6.51; *p* = 0.02; $\eta^2_G$=0.57<br>Time x Condition: *F*(2,70) = 2.08; *p* = 0.18; $\eta^2_G$=0.29<br>Condition: *F*(1,23) = 1.32; *p* = 0.28; $\eta^2_G$=0.11 | *p* = 0.87<br>ES = 0.01 | *p* = 0.31<br>ES = 0.38 | *p* = 0.31<br>ES = 0.26 |
| **30STS (reps)** | -0.42±1.63$^{\S}$<br>[-1.12, 0.29] | 0.92±1.98<br>[0.06, 1.77] | 2.00±2.04*<br>[1.12, 2.88] | -0.38±1.63$^{\S}$<br>[-1.08, 0.33] | 0.79±1.96<br>[-0.05, 1.64] | 2.00±1.71*<br>[1.26, 2.74] | Time: *F*(2,22) = 21.22; *p* = 0.001; $\eta^2_G$=0.81<br>Time x Condition: *F*(2,70) = 1.34; *p* = 0.31; $\eta^2_G$=0.21<br>Condition: *F*(1,23) = 0.01; *p* = 0.94; $\eta^2_G$=0.01 | *p* = 0.93<br>ES = 0.01 | *p* = 0.81<br>ES = 0.06 | *p* = 0.99<br>ES = 0.01 |
| **30BP (reps)** | -0.71±2.2$^{T\S}$<br>[-1.40, 0.05] | 0.58±1.9<br>[-0.03, 1.06] | 1.63±2.1*<br>[0.98, 2.19] | -0.71±2.9$^{T\S}$<br>[-1.43, 0.59] | 1.04±3.0<br>[0.06, 2.07] | 1.88±3.2*<br>[0.47, 2.69] | Time: *F*(2,22) = 15.45; *p* = 0.01; $\eta^2_G$=0.76<br>Time x Condition: *F*(2,70) = 0.96; *p* = 0.42; $\eta^2_G$=0.17<br>Condition: *F*(1,23) = 0.08; *p* = 0.93; $\eta^2_G$=0.01 | *p* = 0.99<br>ES = 0.01 | *p* = 0.56<br>ES = 0.18 | *p* = 0.67<br>ES = 0.09 |

Notes: CI = Confidence Interval; ANOVA = Analysis of Variance.
ES is the Standardised Effect Size presented as Cohen's *d*.
*p < 0.05 and $^{T}$p <0.10 respect to baseline values; $^{\S}$p < 0.01, respect to 48 h values.

5. **Scientific publication in the scientific journal “Nutrients” (Q1; Impact Factor: 5.7), with the title: “Effects of Multi-Ingredient Pre-workout Supplementation across a Five-Day Resistance and Endurance Training Microcycle in Middle-Aged Adults”. DOI: 10.3390/nu12123778. (2020).**

6. **Poster presentation at the 25th Annual European Congress of Sports Science (ECSS), with the title: *“Effects of multi-ingredient pre-workout supplementation across a 5-day resistance and endurance training microcycle in middle-aged adults”*. (2020).**

7. **Poster presentation at the International Sport & Exercise Nutrition Conference 2020, with the title: “Effects of multi-ingredient pre-workout supplementation across a 5-day resistance and endurance training microcycle in middle-aged and pre-menopausal women”. (2020).**

8. **Poster presentation at the 26th Annual European Congress of Sports Science (ECSS), with the title: *“Healthy habits: The status of middle-aged and older adult Spanish population one year after the beginning of covid19 pandemic”*. (2021).**

9. **Poster presentation at the International Sport & Exercise Nutrition Conference 2021, with the title: “the healthy habits and wellbeing status of Spanish and UK middle-aged and older adult population after one year of covid19 pandemic”. (2021).**

10. **Oral communication at the 27th Annual European Congress of Sports Science (ECSS), with the title: *"The recovery effects of a new vegan protein-based multi-ingredient supplement on voluntary muscular contraction and muscle contractile properties in peri-menopausal women".* (2022).**

11. **Scientific publication in the scientific journal "Experimental Gerontology" (Q2 Impact Factor: 4.6), with the title: *"No impact of combining multi-ingredient supplementation with exercise on body composition and physical performance, in healthy middle-aged and older adults: A systematic review and meta-analysis".* DOI: 10.1016/j.exger.2022.112079. (2022).**

12. **Oral communication at the 17th FIEPS European Congress – 100 years of FIEPS, with the title: "*The effects of a vegan multi-ingredient pre-workout supplement in body composition, resting metabolic rate and performance in middle-aged peri- and post-menopausal women".* (2023).**

13. **Poster presentation at the 28th Annual European Congress of Sports Science (ECSS), with the title: *"The effects of a vegan multi-ingredient pre-workout supplement in body composition, resting metabolic rate, and performance in middle-aged peri-and post-menopausal women".* (2023).**

14. **Scientific publication in the scientific journal "Journal of the International Society of Sports Nutrition" (Q1 Impact Factor: 1.6), with the title: *"Pre-Workout Multi-Ingredients or Carbohydrate Alone Promote Similar Resistance Training Outcomes in Middle-aged Adults: A Double-Blind, Randomized Controlled Clinical Trial.".* DOI: 10.1080/15502783.2025.2519515. (2025).**

15. **Scientific publication in the scientific journal “Journal of Dietary Supplements” (Q2 Impact Factor: 1.9), with the title: *“Effect of a Multi-Ingredient Post-Workout Dietary Supplement on Body Composition and Muscle Strength - A Randomized Controlled Trial”*. DOI: 10.1080/19390211.2025.2488811. (2025).**