Document Version

This is the author's preprint.

Citation for published version (APA):

Muñoz, A., de la Rubia, A., Lorenzo-Calvo, J., Karayigit, R., Garcés-Rimón, M., López-Moreno, M., Domínguez, R., Scanlan, A. T., & López-Samanes, Á. (2024). Multiday Beetroot Juice Ingestion Improves Some Aspects of Neuromuscular Performance in Semi-Professional, Male Handball Players: A Randomized, Double-Blind, Placebo-Controlled, Crossover Study. International Journal of Sport Nutrition and Exercise Metabolism (published online ahead of print 2024). Retrieved Nov 29, 2024, from https://doi.org/10.1123/ijsnem.2024-0113

Citing this paper

Please note that where the full-text provided on Comillas' Research Portal is the Preprint versión, preprints are early stage research papers that have not been peer-reviewed.

General rights

Copyright and moral rights for the publications made accessible in the Research Portal are retained by the authors and/or other copyright owners nd it is a condition of accessing publications that users recognize and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the Research Portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain.
- You may freely distribute the URL identifying the publication in the Research Portal.

Take down policy

If you believe that this document breaches copyright please contact Comillas providing details, and we will remove access to the work immediately and investigate your claim

INTERNATIONAL JOURNAL OF SPORT NUTRITION AND EXERCISE METABOLISM

Multi-day beetroot juice ingestion improves some aspects of neuromuscular performance in semi-professional, male handball players: A randomized, double-blind, placebo-controlled, crossover study

Journal:	*International Journal of Sport Nutrition & Exercise Metabolism*
Manuscript ID	IJSNEM.2024-0113.R2
Manuscript Type:	Original Research
Keywords:	nutrition, team-sports, suuplement, nitric oxide, anaer, strength

SCHOLARONE™
Manuscripts

Multi-day beetroot juice ingestion improves some aspects of neuromuscular performance in semi-professional, male handball players: A randomized, double-blind, placebo-controlled, crossover study

Preferred Running Head: Multi-day beetroot juice and neuromuscular performance in handball

Abstract Word Count: 197

Number of References: 44

Text-Only Word Count: 4406

Number of Figures and Tables: 2 Figures, 1 Table

For Peer Review

ABSTRACT

The aim of this study was to examine the effects of multi-day beetroot juice ingestion on neuromuscular performance in semi-professional, male handball players. Twelve handball players competing in the second [redacted] national division received 70 mL of beetroot juice (6.4 mmol of nitrate [NO_3^-]) or 70 mL of a placebo beetroot juice (0.04 mmol NO_3^-) for three consecutive days in a randomized, double-blind, crossover manner with a one-week wash-out between conditions. Following supplementation in each condition, players completed a neuromuscular test battery involving handball throwing, isometric handgrip strength, countermovement jump, change-of-direction speed, and repeated-sprint assessments, with side effects. Countermovement jump (4.7%; $P = 0.038$; Hedge's $g_{av} = 0.29$) and isometric handgrip strength (7.8%; $P = 0.021$; $g_{av} = 0.59$) were significantly superior with beetroot juice ingestion compared to the placebo. In contrast, non-significant differences were evident between conditions for all other neuromuscular performance variables ($P > 0.05$; $g_{av} = 0.00–0.27$). Red urine production was the only side effect demonstrating a significantly higher prevalence ($P = 0.046$) with beetroot juice ingestion. Three days of beetroot juice supplementation may be a useful nutritional strategy in semi-professional, male handball players given its ergogenic benefit to some aspects of neuromuscular performance.

Keywords: team-sports, nutrition, nitric oxide, inorganic nitrate, power, strength

For Peer Review

INTRODUCTION

Handball is a court-based team sport characterized by intermittent, repeated, intense neuromuscular actions such as jumping, changing direction, sprinting, and throwing alongside technical-tactical actions such as blocking, body contacts, and passing (Karcher & Buchheit, 2014). Although handball has similarities with other team sports, the time-outs and unlimited number of substitutions available to coaching staff provides frequent recovery opportunities for players to optimally maintain execution of high-intensity movements during key moments throughout games (Gutiérrez-Aguilar et al., 2016). In addition, the number of players competing at any time per team (i.e., 6 field players plus a goalkeeper) combined with the relatively small playing area (i.e., 40-m long and 20-m wide), places strong emphasis on the ability to accelerate and decelerate rapidly, jump explosively, and sprint repeatedly during offensive and defensive scenarios in games (Karcher & Buchheit, 2014) Consequently, various neuromuscular physical qualities must be adequately developed in line with these intense game demands among handball players, such as muscle strength, muscle power, and repeated-sprint ability (Munoz et al., 2020).

To optimize performance among their players, handball coaching and performance staff may consider nutritional strategies that could enhance neuromuscular physical qualities important for success in handball. Regarding nutritional supplements, beetroot juice is a good source of nitrate (NO_3^-), which is a precursor of nitric oxide (NO) through the NO_3^- to nitrite (NO_2^-) to NO pathway (Lundberg et al., 2008). This pathway is thought to promote increased sarcoplasmic reticulum calcium release and re-uptake with ensuing enhancements in force output within type II muscle fibers (Hernandez et al., 2012), as well as increased neurotransmitter release (Esen et al., 2022) and attenuated muscle potassium efflux (Wylie et al., 2013) that could benefit neuromuscular performance. Consequently, it is thought that a minimum threshold may exist for dietary NO_3^- consumption of >5 mmol to enhance athletic performance (Senefeld et al., 2020). In turn, systematic reviews have documented that the use of beetroot juice supplementation enhances muscle strength and power-related attributes (Gonzalez et al., 2023), as well as performance in repeated high-intensity activity bouts (Alsharif et al., 2023; Dominguez et al., 2018). However, beetroot juice supplementation has been shown to have equivocal effects on neuromuscular performance specifically among team-sport athletes, which appears to be dependent upon the timeframe across which ingestion occurred (Fernandez-Elias et al., 2022; Thompson et al., 2016). More precisely, studies have mostly reported no significant impact on neuromuscular physical qualities with single-dose, acute beetroot juice supplementation in professional, female field-hockey players (Lopez-Samanes et al., 2023), male team-sport

For Peer Review

athletes (Reynolds et al., 2020), and junior (15.6 ± 0.5 years), regional, male basketball players (Lopez-Samanes et al., 2020), while other studies have reported benefits to occur with supplementation followed across five to seven days in recreational or amateur male athletes pooled across various team sports (Nyakayiru et al., 2017; Thompson et al., 2016; Thompson et al., 2015). However, while initial evidence is promising, research into the effects of multi-day beetroot juice supplementation on neuromuscular performance is lacking overall in professional or semi-professional team-sport athletes, with no sport-specific data provided for handball players. In this regard, existing findings for multi-day beetroot supplementation reported in recreational and amateur team-sport athletes cannot be simply translated to higher playing levels or specifically to handball players given these factors likely predispose to unique fitness profiles and physical attributes, which in turn can impact the effectiveness of beetroot supplementation (Zamani et al., 2021).

To our knowledge, only one study has analyzed the effects of an acute, single dose of beetroot juice among adolescent (13.0 ± 0.2 years), amateur, female handball players (Nogueira & De Viebig, 2016). In this study, 500 mL of beetroot juice ingested 3 h prior to testing significantly (P <0.05) improved repeated-sprint performance compared to a placebo, while no differences were reported in blood pressure, heart rate, and perceived exertion. However, this research only examined repeated-sprint performance in 10 adolescent, female handball players, which limits the scope of neuromuscular qualities for which evidence is provided and the transfer to adult, male players given the NO_3^- response may vary according to age, training status, and sex (Shannon et al., 2021). Moreover, the effects of multi-day beetroot juice supplementation as opposed to a single acute dose remains to be investigated among handball players. Thus, the aim of this study was to determine the effects of three days of beetroot juice ingestion on neuromuscular performance in semi-professional, male handball players.

For Peer Review

METHODS

Subjects

A total of nineteen male handball players from the same team competing in the Second Division of the [redacted] National League were assessed for eligibility and seventeen semi-professionals were recruited for this study. However, five players were excluded due to failure in attending both experimental sessions (n = 3) or non-adherence to the prescribed supplementation protocol (n = 2) (Figure 1). Consequently, the sample consisted of 12 players (age: 21.5 ± 5.7 years; height: 1.85 ± 0.04 m; body mass: 83.2 ± 11.3 kg; competitive handball experience: 12.8 ± 6.3 years; training duration per week: 9.8 ± 2.6 h). An a priori sample size calculation using G*Power (version 3.1.9.2; University of Dusseldorf; Dusseldorf, Germany) indicated that nine players were needed to obtain statistically significant differences in neuromuscular performance (i.e., using countermovement jump) between two groups using an effect size of 1.20 (Clifford et al., 2016), β = 0.80, and two-tailed $\alpha = 0.05$. Exclusion criteria for participation included intolerance to beetroot juice or NO_3^- derivatives, suffering from any chronic pathology or injury in the month prior to the investigation, and/or use of medication or supplements (e.g., caffeine) during the study. Players (and guardians for the three players who were <18 years) were informed of all experimental procedures, including the risks and benefits of participation, before providing consent to participate. The Bioethics Commission of the [redacted] University approved the study (no: 46/2018), which complied with the Declaration of Helsinki.

*****INSERT FIGURE 1 AROUND HERE*****

Procedures

A randomized, double-blind, placebo-controlled crossover study design was adopted. Although a counterbalanced approach could not be undertaken when allocating subjects to treatments upon the initial trial due to the uneven nature of the sample (n = 17), a counterbalanced design was ultimately followed with the 12 included subjects evenly completing each treatment in the initial trial. Each player participated in two identical experimental trials separated by one week to allow physical recovery between testing occasions and washout from the beetroot juice treatment. For three consecutive days handball players ingested 70 mL of beetroot juice (6.4 mmol of NO_3^-, Beet-It-Pro Elite Shot, James White Drinks Ltd., Ipswich, UK) made from concentrates of beetroot juice (98%) and lemon juice (2%) (nutritional information (per 100 mL), energy: 373 kJ; fat: 0 g; carbohydrates: 18 g [sugars: 17 g]; protein: 3.7 g; salt: 0.48 g) or 70 mL of a placebo drink with an identical composition to the beetroot juice but depleted of nitrates. (i.e., 0.04 mmol of NO_3^-, Beet-It-Pro Elite Shot, James White Drinks Ltd., Ipswich, UK) at the same time on each day (17:00), including the testing day (day 3). This timing was chosen to align with previous studies that established the peak response nitrate/nitrite (NO_3^- and NO_2^-) occurs 2–3 hours after ingestion (Wylie et al., 2013) so that the likely ergogenic response occurred during neuromuscular testing, which was conducted from 19:30–20:15). Fluids ingested in each trial were matched in flavor, appearance, and packaging. Player randomization was conducted using an online tool (https://www.randomizer.org/). An external researcher was responsible for assigning the alphanumeric code for each sequence to blind players and researchers during the trials. The codes were unveiled to the researchers after statistical analysis.

For Peer Review

In addition, two days prior to the initial testing session, players underwent a familiarization session via explanation, demonstration, and completion of the testing battery to negate any learning effects. To ensure standardization of test administration and measurement across sessions, all tests were completed in an identical order on the same, indoor, hardwood handball court where players habitually trained and at the same time of day (19:30) to avoid any circadian variations in performance. Testing was conducted midway through the in-season phase. Across testing sessions, mean ± standard deviation (SD) air temperature was 13.5. ± 0.7 °C and relative humidity was 43.5 ± 2.1% measured with a portable weather station (Meteorological Station, Küken, Spain).

Two days prior to study commencement, dietary NO_3^- intake was restricted by instructing players to avoid NO_3^- rich foods provided in writing (e.g. beetroot, celery, lettuce, arugula, spinach, kale). Players were encouraged to avoid brushing their teeth, using any oral antiseptic rinse or chewing gum, and ingesting sweet foods that could alter their oral microbiota to interfere with NO_3^- concentrations during the whole experimental trial (Burleigh et al., 2019). Players were also instructed to refrain from any type of exercise 24 h before testing and to follow a daily energy intake breakdown of 60% carbohydrates, 30% fats, and 10% proteins during each experimental trial (all players completed 3-day food diaries prior to testing to confirm they followed the macronutrient recommendations) (Yang et al., 2010). Therefore, macronutrient intake was reviewed upon completion by one of the researchers to ensure that subjects adhered to the nutritional recommendations throughout the study. Saliva samples for determining NO_3^- and NO_2^- concentrations were obtained for each player 150 min after beetroot juice or placebo ingestion on the third day in each trial and were stored at -20°C for four months until subsequent analysis (Richard et al., 2018). To confirm the effectiveness of beetroot juice supplementation on $NO_3^-$and NO_2^- levels, saliva concentrations were measured using a nitric oxide assay kit (EMSNO K195325, Thermo Fisher Scientific, Roskilde, Denmark) according to the manufacturer's instructions and as reported previously (Richard et al., 2018). All samples were measured in duplicate and averaged for use in analyses. Rating of perceived exertion (RPE) was measured individually for each player 30 min after completing the testing battery in each trial using the adapted version of Borg's category ratio scale (Foster et al., 2001). Players also completed a questionnaire on the morning following completion of each experimental trial to identify any side effects associated with beetroot juice ingestion using yes/no responses (see Table 1 for list of side effects assessed) as used previously in similar research (Lopez-Samanes et al., 2022).

For Peer Review

Neuromuscular performance testing battery

All neuromuscular performance variables gathered during the testing battery presented acceptable test-retest reliability (intraclass correlation coefficient: 0.90–0.99; coefficient of variation: 0.9–4.1%) according to previous studies (Sassi et al., 2009). The following tests were administered in the order they are presented.

Ball throwing test

Ball throwing was performed using two separate handball-specific distances (i.e., at the 7-m and 9-m penalty lines) with a preparatory three-step run-up before jumping vertically and throwing the ball at each distance as previously reported (Munoz et al., 2020). For the 7-m throw, the test followed the international rules (International Handball Federation) for the penalty throw in handball (Munoz et al., 2020). Handball players performed three throws at each distance separated by 60 s of passive standing recovery. Players were instructed to throw at maximal velocity for all attempts. A radar gun (Stalker Solo 2 Radar Gun, Applied Concepts, Texas, USA) was used to record maximal ball velocity ($km \cdot h^{-1}$), held by an investigator 2 m behind players. The fastest of the three attempts for each distance was used for analysis.

Isometric handgrip strength test

Isometric handgrip strength was measured in the dominant hand using a calibrated handgrip dynamometer (Takei 5101; Takei, Tokyo, Japan). Players sat with their back straight, legs in an extended position in front of the body, tested arm in front of the body in an extended position, the contralateral arm beside the body, and the forearm and hand (of the tested arm) in a neutral position (Lopez-Samanes et al., 2020). Players held the dynamometer handle between the second interphalangeal joint of the four fingers and the base of the thumb. Players were instructed to squeeze the dynamometer handle with maximal effort for 5 s. The highest force output (N) from two attempts, which were separated by a 60-s passive standing recovery period, was used for analysis.

Countermovement jump

Players commenced the countermovement from an erect standing position with a straight torso, knees fully extended, hands on hips, and feet shoulder-width apart maintaining this position for at least 2 s before the descending phase. Players were encouraged to execute a fast downward movement to approximately 90º knee flexion, although the squat depth was self-selected, and immediately follow this movement with a fast upward vertical movement to jump as high as possible in the one sequence (Slinde et al., 2008). When landing, both feet were required to be within the borders of the contact mat. Jump height

For Peer Review

(cm) was measured using a contact platform (Chronojump Boscosystem, Barcelona, Spain), with the highest jump used for analysis. Players completed two maximal countermovement jumps following established methodology with 45 s of passive standing recovery between jumps (Bosco et al., 1983).

Modified Agility T-test

The Modified Agility T-test was administered using the foundation protocol previously described. Players began the test in a split stance with the toes of their leading foot positioned 1 m behind the starting line to avoid inadvertent triggering of timing. The test involves an initial forward sprint for 5 m, then side-shuffle to the left for 2.5 m, side-shuffle to the right for 5 m, side-shuffle back to the left for 2.5, and backpedal for 5 m to the starting point (Sassi et al., 2009). Two electronic timing gates (Polifemo Radio Light, Microgate, Italia) were set 1 m above the ground and positioned 3 m apart on either side of the start/finish line. Timing started when players passed through the initial timing gate. The quickest performance (s) from two attempts separated by 2 min of passive standing recovery was used for analysis.

Repeated-sprint test

The repeated-sprint test consisted of six 30-m shuttle sprints with a 180º change in direction at 15 m (15 m + 15 m) in each shuttle. Players started a new sprint every 20 s (e.g., if a shuttle sprint was performed in 6 s, there was a 14-s recovery interval before the next shuttle (Okuno et al., 2013)). Players began each sprint in a split stance with the toes of their leading foot positioned 1 m behind the starting line. Players triggered a set of timing gates at the starting line before sprinting for 15 m, changing direction, and then returning to the starting line. Performance time was measured using the same electronic timing gates placed 1 m above the ground and 3 m apart (Polifemo Radio Light, Microgate, Italia). The best sprint time (s), mean sprint time (s), and percentage (%) sprint decrement (i.e., repeated-sprint ability index [RSA $_{index}$]) were determined across all shuttles for analysis. RSA $_{index}$ was calculated as 100 x ((fastest time – slowest time)/fastest time) (Bishop et al., 2011).

Statistical analysis

All variables are presented as mean ± SD given Shapiro-Wilk tests confirmed normal distribution of all data. All variables were compared between beetroot juice and placebo conditions using paired sample t-tests. Hedge's g_{av} effect sizes (± 95% confidence intervals [CI]) were also determined to quantify the magnitude of differences between conditions for each variable and interpreted as: *trivial* = <0.20; *small* = 0.20–0.49; *medium* = 0.50–0.79; and *large* = ≥0.80 (Lakens, 2013). McNemar's test was also used to detect differences in the prevalence of side

For Peer Review

effects between conditions. Statistical significance was accepted at $P \leq 0.05$. All analyses were conducted using Jamovi (version 1.2.17; www. jamovi.org) and effect sizes were calculated as previously recommended (Lakens, 2013). Figures were developed using Graph Prism software (version 8.0.1, GraphPad Software, San Diego, CA, USA).

RESULTS

Salivary nitrate and nitrite concentrations

Salivary NO_3^- (756 ± 642 µM vs. 3274 ± 2068 µM; P <0.001; g_{av} = 1.51 (0.65–2.33)) and NO_2^- concentrations (345 ± 418 µM vs. 2204 ± 1932 µM; P = 0.002; g_{av} = 1.02 (0.30–1.71)) were significantly elevated following beetroot juice ingestion compared to placebo ingestion.

Neuromuscular performance

Figure 2 shows the mean and individual datapoints across placebo and beetroot juice conditions for all neuromuscular performance variables. Players had significantly higher force outputs during the isometric handgrip strength test (475 ± 60.2 N vs. 512 ± 61.7 N; P = 0.021; g_{av} = 0.59 (-0.23–1.40); Figure 2a) and significantly greater countermovement jump heights (36.5 ± 5.6 cm vs. 38.2 ± 5.6 cm; P = 0.037; g_{av} = 0.29 (-0.51–1.10); Figure 2b) with beetroot juice ingestion compared to the placebo condition. In contrast, no significant differences were apparent between conditions for throwing velocity in the 7-m (87.8 ± 4.4 km·h^{-1} vs. 86.6 ± 4.1 km·h^{-1}; P = 0.180; g_{av} = -0.27 (-1.08–0.53); Figure 2c) and 9-m (89.6 ± 4.5 km·h^{-1} vs. 90.2 ± 3.6 km·h^{-1}; P = 0.526; g_{av} = 0.14 (-0.66–0.94); Figure 2d) throwing tests, Modified Agility T-test performance time (5.57 ± 0.22 s vs. 5.57 ± 0.21 s; P = 0.979; g_{av} = 0.00 (-0.80–0.80); Figure 2e), and repeated-sprint performance in the form of best time (5.81 ± 0.28 s vs. 5.79 ± 0.26 s; P = 0.602; g_{av} = -0.09 (-0.89–0.71)), mean time (6.07 ± 0.20 s vs. 6.04 ± 0.20 s; P = 0.509; g_{av} = -0.13 (-0.93–0.67)); Figure 2f), and RSI_{index} (4.37 ± 1.9% vs. 4.43 ± 1.2%; P = 0.904; g_{av} = 0.04 (-0.76–0.84)).

*****INSERT FIGURE 2 AROUND HERE*****

Rating of perceived exertion

No significant differences in ratings of perceived exertion were apparent between conditions (placebo: 6.25 ± 1.14; beetroot juice: 6.17 ± 1.03; P = 0.820; g_{av} = -0.07 (-0.87–0.73)).

Side effects

The proportion of players reporting various side effects in each condition is shown in Table 1. Red urine was the only side effect to have a significantly higher prevalence with beetroot juice ingestion compared to the placebo condition (P = 0.046), with all other items presenting non-significant differences between

For Peer Review

conditions (*P* = 0.082–0.655) (Table 1). Only 41.6% (5 out of 12) players correctly guessed the order of the trials, indicating successful blinding to the interventions.

*****INSERT TABLE 1 AROUND HERE*****

DISCUSSION

The aim of this study was to determine the effects of three days of beetroot juice ingestion on neuromuscular performance in semi-professional, male handball players. Accordingly, multi-day beetroot ingestion (3 days of 70 mL at 6.4 mmol NO_3^-) significantly improved handgrip strength (7.8%) and jump (4.7%) performance compared to a placebo, while non-significant differences were evident between conditions for ball throwing speed (-1.4–0.7%), change-of-direction speed (0.1 %), and repeated-sprint performance (-0.5–0.3%). These outcomes indicate supplementation with beetroot juice may induce meaningful improvements in strength and jumping abilities that are important in handball, without any deleterious side effects.

Ball throwing (i.e., passing or shooting) is a common offensive action performed >100 times per game in some positions among handball players (Karcher & Buchheit, 2014), with faster throwing velocities likely advantageous to move the ball across the court more quickly and past the goalkeeper to score (Pueo et al., 2022). In turn, our data showed no significant differences in 7-m and 9-m ball throwing velocities between conditions. Although no studies have previously examined the effects of beetroot juice supplementation on ball throwing performance in handball, our results align with those reported for single-dose, acute beetroot supplementation (i.e., 70 mL at 6.4 mmol of NO_3^-) on other upper-body, technical athletic actions such as serving velocity in tennis (Fernandez-Elias et al., 2022).

While isometric handgrip strength is commonly used as an indicator of whole-body strength (Wind et al., 2010), optimal handgrip strength may be important among handball players given the ability to securely grasp the ball is necessary to control possession and during shooting or passing on offence, as well as to withstand forceful contacts with opponents and when blocking the ball reasons (Tsakalou et al., 2015). Likewise, vertical jumping is important in handball given most throws involve jumping actions to optimize positioning, evade opponents, and increase decision-making time when on offence, as well as to block opponent throws on defense (Wagner et al., 2014). In this way, the significant improvements in isometric handgrip strength and countermovement jump height with three days of beetroot juice ingestion we observed may be attributed to various potential mechanisms associated with NO_3^- supplementation. Among these mechanisms, attenuated muscle potassium efflux (Wylie et al., 2013), increased neurotransmitter release (Esen et al., 2022) and augmented skeletal muscle nitrate concentrations

For Peer Review

(Kadach et al., 2023) could have played a role. These findings contrast those made in previous studies, where single-dose, acute beetroot juice ingestion with 70–140 mL (i.e., 6.4–12.8 mmol NO_3^-) has predominantly been examined and shown to elicit non-significant effects on handgrip strength among other samples of team-sport athletes (Fernandez-Elias et al., 2022; Lopez-Samanes et al., 2022). Consequently, our findings add novel evidence to this area demonstrating more extensive multi-day beetroot juice supplementation may better impact strength properties than single-dose supplementation in semi-professional, male team-sports athletes. However, the benefits to countermovement jump height we observed with three days of beetroot juice ingestion contrast previous research examining recreationally-active adults who received six days of beetroot juice supplementation (140 mL at 12.8 mmol NO_3^-) (Jonvik et al., 2021). However, the differences between studies could be attributed to variations in the training status of subjects examined (semi-professional vs. recreational athletes). In this regard, players in our study were accustomed to performing intense explosive actions as part of their regular training and game requirements, as opposed to the recreational athletes examined by Jonvik et al. (2021). However, variations in neuromuscular performance in response to multi-day beetroot juice supplementation according to training status should be directly investigated in future research to confirm this notion.

Competitive handball involves players performing several changes in direction across games. Therefore, isolated change-of-direction speed and the ability to repeatedly execute sprints incorporating directional changes are crucial attributes during competitive situations in handball (Karcher & Buchheit, 2014). However, beetroot juice supplementation did not improve either of these attributes in our study. Our findings for the Modified Agility T-test align with those reported in other research exploring single-dose beetroot juice supplementation and using this test in team-sport athletes (Lopez-Samanes et al., 2020; Lopez-Samanes et al., 2022). In this regard, the high coordinative and technical requirements of the Modified Agility T-test may have negated any potential muscular-induced ergogenic responses with beetroot juice supplementation given strength and power-related attributes have been shown to contribute <50% to performance in this test among team-sport athletes (Scanlan et al., 2021). However, further research is needed to clearly understand the interactions between beetroot juice supplementation and movement complexity during change-of-direction tasks in athletes to better understand these effects. Furthermore, beetroot juice supplementation may be postulated to improve maintenance of sprint performance across repeated bouts as frequently documented for team-sport athletes competing at recreational or amateur levels (Thompson et al., 2015; Wylie et al., 2016), potentially due to a reduced oxygen

For Peer Review

cost of muscle force production (Bailey et al., 2009) and enhanced phosphocreatine resynthesis during recovery (Bailey et al., 2010). However, any oxidative-related ergogenic effects of beetroot juice supplementation on repeated-sprint performance in our study may have been countered by the strong aerobic fitness likely held in the sample of semi-professional handball players we examined. Although aerobic fitness was not directly measured, the players we examined had a strong competitive history within the sport and were completing ~10 h of training per week on average, which encompassed aerobic conditioning. Accordingly, possessing high aerobic fitness may affect the ergogenic benefits induced by dietary nitrate supplementation potentially through various mechanisms such as well-developed vascular and cellular adaptations promoting less room for further improvements in metabolic efficiency with supplementation or higher basal levels of nitrite meaning stronger dosages may be needed for ergogenic outcomes (Zafeiridis, 2014; Zamani et al., 2021). Consequently, the lack of benefit to repeated-sprint performance with beetroot supplementation in the semi-professional players we observed may be expected to differ from previous findings reported in amateur and recreational team-sport athletes (Rojas-Valverde et al., 2021).

Alongside these performance-related observations, it should be noted that the appearance of red urine was the only side effect significantly elevated with beetroot juice ingestion compared to the placebo condition. This finding aligns with previous research reporting limited side effects among team-sport athletes when implementing different beetroot juice supplementation regimes (Lopez-Samanes et al., 2022). However, existing evidence encompasses short-term beetroot supplementation strategies, with more research needed to better understand the risks associated with high beetroot juice consumption across longer durations.

Although this study provides new insight into the effects of multi-day beetroot juice supplementation on neuromuscular performance in a sample of handball players, some notable limitations should be mentioned. First, the multi-day supplementation plan was conducted across three days with 70 mL at 6.4 mmol of NO_3^-consumed per day; other ingestion durations and dosages may elicit different effects on neuromuscular performance with more research needed to identify if an optimal supplementation regime exists. Second, although semi-professional, male handball players were examined in this study, wider samples of players encompassing females and other competition levels are encouraged to strengthen the available evidence base on this topic given sex and training status may impact the response to beetroot juice supplementation (Shannon et al., 2021). Third, testing approaches were focused solely on isolated neuromuscular

For Peer Review

physical qualities in this study; however, performance during small-sided games, simulation tests, or actual competitive games are encouraged for greater ecological validity in future evidence on this topic. Fourth, future research should investigate the role of ascorbic acid (which is contained in the beetroot juice) and its potential effects on nitric oxide bioavailability.

Practical applications

Given the potential importance of handgrip strength and jumping ability in executing game tasks in male handball, these findings suggest that multi-day beetroot supplementation may offer some benefit to physical performance in those competing at the semi-professional level. Specifically, a relatively short supplementation plan across three days may be practical to implement given it will not inconvenience players greatly and be cost-effective for most teams. Furthermore, no significant acute side effects apart from red urine were apparent, supporting the safety of relatively brief multi-day beetroot juice supplementation with dosages of 70 mL at 6.4 mmol NO_3^-. It should be noted that while beetroot juice appears to benefit aspects of neuromuscular performance, it is unclear if these benefits will translate to improved performance in actual games, which should be investigated further to extend upon the results of this study.

For Peer Review

CONCLUSIONS

This study adds to the limited research examining the effects of any form of beetroot juice ingestion on neuromuscular performance in handball players (Nogueira & De Viebig, 2016), as well as specifically using multi-day supplementation approaches in wider team-sport athletes. Our findings demonstrate multi-day beetroot ingestion (three days with dosages of 70 mL at 6.4 mmol of NO_3^- per day) significantly improved some neuromuscular qualities in the form of isometric handgrip strength and countermovement jump height, with no significant effects on ball throwing velocity, change-of-direction speed, and repeated-sprint ability in semi-professional, male handball players.

ACKNOWLEDGMENTS

The authors wish to thank the players for their invaluable contribution to the study. **Author Contributions:** Conceptualization: Alejandro Muñoz and Álvaro López-Samanes. Data curation: Alejandro Muñoz and Álvaro López-Samanes. Formal analysis: Miguel López-Moreno and Marta Garcés. Funding acquisition: Alejandro Muñoz and Álvaro López Samanes Investigation: Alejandro Muñoz, Alfonso de la Rubia, Jorge Lorenzo-Calvo, Raci Karayigit, Marta Garcés-Rimón, Miguel López-Moreno, Raúl Domínguez, Aaron T. Scanlan and Álvaro López-Samanes Project administration:

Alejandro Muñoz and Álvaro López-Samanes. Resources: Alejandro Muñoz, Aaron Scalan and Álvaro López-Samanes. Software: Raúl Domínguez. Supervision: Alejandro Muñoz, Aaron Scalan and Álvaro López-Samanes. Validation: Alejandro Muñoz, Aaron Scalan and Álvaro López-Samanes. Writing—original draft: Alejandro Muñoz, Raci Karayigit, Aaron Scalan, Raúl Domínguez and Álvaro López-Samanes. Writing—review and editing: Alejandro Muñoz, Alfonso de la Rubia, Jorge Lorenzo-Calvo, Raci Karayigit, Marta Garcés-Rimón, Miguel López-Moreno, Raúl Domínguez, Aaron T. Scanlan and Álvaro López-Samanes. All authors have read and agreed to the published version of the manuscript.

Conflict of interest

The authors declare no support from any organization for the submitted work; no financial relationships with any organizations that might have an interest in the submitted work in the previous three years; and no other relationships or activities that could appear to have influenced the submitted work.

Funding

This work is part of the research project entitled "Isolated and combined effect of supplementation with nitric oxide precursors (i.e., beetroot juice) and buffering agents (i.e., beta-alanine) on neuromuscular and inflammatory markers in elite/recreational athletes" within the framework of the Financing of Internal Research Projects [redacted]

References

Alsharif, N. S., Clifford, T., Alhebshi, A., Rowland, S. N., & Bailey, S. J. (2023). Effects of Dietary Nitrate Supplementation on Performance during Single and Repeated Bouts of Short-Duration High-Intensity Exercise: A Systematic Review and Meta-Analysis of Randomised Controlled Trials. *Antioxidants (Basel)*, *12*(6). 1194. https://doi.org/10.3390/antiox12061194

Bailey, S. J., Fulford, J., Vanhatalo, A., Winyard, P. G., Blackwell, J. R., DiMenna, F. J., Wilkerson, D. P., Benjamin, N., & Jones, A. M. (2010). Dietary nitrate supplementation enhances muscle contractile efficiency during knee-extensor exercise in humans. *J Appl Physiol (1985)*, *109*(1), 135-148. https://doi.org/10.1152/japplphysiol.00046.2010

Bailey, S. J., Winyard, P., Vanhatalo, A., Blackwell, J. R., Dimenna, F. J., Wilkerson, D. P., Tarr, J., Benjamin, N., & Jones, A. M. (2009). Dietary nitrate supplementation reduces the O2 cost of low-intensity exercise and enhances tolerance to high-intensity exercise in humans.

For Peer Review

J Appl Physiol (1985), *107*(4), 1144-1155. https://doi.org/10.1152/japplphysiol.00722.2009

Bishop, D., Girard, O., & Mendez-Villanueva, A. (2011). Repeated-sprint ability - part II: recommendations for training. *Sports Med*, *41*(9), 741-756. https://doi.org/10.2165/11590560-000000000-00000

Burleigh, M., Liddle, L., Muggeridge, D. J., Monaghan, C., Sculthorpe, N., Butcher, J., Henriquez, F., & Easton, C. (2019). Dietary nitrate supplementation alters the oral microbiome but does not improve the vascular responses to an acute nitrate dose. *Nitric Oxide*, *89*, 54-63. https://doi.org/10.1016/j.niox.2019.04.010

Clifford, T., Bell, O., West, D. J., Howatson, G., & Stevenson, E. J. (2016). The effects of beetroot juice supplementation on indices of muscle damage following eccentric exercise. *Eur J Appl Physiol*, *116*(2), 353-362. https://doi.org/10.1007/s00421-015-3290-x

Dominguez, R., Mate-Munoz, J. L., Cuenca, E., Garcia-Fernandez, P., Mata-Ordonez, F., Lozano-Estevan, M. C., Veiga-Herreros, P., da Silva, S. F., & Garnacho-Castano, M. V. (2018). Effects of beetroot juice supplementation on intermittent high-intensity exercise efforts. *J Int Soc Sports Nutr*, *15*, 2. https://doi.org/10.1186/s12970-017-0204-9

Esen, O., Faisal, A., Zambolin, F., Bailey, S. J., & Callaghan, M. J. (2022). Effect of nitrate supplementation on skeletal muscle motor unit activity during isometric blood flow restriction exercise. *Eur J Appl Physiol*, *122*(7), 1683-1693. https://doi.org/10.1007/s00421-022-04946-y

Fernandez-Elias, V., Courel-Ibanez, J., Perez-Lopez, A., Jodra, P., Moreno-Perez, V., Coso, J. D., & Lopez-Samanes, A. (2022). Acute Beetroot Juice Supplementation Does Not Improve Match-Play Activity in Professional Tennis Players. *J Am Nutr Assoc*, *41*(1), 30-37. https://doi.org/10.1080/07315724.2020.1835585

Foster, C., Florhaug, J. A., Franklin, J., Gottschall, L., Hrovatin, L. A., Parker, S., Doleshal, P., & Dodge, C. (2001). A new approach to monitoring exercise training. *J Strength Cond Res*, *15*(1), 109-115. https://www.ncbi.nlm.nih.gov/pubmed/11708692

Gonzalez, A. M., Townsend, J. R., Pinzone, A. G., & Hoffman, J. R. (2023). Supplementation with Nitric Oxide Precursors for Strength Performance: A Review of the Current Literature. *Nutrients*, *15*(3). https://doi.org/10.3390/nu15030660

Gutiérrez-Aguilar, Ó., Montoya-Fernández, M., Fernández-Romero, J., & Saavedra-García, A. (2016). Analysis of time-out use in handball and its influence on the game performance. *Int J Perform Anal Sport.* , *16*(1), 1-11.

For Peer Review

Hernandez, A., Schiffer, T. A., Ivarsson, N., Cheng, A. J., Bruton, J. D., Lundberg, J. O., Weitzberg, E., & Westerblad, H. (2012). Dietary nitrate increases tetanic [Ca2+]i and contractile force in mouse fast-twitch muscle. *J Physiol*, *590*(15), 3575-3583. https://doi.org/10.1113/jphysiol.2012.232777

Jonvik, K. L., Hoogervorst, D., Peelen, H. B., de Niet, M., Verdijk, L. B., van Loon, L. J. C., & van Dijk, J. W. (2021). The impact of beetroot juice supplementation on muscular endurance, maximal strength and countermovement jump performance. *Eur J Sport Sci*, *21*(6), 871-878. https://doi.org/10.1080/17461391.2020.1788649

Kadach, S., Park, J. W., Stoyanov, Z., Black, M. I., Vanhatalo, A., Burnley, M., Walter, P. J., Cai, H., Schechter, A. N., Piknova, B., & Jones, A. M. (2023). (15) N-labeled dietary nitrate supplementation increases human skeletal muscle nitrate concentration and improves muscle torque production. *Acta Physiol (Oxf)*, *237*(3), e13924. https://doi.org/10.1111/apha.13924

Karcher, C., & Buchheit, M. (2014). On-court demands of elite handball, with special reference to playing positions. *Sports Med*, *44*(6), 797-814. https://doi.org/10.1007/s40279-014-0164-z

Lakens, D. (2013). Calculating and reporting effect sizes to facilitate cumulative science: a practical primer for t-tests and ANOVAs. *Front Psychol*, *4*, 863. https://doi.org/10.3389/fpsyg.2013.00863

Lopez-Samanes, A., Gomez Parra, A., Moreno-Perez, V., & Courel-Ibanez, J. (2020). Does Acute Beetroot Juice Supplementation Improve Neuromuscular Performance and Match Activity in Young Basketball Players? A Randomized, Placebo-Controlled Study. *Nutrients*, *12*(1). https://doi.org/10.3390/nu12010188

Lopez-Samanes, A., Perez-Lopez, A., Morencos, E., Munoz, A., Kuhn, A., Sanchez-Migallon, V., Moreno-Perez, V., Gonzalez-Frutos, P., Bach-Faig, A., Roberts, J., & Dominguez, R. (2023). Beetroot juice ingestion does not improve neuromuscular performance and match-play demands in elite female hockey players: a randomized, double-blind, placebo-controlled study. *Eur J Nutr*, *62*(3), 1123-1130. https://doi.org/10.1007/s00394-022-03052-1

Lopez-Samanes, A., Ramos-Alvarez, J. J., Miguel-Tobal, F., Gaos, S., Jodra, P., Arranz-Munoz, R., Dominguez, R., & Montoya, J. J. (2022). Influence of Beetroot Juice Ingestion on Neuromuscular Performance on Semi-Professional Female Rugby Players: A Randomized, Double-Blind, Placebo-Controlled Study. *Foods*, *11*(22). https://doi.org/10.3390/foods11223614

Lundberg, J. O., Weitzberg, E., & Gladwin, M. T. (2008). The nitrate-nitrite-nitric oxide pathway in physiology and therapeutics. *Nat Rev Drug Discov*, *7*(2), 156-167. https://doi.org/10.1038/nrd2466

Munoz, A., Lopez-Samanes, A., Perez-Lopez, A., Aguilar-Navarro, M., Moreno-Heredero, B., Rivilla-Garcia, J., Gonzalez-Frutos, P., Pino-Ortega, J., Morencos, E., & Del Coso, J. (2020). Effects of Caffeine Ingestion on Physical Performance in Elite Women Handball Players: A Randomized, Controlled Study. *Int J Sports Physiol Perform*, *15*(10), 1406-1413. https://doi.org/10.1123/ijspp.2019-0847

Nogueira, T., & De Viebig, R. (2016). Efeitos ergogênicos do consumo de suco de beterraba em adolescentes do gênero feminino praticantes de handebol. *Revista Brasileira De Prescrição E Fisiologia Do Exercício.*, *56*(9), 635-642.

Nyakayiru, J., Jonvik, K. L., Trommelen, J., Pinckaers, P. J., Senden, J. M., van Loon, L. J., & Verdijk, L. B. (2017). Beetroot Juice Supplementation Improves High-Intensity Intermittent Type Exercise Performance in Trained Soccer Players. *Nutrients*, *9*(3). https://doi.org/10.3390/nu9030314

Okuno, N. M., Tricoli, V., Silva, S. B., Bertuzzi, R., Moreira, A., & Kiss, M. A. (2013). Postactivation potentiation on repeated-sprint ability in elite handball players. *J Strength Cond Res*, *27*(3), 662-668. https://doi.org/10.1519/JSC.0b013e31825bb582

Pueo, B., Tortosa-Martinez, J., Chirosa-Rios, L. J., & Manchado, C. (2022). Throwing performance by playing positions of male handball players during the European Championship 2020. *Scand J Med Sci Sports*, *32*(3), 588-597. https://doi.org/10.1111/sms.14100

Reynolds, C. M. E., Evans, M., Halpenny, C., Hughes, C., Jordan, S., Quinn, A., Hone, M., & Egan, B. (2020). Acute ingestion of beetroot juice does not improve short-duration repeated sprint running performance in male team sport athletes. *J Sports Sci*, *38*(18), 2063-2070. https://doi.org/10.1080/02640414.2020.1770409

Richard, P., Koziris, L. P., Charbonneau, M., Naulleau, C., Tremblay, J., & Billaut, F. (2018). Time-Trial Performance in World-Class Speed Skaters After Chronic Nitrate Ingestion. *Int J Sports Physiol Perform*, *13*(10), 1317-1323. https://doi.org/10.1123/ijspp.2017-0724

Rojas-Valverde, D., Montoya-Rodriguez, J., Azofeifa-Mora, C., & Sanchez-Urena, B. (2021). Effectiveness of beetroot juice derived nitrates supplementation on fatigue resistance during repeated-sprints: a systematic review. *Crit Rev Food Sci Nutr*, *61*(20), 3395-3406. https://doi.org/10.1080/10408398.2020.1798351

For Peer Review

Sassi, R. H., Dardouri, W., Yahmed, M. H., Gmada, N., Mahfoudhi, M. E., & Gharbi, Z. (2009). Relative and absolute reliability of a modified agility T-test and its relationship with vertical jump and straight sprint. *J Strength Cond Res*, *23*(6), 1644-1651. https://doi.org/10.1519/JSC.0b013e3181b425d2

Scanlan, A. T., Wen, N., Pyne, D. B., Stojanovic, E., Milanovic, Z., Conte, D., Vaquera, A., & Dalbo, V. J. (2021). Power-Related Determinants of Modified Agility T-test Performance in Male Adolescent Basketball Players. *J Strength Cond Res*, *35*(8), 2248-2254. https://doi.org/10.1519/JSC.0000000000003131

Senefeld, J. W., Wiggins, C. C., Regimbal, R. J., Dominelli, P. B., Baker, S. E., & Joyner, M. J. (2020). Ergogenic Effect of Nitrate Supplementation: A Systematic Review and Meta-analysis. *Med Sci Sports Exerc*, *52*(10), 2250-2261. https://doi.org/10.1249/MSS.0000000000002363

Shannon, O. M., Easton, C., Shepherd, A. I., Siervo, M., Bailey, S. J., & Clifford, T. (2021). Dietary nitrate and population health: a narrative review of the translational potential of existing laboratory studies. *BMC Sports Sci Med Rehabil*, *13*(1), 65. https://doi.org/10.1186/s13102-021-00292-2

Slinde, F., Suber, C., Suber, L., Edwen, C. E., & Svantesson, U. (2008). Test-retest reliability of three different countermovement jumping tests. *J Strength Cond Res*, *22*(2), 640-644. https://doi.org/10.1519/JSC.0b013e3181660475

Thompson, C., Vanhatalo, A., Jell, H., Fulford, J., Carter, J., Nyman, L., Bailey, S. J., & Jones, A. M. (2016). Dietary nitrate supplementation improves sprint and high-intensity intermittent running performance. *Nitric Oxide*, *61*, 55-61. https://doi.org/10.1016/j.niox.2016.10.006

Thompson, C., Wylie, L. J., Fulford, J., Kelly, J., Black, M. I., McDonagh, S. T., Jeukendrup, A. E., Vanhatalo, A., & Jones, A. M. (2015). Dietary nitrate improves sprint performance and cognitive function during prolonged intermittent exercise. *Eur J Appl Physiol*, *115*(9), 1825-1834. https://doi.org/10.1007/s00421-015-3166-0

Tsakalou, L., Kotsampouikidou, Z., Papa, M., & Zapartidisi, I. (2015). Handgrip strength and ball velocity of young male and female handball players. *J Phys Educ Sport.*, *15*(4), 800–804.

Wagner, H., Finkenzeller, T., Wurth, S., & von Duvillard, S. P. (2014). Individual and team performance in team-handball: a review. *J Sports Sci Med*, *13*(4), 808-816. https://www.ncbi.nlm.nih.gov/pubmed/25435773

Wind, A. E., Takken, T., Helders, P. J., & Engelbert, R. H. (2010). Is grip strength a predictor for total muscle strength in healthy children, adolescents, and young

For Peer Review

adults? *Eur J Pediatr*, *169*(3), 281-287. https://doi.org/10.1007/s00431-009-1010-4

Wylie, L. J., Bailey, S. J., Kelly, J., Blackwell, J. R., Vanhatalo, A., & Jones, A. M. (2016). Influence of beetroot juice supplementation on intermittent exercise performance. *Eur J Appl Physiol*, *116*(2), 415-425. https://doi.org/10.1007/s00421-015-3296-4

Wylie, L. J., Kelly, J., Bailey, S. J., Blackwell, J. R., Skiba, P. F., Winyard, P. G., Jeukendrup, A. E., Vanhatalo, A., & Jones, A. M. (2013). Beetroot juice and exercise: pharmacodynamic and dose-response relationships. *J Appl Physiol (1985)*, *115*(3), 325-336. https://doi.org/10.1152/japplphysiol.00372.2013

Yang, Y. J., Kim, M. K., Hwang, S. H., Ahn, Y., Shim, J. E., & Kim, D. H. (2010). Relative validities of 3-day food records and the food frequency questionnaire. *Nutr Res Pract*, *4*(2), 142-148. https://doi.org/10.4162/nrp.2010.4.2.142

Zafeiridis, A. (2014). The effects of dietary nitrate (beetroot juice) supplementation on exercise performance: A review *American Journal of Sports Science*, *2*(4), 97-110.

Zamani, H., de Joode, M., Hossein, I. J., Henckens, N. F. T., Guggeis, M. A., Berends, J. E., de Kok, T., & van Breda, S. G. J. (2021). The benefits and risks of beetroot juice consumption: a systematic review. *Crit Rev Food Sci Nutr*, *61*(5), 788-804. https://doi.org/10.1080/10408398.2020.1746629

For Peer Review

Tables

Side effect	Condition (%)		*P*
	Placebo	***Beetroot juice***	
Gastrointestinal problems	17	25	0.564
Red urine	8	42	0.046*
Gastroesophageal reflux	25	42	0.317
Nausea	25	42	0.317
Muscular pain	33	25	0.655
Headache	0	25	0.082
Increased urination	33	17	0.157
Fatigue	17	8	0.564
Nervousness	8	0	0.339

Table 1. Prevalence (percentage of players) of side effects reported after beetroot juice or placebo ingestion in semi-professional, male handball players (n = 12).

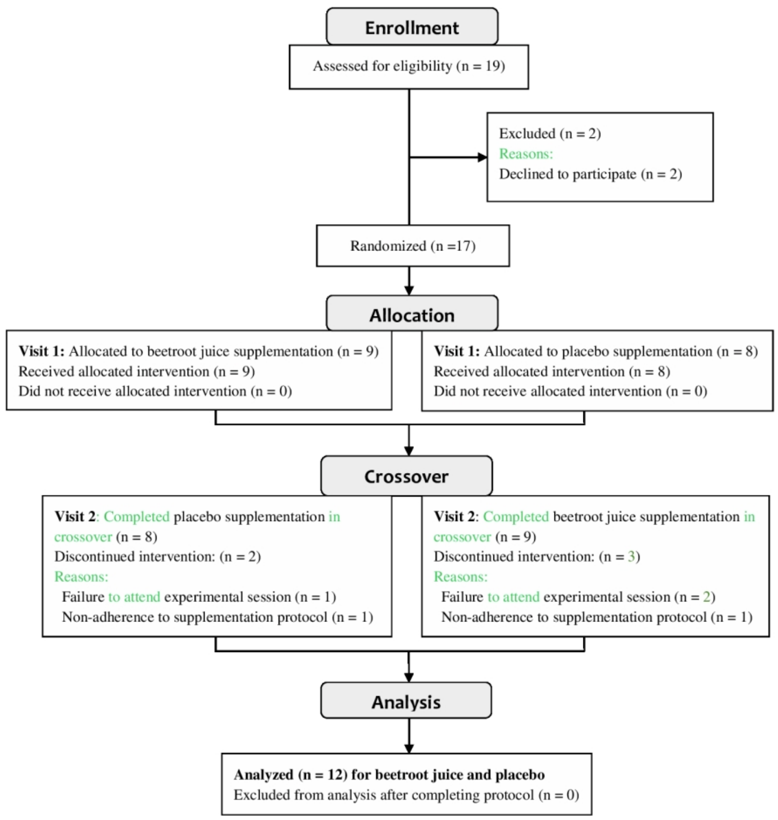
For Peer Review

Figures

Figure 1: CONSORT flowchart of randomized, double-blind, crossover design.

Figure 2: Isometric handgrip strength (A), countermovement jump height (B), 7-m throwing velocity (C), 9-m throwing velocity (D), Modified Agility T-test time (E), and mean repeated-sprint time (F) in semi-professional male handball players. Means are represented as the black line and each individual player is shown as dotted grey lines in each graph. *Statistically significant differences occurred at $P < 0.05$.

For Peer Review



CONSORT flowchart of randomized, double-blind, crossover design.

215x279mm (200 x 200 DPI)

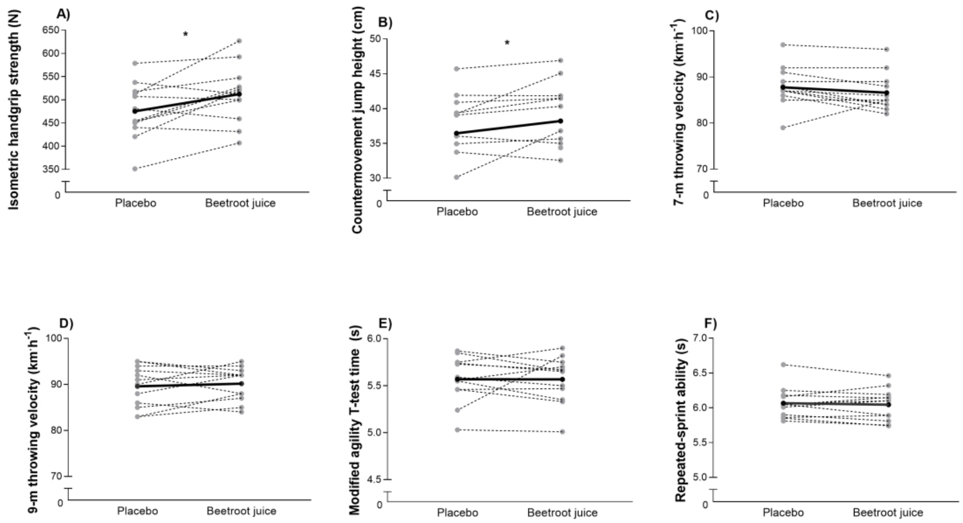


Figure 2: Isometric handgrip strength (A), countermovement jump height (B), 7-m throwing velocity (C), 9-m throwing velocity (D), Modified Agility T-test time (E), and mean repeated-sprint time (F) in semi-professional male handball players. Means are represented as the black line and each individual player is shown as dotted grey lines in each graph. *Statistically significant differences occurred at P <0.05.

268x151mm (300 x 300 DPI)