03 03 2026

Dear Sir/Madam,

Please find attached the Study Protocol and Statistical Analysis Plan entitled:

Brief Title:

SHORT-TERM BROCCOLI SUPPLEMENTATION AND ACUTE OXIDATIVE STRESS RECOVERY

Unique Protocol ID: BI-TRS (M)-2024-703

NCT Number: NCT ID not yet assigned

Document Date: 03 March 2026

In accordance with the requirements, a cover page including the official title, NCT number (pending assignment), unique protocol identification number, and document date has been prepared and included in the submitted document.

The document provides a comprehensive description of the study methodology, including participant characteristics, study design, intervention and placebo conditions, dosing schedule, biological sample collection and analysis procedures, exercise testing protocols, biochemical assessments, and the detailed statistical analysis plan.

Should an NCT number be assigned, the document will be updated accordingly to reflect the official registration identifier.

Sincerely,

Sigitas Kamandulis

Director, Institute of Sports Science and Innovation

Lithuanian Sports University

Study Protocol and Statistical Analysis Plan

Methods

Participants

Twenty healthy males aged 18–37 years were recruited. They had no illnesses or injuries in the month preceding data collection and were engaged in organized physical activity no more than twice per week. Three participants dropped out due to acute infectious disease or changed life circumstances, leaving 17 participants for the analyses (mean ± SD: age 23.8 ± 4.9 years, height 182.3 ± 6.1 cm, weight 80.0 ± 12.8 kg). Participants maintained their regular diets and daily routine. Each participant read and signed a written informed consent form. The study was approved by the Lithuanian Sports

University Ethics Committee (BI-TRS (M)-2024-703) and conducted in accordance with the principles outlined in the Declaration of Helsinki.

Study design and measurements

The study was a double-blind, crossover design, with neither the participants nor the investigators directly in contact with the participants aware of the intervention condition. The input order was randomized, and a 3-week washout period was applied between conditions. Participants were asked to complete questionnaires regarding their consumption of stimulating drinks (coffee, tea, energy drinks, alcohol), broccoli-related foods (Brussels sprouts, mustard greens, leafy cabbage, turnips, cabbage, cauliflower, horseradish, arugula, radishes), and supplements (proteins, BCAA, omega-3, carbohydrates, creatine) within the 24 hours preceding each exercise test.

Intervention arm: The intervention consisted of a single 35-mL scoop of broccoli powder (BrocAffex; 99.5% broccoli powder and 0.5% mustard seed powder), corresponding to 10 g of supplement and delivering 320 μg of glucoraphanin per serving. The supplement was mixed with 300 mL of chocolate oat milk (13 participants) or 300 mL of orange juice (4 participants).

Placebo arm: 1/2 x 35 ml scoop of dried spinach powder blended with 300 ml of chocolate oat milk (13 participants) or orange juice (4 participants).

Dosing schedule (10 doses in total): 14, 12, 10, 8, 6, 4, 3, 2, and 1 day, and 3 h before the exercise challenge.

A fresh beverage was made as a cocktail using a shaker just before each consumption, was drunk within several minutes, and a glass of water (300–500ml) was taken within 30 min after consumption.

Urine collection and analysis

All urine produced by the participant was collected over a 24 h period after ingestion of the first supplementation dose. The urine was collected into a clean plastic container and kept in a cool place, then delivered to the lab within 2 hours at the end of 24 h period of collection. The urine volume was measured, and aliquots were taken and kept at –80 °C for subsequent sulforaphane (SFN) analysis by LC-MS (liquid chromatograph Shimadzu LC-30AD, mass spectrometer LCMS-2020). Urine was analysed in all twenty participants from the Broccoli supplementation condition and in five randomly selected participants from the Placebo condition.

Exercise tests

Metabolically demanding exercise task. Incremental ramp cycling exercise up to voluntary exhaustion was implemented using the following protocol: 4 minutes at 40W, after which power was continuously increasing by 1W/3sec ramp (i.e., by 20W each minute). A portable breath-by-breath analyzer (Cortex Metamax 3B, Germany) was used, and heart rate (HR) was monitored (Polar H10, Kempele, Finland) throughout the test. Participants were pedaling at ~70 rpm and were required to cycle until exhaustion with standardized verbal encouragement during the last stages of the test. The following criteria were used to verify that the maximal oxygen uptake (VO_2max) was attained: peak HR reached at least 90% of the age-predicted maximal HR (220 minus individual ages in years), and the respiratory exchange ratio reached > 1.1. The following peak parameters were measured: HR, oxygen consumption (VO_2), pulmonary ventilation (VE), breathing frequency, and respiratory exchange ratio (RER, CO_2/VO_2). VO_2max and HRmax were determined as the highest values of 20-s rolling average during the latest stages of the test. The test failure criterion was the inability to maintain cadence above 60 rpm for longer than 5 seconds despite verbal encouragements.

Muscle power test. Countermovement jump (CMJ) height was measured before (baseline), and then 1min, 30min, and 60min after the metabolically demanding exercise task. Before the first (baseline)

and the last (60 min after the metabolically demanding exercise task) muscle power testing time point, a short, standardized warm-up consisting of 5 min of very light cycling at 50 W on the cycle ergometer (Ergo Line, Medical Measurement Systems, Germany) and 2 min of low-intensity dynamic stretching was conducted. Each jump began from an upright standing position, followed by squatting to approximately 90° knee angle before immediately jumping vertically off the ground as high as possible. A 20-second interval was maintained between each of the attempts, and participants kept their hands on their waist during the jumps. The CMJs were performed using a photoelectric cell system (Optojump, Microgate, Italy), and the best result from three to four attempts was taken for analysis.

Blood analyses

Capillary blood lactate was measured before the metabolically demanding exercise task, and then 1min, 3min, 5min, 30min and 60min post-exercise. During the recovery after exercise, the participant assumed a supine position on a nearby couch and was resting passively until the blood lactate analysis was over (except for the time periods required to conduct muscle power vertical jump test). After carefully cleansing the fingertip skin with an alcohol swab and piercing it with a sterile disposable lancet, 0.3 µL of blood was drawn into a reagent strip of a portable analyzer (Pro2, Arkray Inc., Kyoto, Japan) for an immediate read-out of the lactate value.

Plasma malondialdehyde (MDA) concentration was analyzed before incremental cycling and 1 h post-test. Blood samples were taken by puncture of an antecubital vein, dispensed into EDTA-containing tubes. Plasma was then immediately harvested by 1500 g centrifugation for 15 min at 4 °C. Then, plasma aliquots were stored at −80 °C until the analysis. Spectrophotometric analysis (Spark 10M Tecan, Männedorf, Switzerland) of the samples was carried out at 450 nm wavelength to measure MDA concentration using commercially available kits (Ref. #E1371Hu, BT Lab, Zhejiang, China) following the provided instructions. To ensure accuracy, samples were analyzed in duplicate, and average values were reported. The coefficient of variation of the assay was less than 10%.

Statistical analysis

Descriptive statistics, presented as the mean ± standard deviation (SD), were calculated for each measured variable. The preliminary analysis of data distribution normality was conducted using the Shapiro-Wilk test. To assess the main and interaction effects of condition and time on blood lactate and countermovement jump (CMJ) height, a two-way repeated measures analysis of variance (RM ANOVA) was employed. When a statistically significant effect was identified, Tukey's honestly significant difference (HSD) post-hoc test was subsequently performed to conduct pairwise multiple comparisons and pinpoint specific mean differences. For any other comparisons involving paired measurements outside of the RM ANOVA structure, a dependent samples t-test was utilized to determine significant differences. All statistical analyses were performed using IBM SPSS Statistics (version 30.0; IBM Corp., Armonk, NY, USA), with the threshold for statistical significance set a priori at $p < .05$.