Experimental Approach to the Problem

Research Design

Twenty-four to Thirty healthy, resistance-trained men (n=12-15) and women (n=12-15) between the ages of 18 and 35 will be recruited for this randomized, cross-over investigation. In order to be considered resistance trained, participants will need to have been engaged in resistance trained for ≥ 1 year prior to study enrollment and must be able to leg press at least 2x their bodyweight. Participants must also be free of any physical limitations, metabolic or cardiovascular disorders, and musculoskeletal injuries. Females will be excluded if they are not eumenorrheic and/or report the use of hormone-based contraceptives (e.g. oral contraceptive pill) within the previous 3 months. For female participants, all experimental sessions will take place within the same phase of their menstrual cycle. Furthermore, all participants will be asked not take any other nutritional supplements, performance enhancing drugs, and non-steroidal anti-inflammatory drugs (NSAIDs) throughout the duration of their enrolment in the study. Additionally, participants will be excluded if they have a history of antibiotics or intentionally consumed probiotics in the two months prior to enrollment in the study. If a participant has recently consumed probiotics, an 8-week washout period will be required before the participant is enrolled in the study. The study protocol will be approved by the Lipscomb University Institutional Review Board before participant enrollment.

Testing Procedures

Informed Consent & Questionnaires

Following an explanation of all study protocols, benefits, and risks, each participant will provide their written informed consent prior to participation in this study. Furthermore, participants will complete a PARQ2018+, health and activity questionnaire, food frequency questionnaire, and the Gastrointestinal Symptom Rating Scale (GSRS). The GSRS is an instrument of 15 items combined into five symptom groups representing Reflux, Abdominal pain, Indigestion, Diarrhea and Constipation. The GSRS has a seven-point graded Likert-type scale where 1 signifies absence of troublesome symptoms and 7 represents very troublesome symptoms. The reliability and validity of the GSRS has been previously established (3). The food frequency questionnaire will be used as an additional assessment tool for account for macronutrient and fiber intake when making gut composition comparisons.

Anthropometric & Body Composition Assessment

Prior to all body composition and strength measures, height (± 0.1 cm) and body mass (± 0.1 kg) will be measured for each participant using a Health-o-meter scale (Model 500 KL, Pelstar, Alsip, IL, USA) with the participants standing barefoot, with feet together, in their regular exercise attire.

Total body water (TBW) will be determined using multi-frequency bioelectrical impedance analysis (BIA) using the InBody® 570 Body Composition Analyzer device (Biospace, Inc., Seoul, Korea). Body composition from BIA is obtained from the measures of resistance and reactance when an electrical current travels throughout the body. Prior to each assessment the participants' hands and feet will be thoroughly cleaned with InBody® provided tissues. Age, height, and sex will be manually entered, while a scale positioned within the device assessed body mass. The participant will be then instructed from the software to stand fully erect on the measurement electrodes situated on the platform and to hold hand electrodes, with arms extended, without touching the sides of their body. Participants will be asked to

refrain from moving or talking until the assessment will be completed. It has previously been shown that BIA is a valid measurement tool for determining TBW when compared to a deuterium oxide technique [30].

Body mass, non-bone fat-free mass (FFM), and body fat percentage will be determined using whole body–dual energy x-ray absorptiometry (DXA) scans (ProdigyTM; Lunar Corporation, Madison, WI). Total body estimates of percent fat and non-bone FFM (± 0.1 kg) will be determined using company's recommended procedures and supplied algorithms. Daily calibrations of Quality assurance will be completed prior to all DXA scans using the manufacturer supplied calibration block. All DXA assessments will be completed using standardized subject positioning procedures by a single certified radiological technician.

4-Compartment Model (4C)

Percent body fat will be estimated using a criterion 4C model. We will utilize DXA derived body volume and BMC, while TBW from BIA will be used. This has been previously reported as a valid and reliable method of estimating percent body fat, fat mass, and lean mass (7).

1-Repetiton Maximum (1RM) Testing

During the initial visit to the lab, participants will establish their 1-repetition maximal strength (1RM) on the leg press and leg extension exercises. Each participant will complete a standardized warm-up before the 1RM strength session involving 5 minutes of cycling on a cycle ergometer against low resistance, 10 body-weight squats, 10 body weight walking lunges, 10 dynamic walking hamstring stretches, and 10 dynamic walking quadriceps stretches. The 1RM tests will be performed using methods previously described for the leg press and leg extension exercises. Two warm-up sets utilizing a resistance of approximately 40-60% and 60-80% of their estimated maximum for the exercise will be performed. For both exercises, 3-4 subsequent, maximal trials will be completed to determine the 1RM. A 3-5 min rest period will be provided between each trial. Trials which do not meet the range of motion standards for each exercise or where correct technique is not used will be discarded. Foot positioning on leg press sled as well as equipment placement (back, knee, and leg positions) for leg extension will be measured, recorded, and replicated for each participant on subsequent visits. Additionally, any notable attire (such as lifting shoes), which may influence a participant's performance, will be recorded and replicated for each subsequent visit.

Visit 2-3

Study Controls

Following baseline measurements, participants will report to the lab on two additional occasions to complete each experimental condition. On the morning of each visit, participants will be asked to report to the Human Performance Lab (HPL) fasted for 12 hours and asked to refrained from all forms of moderate to vigorous leg exercise over the previous 72 hours. They will also be requested to report after obtaining at least 8-h of sleep and not to have consumed alcohol, nicotine, or any other drug for the previous 36 hours while abstaining from caffeine for the previous 12 hours. To promote hydration, participants will be asked to consume ~500ml of water the night before reporting to the lab, and the morning of each exercise treatment.

Dietary Logs

Participants will be coached to maintain their usual dietary intake in the days preceding the acute resistance exercise trials. Throughout the 24 hours before first experimental trial, each participant will additionally be instructed to record everything they consume in a precise manner. To achieve this, each participant will meet with a registered dietician for a brief education session to aid in accurate content, quantity, and timing of food in their logs. In subsequent trials, participants will be provided their first dietary log and asked to replicate the content, quantity, and timing of their daily diet during the 24 hours prior to the experimental trial in addition to recording everything they consume. Dietary recalls will be analyzed with the nutrition database, Nutritionix (Syndigo LLC, WA, US).

Stool Collection, Handling and Storage

Participants will be provided with a stool sample collection system during their first visit to the lab and instructed to collect a stool sample in the 24-48 hours prior to reporting for their second visit to the lab to complete the first study condition. Upon arrival to the lab, participants will provide the stool sample and investigators will subsequently aliquot and store samples at -80°C.

Resistance Exercise Protocol

Experimental trials will be completed in a balanced, randomized order with each trial being separated by a minimum of one week to warrant adequate recovery. An overview of the acute resistance exercise protocols is presented in Figure 1. All experimental trials will also be performed within a 2-hour window to avoid diurnal variations. Ample hydration status will be verified (USG ≤ 1.020 defined as euhydration) via urine samples collected upon arrival to the HPL and analyzed for specific gravity (USG) by refractometry. During the exercise experimental trial, participants will perform the same standardized warm-up routine described earlier for 1RM testing, followed by either a resistance exercise protocol, consisting of the squat, seated shoulder press, deadlift, bent-over row, and leg press exercises. The exercise protocol will utilize a load of 70% 1-RM for4 sets of 10 repetitions and a 90-second rest period length between sets and exercises. The control session will consist of participants completing assessments but will rest seated for 40 minutes instead of exercising. Treatment order (control or exercise) will be randomized using a random number generator. A similar exercise protocol has been demonstrated to significantly elevate I-FABP, a marker of gut permeability (25). During each protocol, if the participant is unable to complete the desired number of repetitions, spotters will provide assistance until the subject completes the remaining repetitions. Immediately upon completion of the acute resistance exercise bout, participants will complete IP assessments.

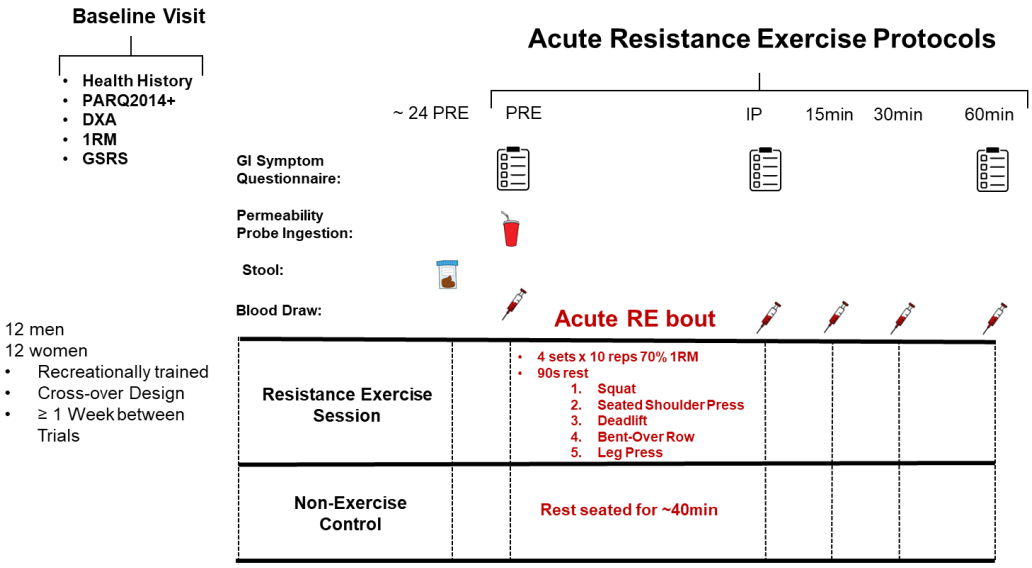

Figure 1. Acute Resistance Exercise Protocol Overview. PRE = Pre-exercise; IP = Immediately-post exercise. RE = Resistance Exercise; GI = Gastrointestinal; 1RM = 1-Repetition Maximum

Acute GI Distress Questionnaires

Before and after the acute resistance exercise protocols (PRE, IP, 60min), participants will complete a questionnaire to assess GI symptoms adapted from a previous study (4). The questionnaire is organized into three sections, each including four to seven questions. Section 1 addresses upper abdominal issues (reflux, heartburn, bloating, cramps, vomiting, nausea); Section 2, lower abdominal issues (intestinal cramps, flatulence, urge to defecate, left abdominal pain, right abdominal pain, loose stool, diarrhea); and Section 3, systemic issues (dizziness, headache, muscle cramp, urge to urinate). Each participant will complete the 17 items on a 10-point scale ranging from 0, no problem at all, to 9, the worst it has ever been.

Blood Collection, Handling and Storage

Blood samples during the experimental trials (PRE, IP, 15min, 30min, 60min) will be drawn from a 20g Teflon™ cannula placed in an antecubital vein, which will remain patent via an isotonic saline flush. Blood samples will be obtained from the cannula with a plastic syringe prior to exercise (PRE), immediately-post (IP), 15-, 30-, and 60min post-exercise. Blood samples will be deposited into untreated (for serum collection), as well as EDTA- and heparin-treated (for plasma collection) BD Vacutainer® tubes. Untreated tubes will clot for 30 minutes prior to centrifugation, while treated tubes will be centrifuged immediately for 15 min at 1500 x g at 4ºC. The resulting serum and plasma will be aliquoted and stored at -80ºC until analysis. Samples will be thawed only once for biochemical analysis.

Assessment of intestinal damage, permeability, and inflammation

I-FABP concentrations from EDTA plasma will be measured using an ELISA (RayBioTech Inc., Norcross, GA) according to the manufacturer's instructions. Lactulose/Rhamnose (LR) ratio will be assessed using a previously published protocol (2), with the modification of using L-rhamnose instead of mannitol as the monosaccharide probe (5, 6). Briefly, at baseline, participants will drink a standardized sugar solution containing 5 g lactulose, 2 g rhamnose, 1 g sucrose, and 0.5 g D-xylose in a total of 50

mL water. Saccharide concentrations will then be determined from serum samples at all time-points and the LR ratio will be calculated. Serum will be analyzed for endotoxin concentration using a commercially available kinetic limulus amebocyte lysate (LAL) assay (Lonza; Allendale, NJ). Circulating plasma concentrations of the pro-inflammatory cytokine TNF-α will be assayed via commercially available ELSIA kits (ALPCO, Salem, NH, USA).

For fecal analysis, feces will be thawed, and 100 mg will be added added to 4.9 ml of extraction buffer (0.1 M Tris, 0.15 M NaCl, 1.0 M urea, 10 mM CaCl2 $2H_2O$, 0.1 M citric acid, 0.5% BSA, pH 8.0). After 30 minutes shaking, 1 ml of suspension will be centrifuged at 10,000 rpm for 20 minutes at 4°C. Fecal supernatants will be assayed for calprotectin via ELISA (Eagle Biosciences, Nashua, NH) using manufacturer's instructions. A summary of the markers of interest is presented in Table 1.

Table 1. Biochemical Markers of Interest

Marker	~24H Pre	Pre	IP	15min	30min	60min	~24H Post
Blood							
I-FABP		X	X	X	X	X	
Endotoxin (LAL)		X	X	X	X	X	
Lactulose/Rhamnose Ratio		X	X	X	X	X	
Fecal							
Shallow Shotgun Metagenomics	X						
SCFA	X						

I-FABP = Intestinal Fatty-acid Binding Protein, LAL = limulus amebocyte lysate, SCFA = Short-Chain Fatty Acids. 24H Pre= 24hours before exercise, Pre= Pre-exercise, IP = Immediately post-exercise, 24H = 24-hours post-exercise.

Fecal Shallow Shotgun Metagenomics

After collection, 10-20g feces will be separated into sterile collection containers and immediately frozen at -20°C. After thawing, DNA will be extracted from samples using the Qiagen MagAttract PowerSoil DNA KF Kit (formerly MO BIO PowerSoil DNA Kit) optimized for the Thermofisher KingFisher robot according to manufacturer's instructions. Whole DNA will be prepared for sequencing using the Illumina Nextera Flex DNA library prep kit. Samples will be barcoded and mixed together for sequencing. The mix will then be subjected to paired-end sequencing using Illumina sequencers. Paired reads will be concatenated and processed to remove adaptors, barcodes and low-quality reads as well as contaminant sequences (typically host DNA). Sequences will then then mapped to databases to obtain taxonomic and functional profiles. Sequencing will be performed on the Illumina NextSeq. The number of reads targeted ranges from 0.5 to 3 million.

Fecal Short-Chain Fatty Acid Analysis

After collection, 10-20g feces will be separated into sterile collection containers and immediately frozen at -80°C. After thawing, Short chain fatty acids will be extracted from stool in an aqueous solution, analyzed in a gas chromatograph (GC) coupled with a flame ionization detector (FID), using a Thermo TG-WAXMS A GC Column. The analytes will be quantified against a series of stock standard solutions.

Statistical Analysis

Prior to analysis all data will be assessed to ensure normal distribution, homogeneity of variance and sphericity. Non-normally distributed data will be transformed using the natural log (LN) and if sphericity is violated, a Greenhouse Geisser correction will be applied. General Linear Model (GLM) repeated measures analyses [time x trial x sex] will be used to compare trials (RE, CON) for all variables. Following a significant F ratio, separate one-way repeated-measures ANOVA will be performed to assess the effect of time during each trial, and separate paired-samples t-tests will be used to compare trials at each time point. Separate independent t-tests will also be used to evaluate specific differences between sexes when applicable. An alpha level of $p < 0.05$ will be considered statistically significant for all comparisons. Based on a standard deviation of 511 $pg \cdot ml^{-1}$ and 347 $pg \cdot ml^{-1}$ for post-exertional I-FABP for males and females, respectively (8) and using a standard alpha (0.05) and beta value (0.8), a sample size of n= 4 per group was calculated to have adequate statistical precision to detect a > 110% increase in I-FABP post-exercise. Such increases in I-FABP after exercise have also been correlated with the magnitude of intestinal permeability (9). Current participant numbers are also in accordance with sufficient statistical precision to detect significant differences in gastrointestinal symptoms (1). From the metagenome sequencing the group comparisons of gut microbiota will be analyzed with non-parametric Wilcoxon matched-pair signed rank test and corrected for multiple comparison with Benjamini-Hochberg procedure. Regarding the taxonomic data, all analyses will be made with Quantitative Insights Into Microbial Ecology (QIIME) open-sourced software from the randomly subsampled Operational Taxonomic Unit (OUT) table with rarefaction level matching the sample with the lowest total OTU count. The bacterial diversity of the samples (α-diversity metrics) and statistically significant differences in the OTU abundances will be computed with QIIME. Any correlations between non-normally distributed gut microbiota and other variables will be determined using Spearman's rank correlation coefficient in SPSS. The GLM in SPSS will be used to determine whether differences in taxa occur due to biological sex or whether they are dependent on age, weight, body fat %, total energy intake, macronutrient composition, or fiber.

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