

1 **Changes in intestinal microbiota composition and metabolism coincide with increased**
2 **intestinal permeability in young adults under prolonged physiologic stress**

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30 read and approved the final manuscript.

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34 **ABSTRACT**

35 The magnitude, temporal dynamics, and physiologic effects of intestinal microbiome
36 responses to physiologic stress are poorly characterized. This study used a systems biology
37 approach and multiple-stressor military training environment to determine the effects of
38 physiologic stress on intestinal microbiota composition and metabolic activity, and intestinal
39 permeability (IP). 73 Soldiers were provided three rations/d with or without protein- or
40 carbohydrate-based supplements during a four day cross-country ski march (STRESS). IP was
41 measured before and during STRESS. Blood and stool samples were collected before and after
42 STRESS to measure inflammation, stool microbiota, and stool and plasma global metabolite
43 profiles. IP increased $62\% \pm 57\%$ (mean \pm SD, $P < 0.001$) during STRESS independent of diet
44 group, and was associated with increased inflammation. Intestinal microbiota responses were
45 characterized by increased α -diversity, and changes in the relative abundance of $>50\%$ of
46 identified genera, including increased abundances of less dominant taxa at the expense of more
47 dominant taxa such as *Bacteroides*. Changes in intestinal microbiota composition were linked to
48 23% of metabolites that were significantly altered in stool after STRESS. Pre-STRESS
49 *Actinobacteria* relative abundance, and changes in serum IL-6 and stool cysteine concentrations,
50 collectively, accounted for 84% of the variability in the change in IP. Findings demonstrate that
51 a multiple-stressor military training environment induced increases in IP that were associated
52 with alterations in markers of inflammation, and with intestinal microbiota composition and
53 metabolism. Observed associations between IP, the pre-stress microbiota, and microbiota
54 metabolites suggest targeting the intestinal microbiota could provide novel strategies for
55 preserving IP during physiologic stress.

56

57 **Keywords:** microbiology, gut barrier, exercise, energy metabolism, metabolomics

58

59 **New and Noteworthy:** Military training, a unique model for studying temporal dynamics of
60 intestinal barrier and intestinal microbiota responses to stress, resulted in increased intestinal
61 permeability concomitant to changes in intestinal microbiota composition and metabolism. Pre-
62 stress intestinal microbiota composition and changes in fecal concentrations of metabolites
63 linked to the microbiota were associated with increased intestinal permeability. Findings suggest
64 that targeting the intestinal microbiota could provide novel strategies for mitigating increases in
65 intestinal permeability during stress.

66 INTRODUCTION

67 The intestinal barrier is a selective physical and immunological barrier that facilitates
68 fluid and nutrient absorption while deterring translocation of potentially harmful luminal
69 antigens into circulation (3). Disruption or dysfunction in the intestinal barrier increases
70 intestinal permeability (IP), initiating a cycle in which translocation of luminal compounds (e.g.,
71 bacterial cell wall LPS) can induce immune and inflammatory responses that exacerbate
72 intestinal barrier damage and further increase IP (3, 15, 54). Sequelae of increased IP and
73 subsequent inflammation can include gastrointestinal distress (54), impaired nutrient absorption
74 and metabolism (35), increased susceptibility to illness and infection (53), decrements in
75 cognitive function and physical performance (12), and, if chronic, increased disease risk (19, 53).

76 The intestinal microbiota and its metabolites are integral mediators of intestinal barrier
77 function and IP, capable of both perturbing and enhancing intestinal barrier integrity by
78 modulating immune responses, oxidative stress, inflammation, vagal signaling, and nutrient
79 availability (40). Intestinal microbiota composition and activity are malleable, influenced by the
80 availability of undigested dietary components (13, 40) and the intestinal environment (e.g., pH,
81 motility, inflammation, immune activity) (48). Dietary ratios of fiber, carbohydrate, protein, and
82 fat are also important as low fiber, high protein, and high fat diets reportedly increase intestinal
83 inflammation and IP by altering ratios of microbes and metabolites that modulate inflammation
84 (13, 14, 41). Severe physical stress (12, 15, 54), psychological stress(34), sleep deprivation and
85 circadian disruption (17, 50), and environmental stressors (9, 24) have also been independently
86 associated with altered intestinal microbiota composition and increased IP. However, current
87 understanding of the intestinal microbiota's role in mediating effects of physical, psychological,

88 and environmental stressors on the intestinal barrier is largely limited to information derived
89 from animal models which may not fully represent the human condition (12, 34).

90 Military training environments offer opportunity for novel insights into the magnitude,
91 temporal dynamics, and health effects of stress responses within the human intestinal
92 microbiome as military personnel commonly endure combinations of prolonged physical
93 exertion, psychological stress, sleep deprivation, and environmental extremes during training and
94 combat (31, 51). In support, transient and chronic gastrointestinal distress (46), suboptimal
95 micronutrient status (21, 36), and cognitive decrements (31) have been reported in military
96 personnel during training and combat. Although underlying etiologies are multifactorial, all are
97 possible sequelae of increased IP, suggesting that intestinal barrier dysfunction and the intestinal
98 microbiota may play a role. In support, a recent study reported gastrointestinal distress during
99 combat-training was linked to stress, anxiety, inflammation, and increased intestinal and blood
100 brain barrier permeability (29, 30). Changes in urinary concentrations of several metabolites
101 potentially derived from the intestinal microbiota were also observed, and were associated with
102 gastrointestinal symptomology and IP (44). Although the authors speculated that changes in
103 intestinal microbiota composition may have contributed to these findings, microbiota
104 composition was not assessed.

105 The present study used a physically demanding military training exercise as a model for
106 elucidating the effects of physiologic and metabolic stress on IP and intestinal microbiota
107 composition and activity, and to identify associations between dietary intake, IP, inflammation
108 and the intestinal microbiota. The data were collected during a trial designed to determine to
109 what extent dietary carbohydrate and protein supplementation spare whole-body protein and
110 attenuate decrements in physiologic status during military training (32, 43). We hypothesized

111 that the multiple-stressor environment, which was expected to induce negative energy balance
112 and body weight loss, would adversely affect intestinal microbiota composition (e.g., decrease
113 diversity, increase abundance of pro-inflammatory taxa, and decrease abundance of putatively
114 beneficial taxa), and increase IP. We further hypothesized, that supplemental protein would
115 exacerbate these decrements by promoting the generation of potentially harmful bacterially-
116 derived metabolites, whereas carbohydrate supplementation would attenuate these decrements by
117 reducing the magnitude of negative energy balance.

118

119 **METHODS**

120 **Participants and experimental design**

121 Seventy three Norwegian Army Soldiers (71M, 2F) participating in a 4-d arctic military
122 training exercise consented to participate in this randomized, controlled trial in January 2015 (32,
123 43). All Soldiers >18 years of age participating in the training were eligible for the study. The
124 study was approved by the Institutional Review Board at the US Army Research Institute of
125 Environmental Medicine (Natick, MA, USA) and the Regional Committees for Medical and
126 Health Research Ethics (REK sør-øst, Oslo, NO). Investigators adhered to the policies for
127 protection of human subjects as prescribed in 32 CFR Part 219, US Department of Defense
128 Instruction 3216.02 (Protection of Human Subjects and Adherence to Ethical Standards in DoD-
129 Supported Research) and Army Regulation 70-25. The trial was registered on
130 www.clinicaltrials.gov as NCT02327208.

131 Study staff block randomized volunteers by body weight to a control (CNTRL, n=18),
132 protein-supplement (PRO, n=28), or carbohydrate supplement (CHO, n=27) group in a 1:3
133 (control:intervention) ratio. All volunteers were provided three Norwegian arctic rations/d to

134 consume during the 4-d training exercise. The PRO group was also provided four whey protein-
135 based snack bars/d, while the CHO group was provided four carbohydrate-based snack bars/d.
136 Bars were similar in appearance, taste and texture enabling investigators, study staff and
137 volunteers to remain blind to the macronutrient composition. The training consisted of a 51 km
138 cross-country ski-march during which volunteers skied in 50:10 min work-to-rest ratios while
139 carrying a ~45 kg pack (STRESS). Stool samples were collected over the 2 d prior to STRESS,
140 and the night of or day after completing STRESS in a self-selected subset of volunteers. 24-hr
141 urine collections were completed the day prior to STRESS and on the 3rd day of STRESS. Blood
142 samples were collected the morning before and the morning after STRESS. Primary study
143 objectives were to determine the effects of macronutrient supplementation on whole body protein
144 balance, body mass, and physiological status during military training, and are reported elsewhere
145 (32, 43). This report details secondary study objectives of determining the impact of a multiple-
146 stressor military training environment on IP, and intestinal microbiota composition and activity.

147 Volunteers began consuming provided rations 2 d prior to training and the intervention
148 snack bars on day one of STRESS. Three Norwegian field rations provide 14.6 MJ, 141 g
149 protein, 435 g carbohydrate, and 126 g fat. The four protein-based snack bars provided an
150 additional 4.4 MJ kcal, 85 g whey protein, 102 g carbohydrate, 35 g fat, and <1 g fiber, while the
151 four carbohydrate-based snack bars provided an additional 4.4 MJ, 11 g whey protein, 189 g
152 carbohydrate, 29 g fat, and 1 g fiber. All snack bars were manufactured by a third party that did
153 not participate in data collection (Combat Feeding Directorate, Natick Soldier Systems, Center,
154 Natick, MA). Investigators, study staff, and volunteers were blind to the macronutrient
155 composition of the bars. Volunteers were asked to consume the rations and bars as they
156 normally would during training, and to consume only foods and caloric beverages provided to

157 them by the study team. All volunteers were provided with ration-specific food logs which were
158 collected and reviewed daily by study staff, and used to calculate actual intakes (**Table 1**).

159

160 **Intestinal permeability assay**

161 Intestinal permeability was assessed by quantifying the urinary excretion of orally
162 ingested sugar substitutes (29, 38). Fasted volunteers consumed a solution of 2 g sucralose and 4
163 g mannitol dissolved in ~180 mL of water, then collected all urine produced over the subsequent
164 24 hr. Sucralose is not degraded by the colonic microbiota, is excreted in proportion to
165 paracellular permeability, and is a common marker for whole-gut IP (38). In contrast, mannitol
166 is used for small-bowel permeability measurements (3), but is degraded by the colonic
167 microbiota which prevents its use for IP measurements >5hr. Mannitol results are presented
168 herein solely for comparison to a previous study conducted in a military training environment
169 (29). Sucralose and mannitol concentrations were measured by HPLC (Agilent 1100 HPLC,
170 Santa Clara, CA, USA) as previously described (1, 33). Fractional excretion was calculated by
171 multiplying the measured concentration of each probe by the total volume of urine collected and
172 dividing by the dose administered. Logistical constraints and adverse weather precluded more
173 frequent urine collections, and prevented obtaining complete post-STRESS urine collections
174 from 24 volunteers.

175

176 **Blood biochemistries**

177 Blood was collected following an overnight fast by antecubital venipuncture, separated
178 into serum or plasma, and immediately frozen. Samples were then shipped on dry ice to the U.S.
179 Army Research Institute of Environmental Medicine where they were stored at -80°C until being

180 shipped to Pennington Biomedical Research Center (Baton Rouge, LA) or Metabolon, Inc.
181 (Durham, NC) for analysis. Plasma LPS was measured by ELISA (Cusabio, College Park, MD),
182 serum IL-6 by the Milliplex MAP (Millipore, Billerica, MA), serum high-sensitivity C-reactive
183 protein (CRP) by a chemiluminescent immunometric assay (Siemens Immulite 2000; Siemens
184 Inc., Malvern, PA), and serum creatine kinase (a marker of muscle damage) by an automated
185 chemistry analyzer (Beckman Coulter DXC 600 Pro, Beckman Coulter, Brea, CA).

186

187 **Stool microbiota composition**

188 Stool sample collection was optional to encourage maximal participation for primary
189 study outcomes. A self-selected subset of 38 volunteers provided stool samples, 26 of whom
190 provided both pre- and post-STRESS samples.

191 Stool samples were collected into provided collection containers, immediately placed on
192 ice, and frozen in ~500 mg aliquots within 12 hr of collection. Samples were shipped on dry ice
193 to the U.S. Army Research Institute of Environmental Medicine where they were stored at -
194 80°C. Samples were then shipped to Metabolon, Inc. for metabolomics analysis and to the U.S.
195 Army Center for Health and Environmental Research for intestinal microbiota composition
196 analysis.

197 Samples were selected for DNA extraction in random order, and DNA was extracted
198 using the PowerFecal DNA Isolation kit (MO BIO Laboratories, Inc., Qiagen, Carlsbad, CA).
199 Primers designed to amplify the V3-V4 region of the 16S rRNA gene were employed for PCR
200 amplification (22) according to the Illumina 16S Metagenomic Sequencing Library Preparation
201 manual (Part # 15044223 Rev B; Illumina, Inc., San Diego, CA). A limited cycle PCR generated
202 a single amplicon of ~460 bp to which Illumina sequencing adapters and dual-index barcodes

203 were added. Paired 300 bp reads and MiSeq v.3 reagents were used to generate full-length reads
204 of the V3 and V4 region in a single run on the Illumina MiSeq platform.

205 Sequencing data were processed using Quantitative Insights Into Microbial Ecology
206 (QIIME) v.1.9.1 (8). Read quality assessment, filtering, barcode trimming, and chimera
207 detection were performed on de-multiplexed sequences using USEARCH (16). Operational
208 taxonomic units (OTU) were assigned by clustering sequence reads at 97% similarity. The most
209 abundant sequences with a minimum sequence length of 150 bp were aligned against the
210 Greengenes database core set v.gg_13_15 (37) using PyNAST (7). Taxonomic assignment was
211 completed using the RDP classifier v.2.2 (55).

212

213 **Stool and plasma metabolomics**

214 Stool and plasma aliquots from Soldiers providing both pre- and post-STRESS stool
215 samples were submitted for global metabolite profiling (Metabolon, Inc., Durham, NC).
216 Samples were analyzed using two separate reverse phase (RP)/UPLC-MS/MS methods with
217 positive ion mode electrospray ionization (ESI), RP/UPLC-MS/MS with negative ion mode ESI,
218 and HILIC/UPLC-MS/MS with negative ion mode ESI.

219 Several recovery standards were added prior to the first step in the extraction process, and
220 were analyzed with the experimental samples for quality control. All analysis methods utilized a
221 Waters ACQUITY UPLC (Waters Corp., Milford, MA) and a Thermo Scientific Q-Exactive
222 high resolution/accurate mass spectrometer interfaced with a heated ESI-II source and Orbitrap
223 mass analyzer operated at 35,000 mass resolution. Sample extracts were dried and reconstituted
224 in solvents compatible to each of the four methods. Each reconstitution solvent also contained a
225 series of standards at fixed concentrations to ensure injection and chromatographic consistency.

226 One aliquot was analyzed using acidic positive ion conditions, chromatographically optimized
227 for more hydrophilic compounds. In this method, the extract was gradient eluted from a C18
228 column (Waters UPLC BEH C18-2.1x100 mm, 1.7 μ m) using water and methanol containing
229 0.05% perfluoropentanoic acid and 0.1% formic acid. Another aliquot was also analyzed using
230 acidic positive ion conditions; however, it was chromatographically optimized for more
231 hydrophobic compounds. In this method, the extract was gradient eluted from the same
232 aforementioned C18 column using methanol, acetonitrile, water, 0.05% perfluoropentanoic acid,
233 and 0.01% formic acid and was operated at an overall higher organic content. Another aliquot
234 was analyzed using basic negative ion optimized conditions using a separate dedicated C18
235 column. The basic extracts were gradient eluted from the column using methanol and water,
236 however with 6.5mM Ammonium Bicarbonate at pH 8. The fourth aliquot was analyzed via
237 negative ionization following elution from a HILIC column (Waters UPLC BEH Amide 2.1x150
238 mm, 1.7 μ m) using a gradient consisting of water and acetonitrile with 10mM Ammonium
239 Formate, pH 10.8. The MS analysis alternated between MS and data-dependent MSⁿ scans using
240 dynamic exclusion. The scan range varied slightly between methods but covered 70-1000 m/z.

241 Raw data was extracted, peak-identified and quality control-processed using Metabolon's
242 proprietary hardware and software. Compounds were identified by comparison to a library
243 maintained by Metabolon containing entries of purified standards or recurrent unknown entities.
244 Biochemical identifications were based on three criteria: retention index within a narrow
245 retention index window of the proposed identification, accurate mass match to the library +/- 10
246 ppm, and the MS/MS forward and reverse scores between the experimental data and authentic
247 standards. The MS/MS scores were based on a comparison of the ions present in the

248 experimental spectrum to the ions present in the library spectrum. Peaks were quantified using
249 area-under-the-curve.

250

251 **Bioinformatics**

252 Analyses were completed using R v.3.3.1, Multiexperiment Viewer v.4.9.0, SPSS v.21,
253 and XLSTAT v.2015. An average of $140,762 \pm 103,480$ 16S rDNA sequences per stool sample
254 were obtained which clustered into 2,015 OTUs at 97% sequence identity. OTUs could be
255 assigned to 12 phyla and 83 genera. Alpha-diversity (Shannon and Chao1 indices, and observed
256 OTUs) was calculated using the *phyloseq* R bioconductor package, and β -diversity calculated
257 using Bray-Curtis distances. Prior to statistical analysis of sequencing data, phylum, genus, and
258 OTU-level relative abundances were calculated by dividing the number of reads for each taxa by
259 the total number of reads in the sample. Ordination and cluster analyses were conducted on
260 OTU-level relative abundances, whereas differential analyses were conducted on phylum and
261 genus-level relative abundances. For differential analyses, any OTUs that could not be assigned
262 to the genus level were grouped at the next lowest level of classification possible (e.g., family or
263 order). Relative abundances were arcsine square-root transformed prior to differential analysis
264 to stabilize variance and better approximate normality. Prior to analysis of stool and plasma
265 metabolites, any missing values were imputed using the minimum observed value for each
266 compound, normalized to set the median equal to 1, and \log_{10} -transformed.

267 Ordinations were conducted by principal coordinates analysis (PCoA) of the OTU Bray-
268 Curtis dissimilarity matrix, principal components analysis (PCA) of metabolite data, and
269 hierarchical complete-linkage clustering of Euclidean distances (OTU and metabolite data).
270 Supervised classification of pre- and post-STRESS samples was conducted using Random Forest

271 analysis, and the mean decrease accuracy used to identify taxa driving classification. To
272 examine associations between stool microbiota composition and global metabolite profiles,
273 metabolite PCA ordinations were compared to OTU PCoA ordinations using Procrustes analysis
274 implemented in the R package *vegan*.

275 A knowledge-based approach was used to better identify microbially-derived metabolites
276 by predicting changes in stool metabolite profiles based on changes in stool microbiota
277 composition. For these analyses PICRSUSt v.1.0.0 was first used to predict metagenome
278 functional content from 16S rDNA data (26). Final metagenome functional predictions were
279 performed by multiplying normalized OTU abundance by each predicted functional profile.
280 Differences in predicted metagenomic profiles were examined by comparing KEGG Orthologs
281 between pre- and post-STRESS samples and PCA. Changes in metagenome functional counts
282 over time were examined following Trimmed Mean of M component normalization by fitting
283 linear models using moderated standard errors and the empirical Bayes model. Metabolites
284 predicted to derive from significantly altered KEGG Orthologs ($P \leq 0.05$) were annotated using
285 HMDB v.2.5, KEGG v.80.0 (compounds, pathways, orthologs and reactions), SMPDB v.2.0, and
286 FOOB v.1.0. These metabolites were then compared to the list of metabolites in stool that
287 increased or decreased over time ($P < 0.10$). Overlapping metabolites were considered as
288 indicative of functional relationships between changes in the microbiome and the metabolome.

289

290 **Statistical analysis**

291 Sample size calculations were based on primary study outcomes which have been
292 previously reported (32, 43). Statistical analyses were completed using SPSS v.21 and R v.3.3.1.
293 Data were assessed for normality prior to analysis and transformed if necessary to meet model

294 assumptions. When transformation was not successful, non-parametric tests were used.
295 Repeated measures ANOVA was used to test effects of STRESS and diet, and their interaction
296 on study outcomes. Pairwise comparisons of pre- and post-STRESS genus relative abundances
297 were conducted using the Wilcoxon-signed rank test, and between group comparisons of changes
298 in genus relative abundances were conducted using the Kruskal-Wallis test. Spearman's rank
299 correlation (ρ), Pearson's correlation (r), multiple linear regression, and linear mixed models
300 were used to examine associations among variables. Relationships between sucralose excretion,
301 LPS, IL-6, and CRP concentrations with ordinations of stool microbiota composition, and
302 stool/plasma metabolites were also assessed using linear mixed models. All mixed models
303 included subject as a random factor and time as a continuous covariate. Sucralose excretion,
304 LPS, IL-6 or CRP were entered as dependent variables, and scores for the first three principal
305 components of the ordinations were included as independent variables. Finally, backwards
306 stepwise regression was used to identify the strongest predictors of changes in IP. Independent
307 variables included in the regression model were those that were significantly correlated with
308 changes in sucralose excretion, and included dietary parameters (protein intake), changes scores
309 of inflammation markers (IL-6 and CRP), pre-STRESS stool microbiota characteristics (Shannon
310 diversity, *Actinobacteria* and *Proteobacteria* relative abundances), and change scores for stool
311 metabolites linked to changes in microbiota composition changes (cysteine and arginine).
312 Changes in Shannon diversity and pre-STRESS *Sutterella* relative abundance were also
313 considered in place of pre-STRESS Shannon diversity and *Proteobacteria* relative abundance,
314 respectively.

315 The false discovery rate for all tests including taxa or metabolite data was controlled by
316 adjusting P -values using the Benjamini-Hochberg procedure. Adjusted P -values are presented as

317 *Q*-values. Data are presented as mean \pm SD unless otherwise noted. Statistical significance was
318 set at $P \leq 0.05$ or $Q \leq 0.10$.

319

320 **RESULTS**

321 Macronutrient intakes varied across study groups as planned (Table 1). Specifically,
322 mean protein intake was higher in PRO relative to CNTRL and CHO ($P < 0.05$), mean
323 carbohydrate intake was higher in CHO relative to CNTRL and PRO ($P < 0.05$), and fat intake
324 did not differ between groups. Energy intake was higher in CHO relative to CNTRL and PRO
325 ($P < 0.05$; Table 1). Energy expenditure was high, averaging 25.7 ± 2.2 MJ/d, and did not differ
326 between groups (32). The high energy expenditure resulted in a 55% energy deficit and 2.7 ± 1.2
327 kg body mass loss which also did not differ between groups (32, 43). Serum creatine kinase, IL-
328 6, and CRP concentrations have been reported previously (43). All increased during STRESS
329 independent of diet group, indicating muscle damage and inflammation were induced during
330 STRESS.

331 The volunteers choosing to provide stool samples were all males, and did not differ in age
332 ($P = 0.59$), BMI ($P = 0.47$), or body mass loss ($P = 0.98$), change in intestinal permeability ($P =$
333 0.42), energy intake ($P = 0.51$), macronutrient intake ($P \geq 0.11$), or energy expenditure ($P =$
334 0.94) during STRESS relative to volunteers choosing not to provide stool samples.

335

336 **Intestinal permeability, plasma LPS, and inflammation**

337 Sucralose excretion increased $62 \pm 57\%$ during STRESS independent of diet (main effect
338 of time, $P < 0.001$; **Figure 1A**), suggesting increased IP, and was correlated with changes in
339 creatine kinase ($r = 0.34$, $P = 0.02$), CRP ($\rho = 0.36$, $P = 0.01$), IL-6 (**Figure 1B**), and protein

340 intake ($\rho = -0.31$, $P = 0.03$). Mannitol excretion also increased during STRESS independent of
341 diet (Pre: $28 \pm 8\%$ vs Post: $33 \pm 13\%$; main effect of group, $P = 0.01$). Plasma LPS
342 concentrations did not differ from pre- to post-STRESS ($P = 0.79$; **Figure 1C**). However,
343 Soldiers with increased LPS concentrations demonstrated a trend to have greater increases in IL-
344 6 relative to individuals with no change or a decrease in LPS concentrations (**Figure 1D**).

345

346 **Stool microbiota composition**

347 The Shannon α -diversity index increased during STRESS independent of diet (main
348 effect of time, $P = 0.04$), whereas the Chao1 index (main effect of time, $P = 0.42$) and total
349 observed OTUs (main effect of time, $P = 0.45$) were not affected by STRESS or diet, indicating
350 an increase in the evenness but not the richness of the stool microbiota (**Figure 2A**). PCoA
351 (**Figure 2B**) and cluster (**Figure 2C**) analyses demonstrated an effect of STRESS on the
352 microbiota independent of diet. Random forest analysis differentiated pre- and post-STRESS
353 samples with 100% accuracy. The top 10 taxa contributing to the high prediction accuracy were
354 *Peptostreptococcus*, *Christensenella*, *Faecalibacterium*, *Staphylococcus*, unassigned taxa within
355 the *Mogibacteriaceae*, *Christensenellaceae*, and *Planococcaceae*, families, and unassigned taxa
356 within the *CW040* and *RF39* orders (**Supplemental Table 1**). At the phylum-level, decreases in
357 *Bacteroidetes*, and increases in *Firmicutes* and several other phyla were observed ($Q < 0.10$;
358 **Figure 2D**). At the genus-level, changes in the relative abundances of 48 of 83 identified genera
359 were observed ($Q < 0.10$; Supplemental Table 1). Changes in genus relative abundances did not
360 differ by diet group ($Q > 0.75$ for all).

361

362 **Stool and plasma metabolites**

363 A total of 694 compounds were identified in stool. Principal components (**Figure 3A**)
364 and cluster (**Figure 3B**) analyses of these compounds did not suggest an effect of time point or
365 diet. However, random forest analysis correctly differentiated pre- and post-STRESS stool
366 samples with 84% accuracy (**Figure 3C**), and 274 compounds demonstrated statistically
367 significant changes ($Q < 0.10$). Of these, 81% decreased during STRESS, including several
368 metabolites of amino acid, fatty acid, carbohydrate, and energy metabolism (**Supplemental**
369 **Table 2**). Secondary bile acids and amino acid metabolites (**Figure 4**) known to be solely or
370 partially derived from microbial metabolism were generally decreased as well or unchanged,
371 with the notable exception of p-cresol, a microbial metabolite of tyrosine fermentation, which
372 was increased in stool post-STRESS.

373 A total of 737 compounds were identified in plasma, of which 478 demonstrated
374 statistically significant changes during STRESS ($Q < 0.10$). Changes primarily reflected
375 increases in host energy metabolism, lipolysis, fatty acid oxidation, branched-chain amino acid
376 catabolism, and steroid metabolism (data not shown). However, changes in plasma
377 concentrations of several metabolites known to be partially or fully derived from microbial
378 metabolism were also observed. Specifically, mean concentrations of phenylalanine and tyrosine
379 metabolites including p-cresol sulfate (+48%), p-cresol-glucuronide (+79%), phenylacetate
380 (+44%), phenyllactate (+42%), phenylacetylglutamine (+24%), and 3-(4-hydroxyphenyl)lactate
381 (+40%) were increased (**Figure 4**). In contrast, mean concentrations of the benzoate metabolites
382 2-hydroxyhippurate (-22%), 3-hydroxyhippurate (-61%), and 4-hydroxyhippurate (-35%) were
383 decreased ($Q < 0.10$). Mean concentrations of secondary bile acids in plasma demonstrated
384 more variable responses as glycolithocholate sulfate (+21%), glycohyocholate (+6%),
385 tauroolithocholate 3-sulfate (+89%), and taurocholate sulfate (+56%) concentrations increased,

386 while deoxycholate (-66%), ursodeoxycholate (-63%), and isoursodeoxycholate (-51%)
387 concentrations decreased ($Q < 0.10$).

388

389 **Associations between stool microbiota composition, stool and plasma metabolites, intestinal** 390 **permeability, and inflammation**

391 Changes in sucralose excretion were inversely associated with pre-STRESS Shannon
392 diversity ($\rho = -0.43$, $P = 0.05$) and *Actinobacteria* relative abundance ($\rho = -0.53$, $Q = 0.09$), and
393 positively correlated with pre-STRESS *Proteobacteria* ($\rho = 0.64$, $Q = 0.02$) and *Sutterella* ($\rho =$
394 0.68 , $Q = 0.09$) relative abundances (**Figure 5** and Supplemental Table 1) and changes in
395 Shannon diversity ($\rho = 0.58$, $P = 0.02$). No statistically significant correlations between the pre-
396 STRESS relative abundance of any taxa, or the change in relative abundance of any taxa, and
397 changes in LPS, IL-6 or CRP were detected. Additionally, no association between these
398 variables and scores extracted from the first three principal components of the stool microbiota
399 PCoA analysis were detected.

400 Procrustes analysis demonstrated a significant association between the ordinations of
401 stool metabolites and stool microbiota composition ($M^2 = 0.76$, Monte Carlo $P = 0.001$; **Figure**
402 **6A**) indicating an association between stool metabolites and the stool microbiota. Additionally,
403 prediction models linked changes in stool microbiota composition to 69 of the metabolites found
404 to be altered in stool (**Supplemental Table 3**). These models were supported by Procrustes
405 analysis on ordinations of the significantly altered taxa and these metabolites ($M^2 = 0.72$, Monte
406 Carlo $P = 0.001$). Of the 69 metabolites, amino acid and nucleotide metabolites comprised the
407 majority, and were generally lower post- relative to pre-STRESS ($Q < 0.10$). Changes in two,
408 arginine and cysteine, were correlated with changes in sucralose excretion during STRESS

409 (Table 2). Changes in the concentrations of another 14 metabolites were also inversely
410 correlated with changes in sucralose excretion (Table 2). In accord, scores on the 1st principal
411 component from the ordination of stool metabolite data were associated with sucralose excretion
412 ($\beta \pm SE = -0.05 \pm 0.01$, $P = 0.01$) indicating that the effect of STRESS on stool microbiota was
413 associated with IP.

414 Procrustes analysis also demonstrated a significant association between the ordinations of
415 plasma metabolites and stool microbiota composition ($M^2 = 0.49$, Monte Carlo $P = 0.001$;
416 Figure 6B) indicating an association between plasma metabolites and the stool microbiota.
417 Further, plasma concentrations of 30 of the 69 metabolites that linked the stool microbiota to the
418 stool metabolome in prediction models were altered (Figure 6C and Supplemental Table 3).
419 However, plasma metabolite changes were not correlated with changes in sucralose excretion,
420 IL-6 or CRP.

421 Backwards stepwise regression was used to identify the strongest predictors of changes in
422 IP. The final model comprising pre-STRESS *Actinobacteria* relative abundance, change in
423 serum IL-6 concentrations, and changes in stool cysteine concentrations explained 84% of the
424 variability in the change in sucralose excretion (Table 3). Collectively, these findings
425 demonstrated an association between intestinal microbiota composition, stool metabolite
426 concentrations, and changes in IP.

427

428 Discussion

429 The magnitude, temporal dynamics, and physiologic effects of intestinal microbiome
430 responses to stress are poorly characterized. Our findings demonstrate that a multiple-stressor
431 environment characterized by high physical exertion, suboptimal energy intake, muscle damage,

432 and inflammation adversely effects intestinal barrier integrity concomitant to alterations in
433 intestinal microbiota composition and metabolism. Observed associations between increased IP,
434 the pre-stress microbiota, and stool metabolites associated with the microbiota suggest that
435 targeting the intestinal microbiota could provide novel strategies for maintaining intestinal
436 barrier integrity during physiologic stress.

437 The observed increase in IP in association with increased inflammation (Figure 1) is
438 consistent with the only other study to our knowledge that has assessed IP in military personnel
439 during training (29). In these environments, intense or prolonged exercise may reduce
440 splanchnic perfusion which can trigger intestinal hypoxia, inflammation, and oxidative stress that
441 collectively degrade intestinal barrier integrity and increase IP (15, 24, 54). Stress-induced
442 muscle damage may also contribute to inflammation, potentiating increases in IP by inducing
443 tight junction dysfunction (15). Ultimately, the increase in IP is thought to result in mild
444 endotoxemia and inflammation, and contribute to gastrointestinal distress in endurance athletes
445 (4, 15, 20, 24) and possibly military personnel (29). Although gastrointestinal symptoms were
446 not assessed in this study, Li et al. (29) reported that 70% of Soldiers participating in a 6-wk
447 combat training course reported gastrointestinal distress symptomology (i.e., abdominal pain,
448 diarrhea, constipation), those symptoms were more frequent in Soldiers with the largest increases
449 in IP, and symptoms were associated with psychological decrements. Gastrointestinal distress,
450 to include infectious diarrhea, is historically the leading non-battle injury encountered in
451 deployed military personnel, representing a significant burden to military health care and
452 operational readiness (45-47). Identifying mediators of intestinal barrier responses to severe
453 stress, and developing strategies to target those mediators may therefore have substantial benefit
454 for military personnel.

455 Our findings suggest that the intestinal microbiota may be one mediator of IP responses
456 to severe physiologic stress, and that targeting the microbiota before stress exposure may be one
457 strategy for maintaining IP. In particular, increasing microbiota diversity and *Actinobacteria*
458 relative abundance, and decreasing *Proteobacteria* and *Sutterella* relative abundances before
459 stress exposure may be effective in lieu of the observed associations with changes in IP during
460 stress (Figure 5). Greater microbiota diversity is generally considered indicative of a healthy
461 intestinal ecosystem, having been frequently associated with lower chronic disease risk (11, 19).
462 Similarly, species within the *Actinobacteria* phyla including those belonging to the
463 *Bifidobacterium* and *Collinsella* genera have favorable anti-inflammatory and immuno-
464 modulatory effects which may protect the intestinal barrier during stress (2, 42). In support,
465 *Bifidobacterium* strains are included in multi-strain probiotics that have demonstrated some
466 efficacy, albeit weak, for favorably impacting IP in athletes (25, 49). Increasing *Bifidobacterium*
467 relative abundance using prebiotics such as oligofructose has also been shown to promote
468 intestinal barrier integrity in animal models (6). In contrast, *Proteobacteria* are endotoxin
469 producers which have been linked to inflammatory bowel diseases and subclinical inflammation
470 (19, 27). *Sutterella*, a genus within the *Proteobacteria* phyla, have been shown to promote
471 inflammatory bowel disease by inhibiting immunoglobulin-A secretion (39). As such, although
472 findings are correlative and the study design precludes determining causality, the observed
473 associations between the pre-stressed microbiota and changes in IP during stress are plausible
474 and provide potential targets for further study.

475 To our knowledge, this study is the first to examine intestinal microbiota responses
476 during military training, and expands knowledge regarding the temporal effects of exercise and
477 psychological stress on the microbiome, which is largely limited to animal studies at present (12,

478 34). Human studies have demonstrated that drastic changes in diet impact intestinal microbiota
479 composition (13, 41) by altering the availability of metabolic substrates for intestinal microbes
480 (23). Our findings contrast with those reports in demonstrating alterations in microbiota
481 composition that most likely were not solely attributable to diet, and which were more
482 pronounced than is commonly reported in human diet studies (Figure 2). Although potential
483 mechanisms were not directly assessed, changes in immune activity, intestinal inflammation and
484 oxidative stress, and altered hypothalamic-pituitary-adrenal axis and vagal signaling have all
485 been postulated as mechanisms through which physical and psychological stress modulate the
486 microbiome (12, 34).

487 The increase in Shannon α -diversity and the numerous genus-level changes in relative
488 abundance demonstrated that changes in microbiota composition were broadly characterized by
489 an increase in abundance of less dominant taxa at the expense of more dominant taxa such as
490 *Bacteroides* (Figure 2). This included increased relative abundances of several potentially
491 deleterious and infectious taxa (e.g. *Peptostreptococcus*, *Staphylococcus*, *Peptoniphilus*,
492 *Acidaminococcus*, *Fusobacterium*), and decreased relative abundances of several taxa thought to
493 deter pathogen invasion, reduce inflammation and promote immunity (e.g., *Bacteroides*,
494 *Faecalibacterium*, *Collinsella*, *Roseburia*). As such, an increase in the ratio of less-abundant,
495 potentially harmful taxa to beneficial taxa may explain the unexpected observation that greater
496 increases in diversity during training were correlated with larger increases in IP. However,
497 several alternative explanations exist. Individuals with the lowest pre-STRESS Shannon
498 diversity also demonstrated the largest increases in diversity during STRESS ($r = -0.60$, $P =$
499 0.001). Therefore, the association between increased diversity and increased IP may attributable
500 to lower pre-STRESS diversity. Alternately, higher stool microbiota diversity has been

501 correlated with longer intestinal transit time and higher urinary concentrations of potentially
502 harmful degradation products of bacterial protein metabolism (48). In this study, stool and
503 plasma concentrations of protein degradation products did not uniformly change, although they
504 were more commonly decreased in stool and increased in plasma (Figure 4, Supplemental Tables
505 2 and 3). Whether these observations reflect changes in transit time could not be determined
506 from the collected data. Nonetheless, no protein degradation metabolite was independently
507 associated with increased IP or inflammation. This observation contrasts with reports that
508 bacterial protein metabolites induce intestinal barrier damage and inflammation *in vitro* (56), and
509 suggests that the positive association between protein intake and increases in IP during training
510 was not mediated by bacterial metabolism of diet-derived amino acids.

511 Decreased concentrations of several stool metabolites were associated with increased IP
512 (Table 2). Metabolites included two amino acids, arginine and cysteine, which were predicted to
513 be associated with changes in microbiota composition, and which are plausible modulators of IP
514 based on known physiologic functions. Specifically, arginine is a precursor to polyamines
515 required for intestinal mucosal growth and repair, and for nitric oxide, a potent vasodilator that
516 may protect intestinal barrier integrity by improving splanchnic perfusion, deterring pathogen
517 invasion, and modulating inflammation (28, 54). In support, arginine supplementation has
518 preserved intestinal barrier integrity in various animal stress and intestinal injury models (2),
519 although the effects in humans are less clear (5). Cysteine is an essential component of
520 glutathione, an antioxidant tripeptide critical to maintaining a favorable redox balance in the
521 intestine (10). Of note, Phua et al. (44) recently reported that increases in urinary concentrations
522 of a glutathione metabolite, possibly reflecting increased oxidative stress, were associated with
523 gastrointestinal symptomology during military training. Our findings also suggest that

524 interactions between the intestinal microbiota and dietary fat metabolism may impact IP (Table
525 2). 7-ketodeoxycholate and 12-dehydrocholate are secondary bile acids derived from bacterial
526 metabolism of bile acids secreted in response to dietary fat intake. Secondary bile acids are
527 recognized as important signaling molecules with functions that are thought to include promotion
528 of gut barrier integrity (52). Collectively, these findings suggest that changes in intestinal
529 microbiota composition and metabolism may impact IP during physiologic stress by modulating
530 the availability of amino acid precursors critical to moderating inflammation and oxidative
531 stress, and of secondary bile acids.

532 Study strengths include the provision of diets of known composition providing a range of
533 macronutrient intakes, and the integration of physiologic, stool microbiota composition, and
534 metabolomics data. However, results should be interpreted in the context of the study design and
535 several limitations. The physically demanding environment coupled with the physiologic
536 demands imposed by undereating may have masked some associations, and limited
537 generalizability of the findings, but provides unique and novel insights into the temporal
538 dynamics of host-microbiome interactions during prolonged physical stress. While
539 psychological and sleep deprivation stress were likely also present, we did not quantify those
540 responses. Study participants were predominantly young males, and findings may not be
541 generalizable to older populations or females. Limitations include the correlative nature of
542 associations between outcomes from which causality cannot be determined despite evidence of
543 plausibility, and limited statistical power for some analyses, especially those including between-
544 group comparisons, resulting from only a subset of the full cohort participating in stool
545 collections. The method for measuring plasma LPS concentrations is also a limitation as it did
546 not quantify endotoxin activity which is known to vary between LPS forms (18). Nonetheless,

547 the weak association between changes in plasma LPS and IL-6 are consistent with the well-
548 established pro-inflammatory effects of the compound (18). Including metagenomic or
549 transcriptomic analysis of stool samples would have strengthened findings and complemented
550 the metabolomics analysis by allowing more accurate functional predictions of microbiota
551 function. Reliance on stool for measurements of microbiota composition and metabolites is also
552 a limitation as the composition of the stool may be more reflective of the distal colon than the
553 entirety of the gastrointestinal tract. However, the addition of plasma metabolite measurements
554 was included to better capture bacterial metabolism along the full gastrointestinal tract. Finally,
555 logistical constraints prevented more frequent measurements which would have provided
556 additional insight into temporal dynamics.

557 Using a systems biology approach, this study confirmed the hypothesis that a multiple-
558 stressor environment can induce increases in IP that are associated with inflammation, and
559 intestinal microbiota composition and metabolism. Further, these findings extend the current
560 evidence base by demonstrating that such environments can induce rapid and pronounced
561 changes in the intestinal microbiota, and suggest that the pre-stress intestinal microbiota and
562 changes in microbial metabolism may be important for mediating intestinal barrier responses to
563 stress. As such, targeting the intestinal microbiota could provide novel strategies for mitigating
564 increases in IP and associated sequelae induced by physically and psychologically demanding
565 environments.

566

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574

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576 The opinions or assertions contained herein are the private views of the
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582

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587

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768

769 **Figure 1. Intestinal permeability, plasma LPS and inflammation during military training.**

770 **A)** Intestinal permeability measured by 24 hr urine collection following ingestion of 2 g
771 sucralose (n = 49), and **C)** plasma LPS concentrations (n = 67) before (PRE) and after (POST)
772 military training. Boxes are median and interquartile range, whiskers are 1.5 times the
773 interquartile range, or minimum and maximum if no observations within that range, and solid
774 circles are data points >1.5 times the interquartile range. *Repeated measures ANOVA, main
775 effect of time, $P < 0.001$. **B)** Changes in intestinal permeability are correlated with changes in
776 serum interleukin (IL)-6 concentrations; Pearson's correlation (n = 46). **D)** Soldiers experiencing
777 increases in plasma LPS during training demonstrate a trend for larger increases in serum IL-6
778 relative to those experiencing a decrease or no change in plasma LPS; repeated measures
779 ANOVA, time-by- Δ LPS interaction, $P = 0.07$, #significantly different from PRE, $P < 0.05$.
780 CHO, carbohydrate-supplement group; CNTRL, control group (rations only); PRO, protein-
781 supplement group.

782

783

784 **Figure 2. Military training elicits changes in intestinal microbiota composition. A)** Alpha-

785 diversity before (PRE) and after (POST) military training. Boxes are median and interquartile
786 range, whiskers are 1.5 times the interquartile range, or minimum and maximum if no
787 observations within that range, and solid circles are data points >1.5 times the interquartile range.
788 *Repeated measures ANOVA, main effect of time, $P = 0.04$. **B)** Principal coordinates analysis of
789 Bray-Curtis dissimilarity matrix indicates that stool microbiota community composition was
790 more strongly influenced by the training environment than by individual variability or diet group.
791 Data points represent the stool microbiota community of a single individual. Points closer

792 together are more similar. **C)** Hierarchical complete-linkage clustering of Euclidean distances of
793 OTU relative abundances measured in stool collected before and after military training; $n = 38$.
794 Colored bars are data points representing the stool microbiota composition of an individual.
795 Branches (lines) within the same node (points where branches split) reflect similarity in stool
796 microbiota community composition. Clustering of branches by time point indicates that stool
797 microbiota community composition was more strongly influenced by the training environment
798 than by individual variability or diet group. **D)** Phyla-level shifts in gut microbiota composition;
799 bars are mean relative abundances. Arrows indicate direction of change in relative abundance
800 from PRE to POST. *Repeated measures ANOVA, main effect of time, $P < 0.05$. CHO,
801 carbohydrate-supplement group ($n = 9$); CNTRL, control group (rations only; $n = 5$); PRO,
802 protein-supplement group ($n = 12$).

803
804 **Figure 3. Stool metabolomics before (PRE) and after (POST) military training.** **A)** Principal
805 components, **B)** hierarchical complete-linkage clustering of Euclidean distances; and **C)** random
806 forest analyses of stool metabolites; $n = 25$. **A)** Individual data points represent the metabolite
807 composition within a single individual. Points closer together are more similar. **B)** Columns are
808 individuals and rows are metabolites shaded by abundance within sample. Branches (lines)
809 within the same node (points where branches split) reflect similarity in metabolite composition.
810 Stool metabolites did not demonstrate any distinct clustering pattern. **C)** Top 30 metabolites
811 with the strongest influence on prediction accuracy of the random forest analysis are presented in
812 order of importance (top to bottom). Random forest analysis used individual metabolite profiles
813 to predict whether the samples were from PRE or POST training. Mean decrease in prediction
814 accuracy is the mean decrease in the percentage of observations classified correctly when that

815 metabolite is assigned a random value. Arrows indicate direction of metabolite change from PRE
816 to POST. CHO, carbohydrate-supplement group; CNTRL, control group (rations only); PRO,
817 protein-supplement group.

818

819 **Figure 4. Qualitative changes in phenylalanine and tyrosine (A) and tryptophan (B)**

820 **metabolites in stool and plasma during military training.** Arrows indicate direction of
821 change in stool (brown) and plasma (red) from pre- to post-training (repeated measures ANOVA,
822 main effect of time, $Q < 0.10$). Metabolites circled by dashed line are compounds known to be
823 wholly or partially derived from microbial metabolism. Compounds without arrows were either
824 unchanged ($Q > 0.10$) or not detected.

825

826 **Figure 5. Factors associated with increased intestinal permeability during military training.**

827 Intestinal permeability measured by 24 hr urine collection following ingestion of 2 g sucralose.
828 Spearman's correlation (ρ) ($n = 21$). P -values for correlations with taxa adjusted using
829 Benjamini-Hochberg correction (Q).

830

831 **Figure 6. Stool microbiota composition is associated with stool metabolite, and plasma**

832 **metabolite concentrations.** Procrustes analysis of stool microbiota data ordinated using
833 principal coordinates analysis of Bray-Curtis distances, and stool (A) and plasma (B) metabolite
834 profiles ordinated using principal components analysis. The first three components of each
835 ordination were extracted and analyzed using Procrustes rotation which attempts to rotate
836 ordinations to maximal similarity. Open circles represent the stool microbiota community of a
837 single individual before or after military training. Arrowheads represent the stool or plasma

838 metabolite profile of a single individual before or after military training. Vectors connect
839 microbiota composition with metabolite profiles of the same individual for each time point.
840 Longer vectors indicate greater intra-individual dissimilarity. The fit of each Procrustes rotation
841 over the first three dimensions is reported as the M^2 value. P-values were calculated after 1000
842 permutations. Results indicate similar clustering patterns between stool microbiota composition
843 and stool metabolites, and between stool microbiota composition and plasma metabolites. C)
844 Venn diagram of stool and plasma metabolites that were significantly altered during military
845 training ($Q \leq 0.10$). Diagram indicates that prediction models linked changes in stool microbiota
846 composition to 69 of the metabolites found to be altered in stool, 30 of which were also
847 significantly altered in plasma.

848 **Table 1** Volunteer characteristics, energy expenditure, and dietary intake.
849

	CNTRL (n = 18)	CHO (n = 27)	PRO (n = 28)
Age (y)	19 ± 2	20 ± 1	20 ± 1
BMI (kg/m ²)	23.6 ± 1.8	24.1 ± 2.3	23.3 ± 2.1
Energy expenditure (MJ/d)	25.5 ± 1.7	25.8 ± 2.1	25.8 ± 2.5
Energy intake (MJ/d)	10.5 ± 1.7 (6.5 – 13.0)	13.1 ± 2.6* (7.7 – 16.4)	11.8 ± 2.5 (7.1 – 16.8)
Carbohydrate (g/d)	312 ± 47 (193 – 385)	434 ± 86* (253 – 543)	321 ± 77 [†] (171 – 490)
Protein (g/d)	100 ± 15 (65 – 124)	98 ± 22 (58 – 130)	148 ± 25* [†] (96 – 191)
Fat (g/d)	91 ± 20 (57 – 117)	107 ± 24 (56 – 146)	102 ± 23 (59 – 141)
Fiber (g/d)	25 ± 4 (16 – 32)	25 ± 6 (13 – 33)	22 ± 5 [†] (12 – 33)

850
851 Table adapted from Margolis et al. (32) and Pasiakos et al. (43). Values are mean ± SD and
852 range (min-max). Volunteers received 3 rations/d (CNTRL) or 3 rations/d and 4 carbohydrate-
853 based snacks bars/d (CHO) or 4 protein-based snack bars/d (PRO). n = 1 from CNTRL, and n =
854 2 from PRO excluded due to incomplete food logs.

855 *,[†]Means were compared by one-way ANOVA; *different from CNTRL ($P < 0.05$), [†]different
856 from CHO ($P < 0.05$).

857

858

859 **Table 2.** Stool metabolites associated with changes in intestinal permeability during military
 860 training.

Super pathway	Sub pathway	Biochemical name	ρ	<i>P</i> -value	<i>Q</i> -value
Amino acid	Leucine, isoleucine and valine metabolism	3-methylglutaconate	-0.75	0.001	0.05
		Methionine, cysteine, SAM and taurine metabolism	N-acetyltaurine	-0.73	0.001
		L-Cysteine	-0.70	0.003	0.07
		Taurine	-0.68	0.004	0.08
		N-acetylmethionine sulfoxide	-0.67	0.005	0.09
	Polyamine metabolism	N-acetylputrescine*	-0.78	<0.001	0.05
	Urea cycle; arginine and proline metabolism	L-Arginine	-0.70	0.002	0.06
Carbohydrate	Aminosugar metabolism	Glucuronate	-0.68	0.004	0.08
Cofactors & vitamins	Nicotinate and nicotinamide metabolism	Nicotinate ribonucleoside	-0.69	0.003	0.07
Lipid	Endocannabinoid	Linoleoyl ethanolamide	-0.75	0.001	0.05
		Oleoyl ethanolamide	-0.71	0.002	0.06
	Mevalonate metabolism	Mevalonate	-0.71	0.002	0.06
	Phospholipid metabolism	Trimethylamine N-oxide	-0.71	0.002	0.06
	Secondary bile acid metabolism	7-ketodeoxycholate	-0.86	<0.001	0.01
	12-dehydrocholate	-0.71	0.002	0.06	
Xenobiotics	Xanthine metabolism	1-methylxanthine	-0.76	0.001	0.05

861 Data are Spearman's correlation (ρ) of change in metabolite versus change in sucralose excretion
 862 (post – pre). *P*-values adjusted using the Benjamini-Hochberg correction (*Q*-value).

863 *Significantly increased from pre- to post-training. All other listed metabolites decreased (*Q* <
 864 0.10; see also Supplemental Table 2).

865 **Table 3.** Model predicting changes in intestinal permeability during military training (STRESS).
 866

	$\beta \pm SE$	Standardized β	<i>P</i>-value
<i>Actinobacteria</i> relative abundance (pre-STRESS)	-45.0 \pm 8.5	-0.59	< 0.001
$\Delta\text{Log}_{10}\text{IL-6}$ (pg/mL)	0.4 \pm 0.6	0.43	0.003
$\Delta\text{Log}_{10}\text{Stool cysteine}$	-2.4 \pm 0.6	-0.43	< 0.001
Intercept	1.4 \pm 0.3		< 0.001
Adjusted $R^2 = 0.84$			< 0.001

867
 868 Dependent variable is change in sucralose excretion (post – pre) measured from 24 hr urine
 869 collection following ingestion of 2 g sucralose and expressed as percent of ingested dose (n =
 870 15).
 871

Figure 1

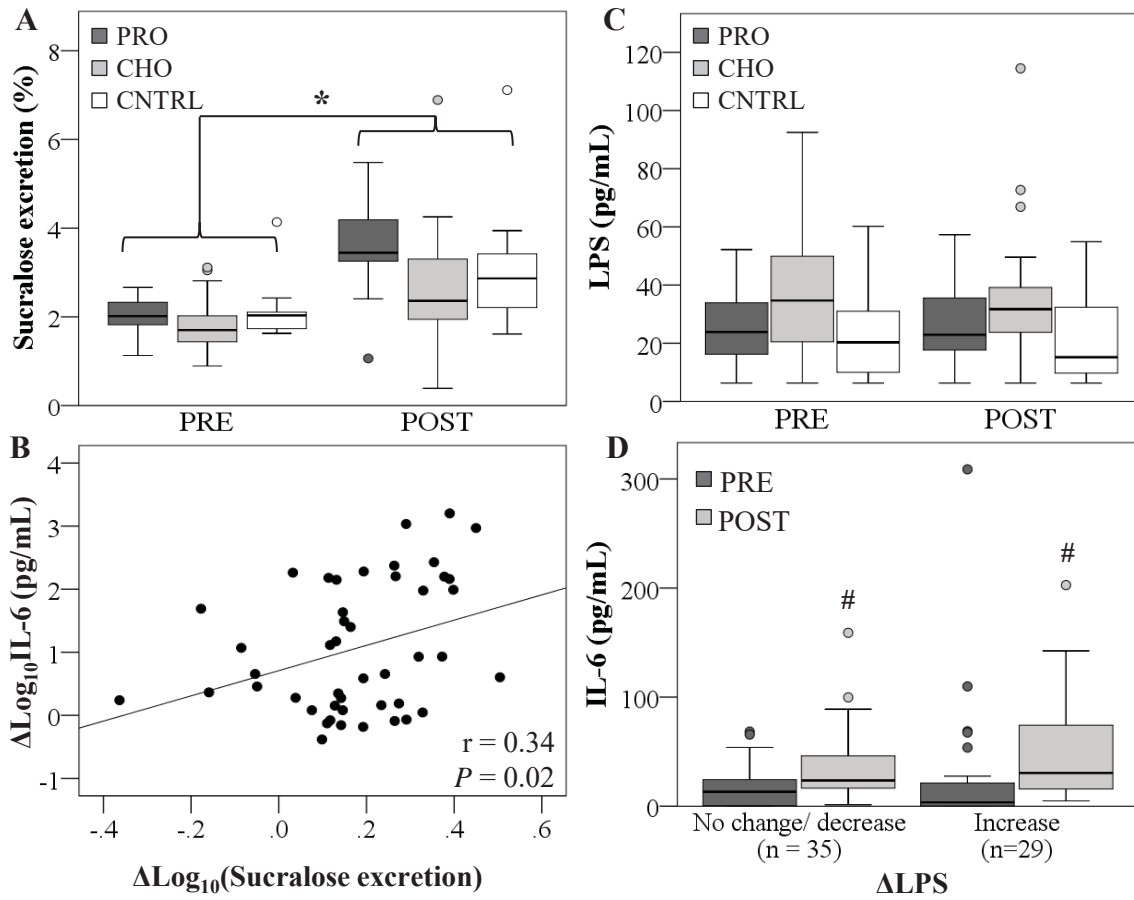


Figure 2

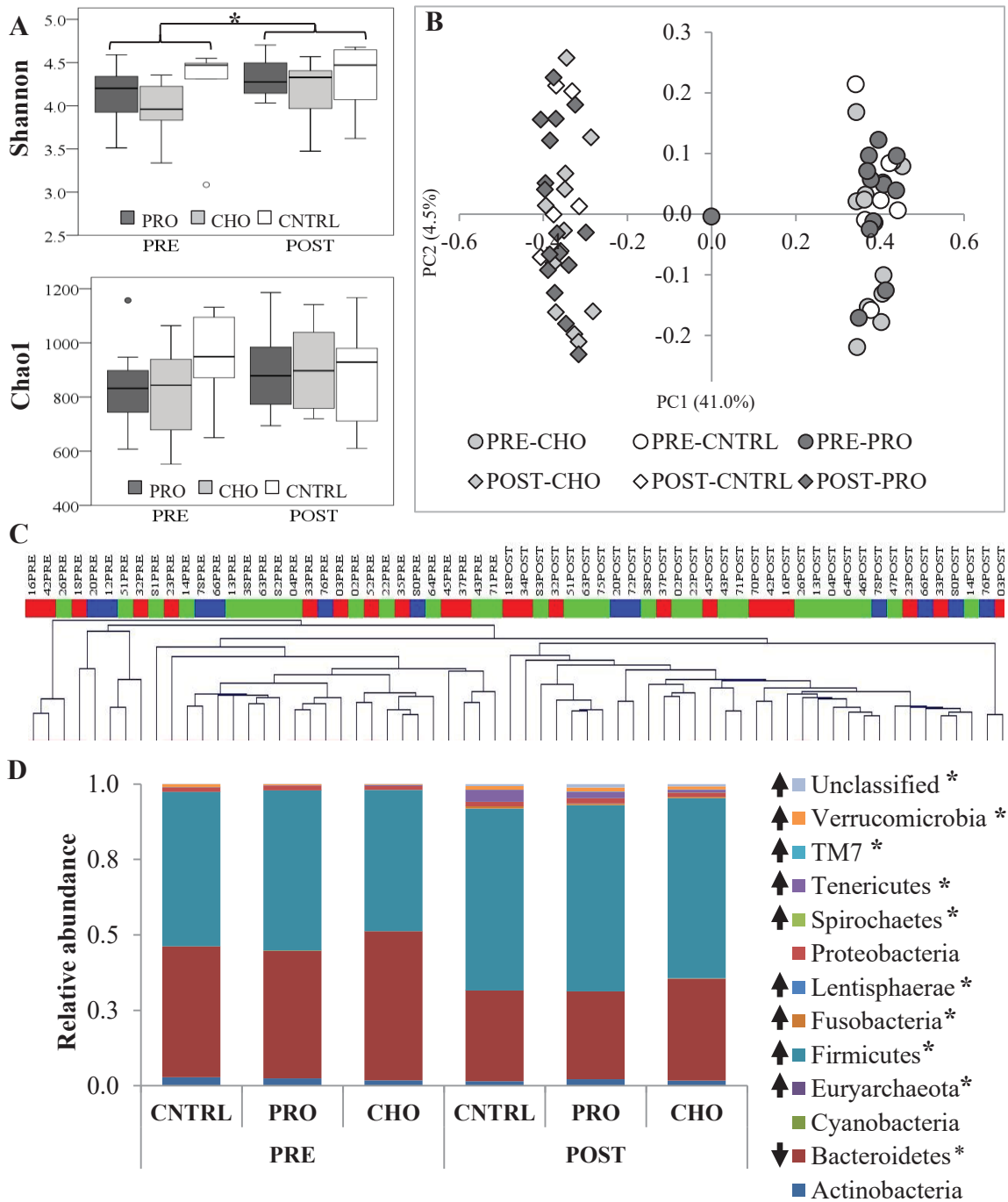


Figure 3

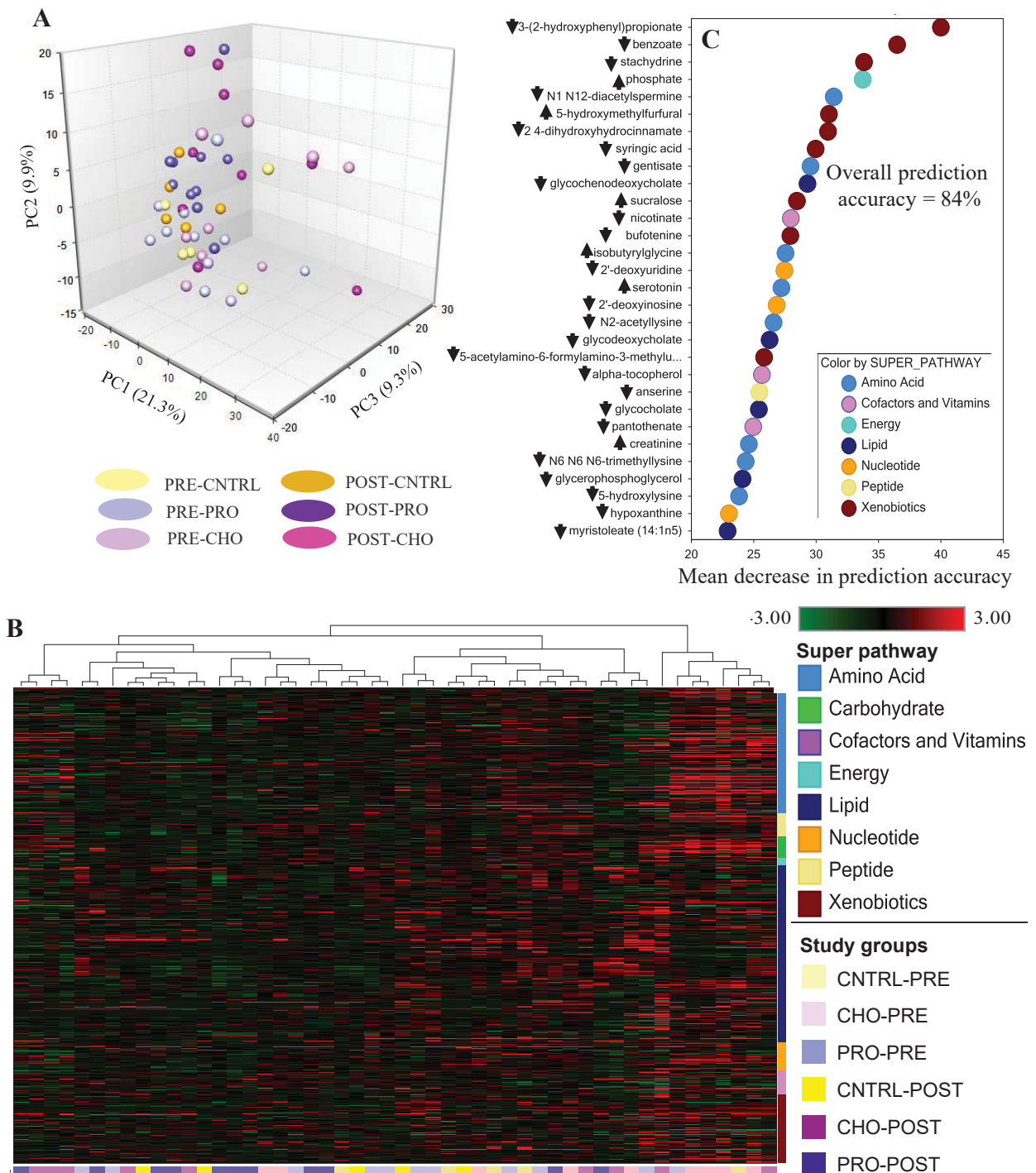


Figure 4

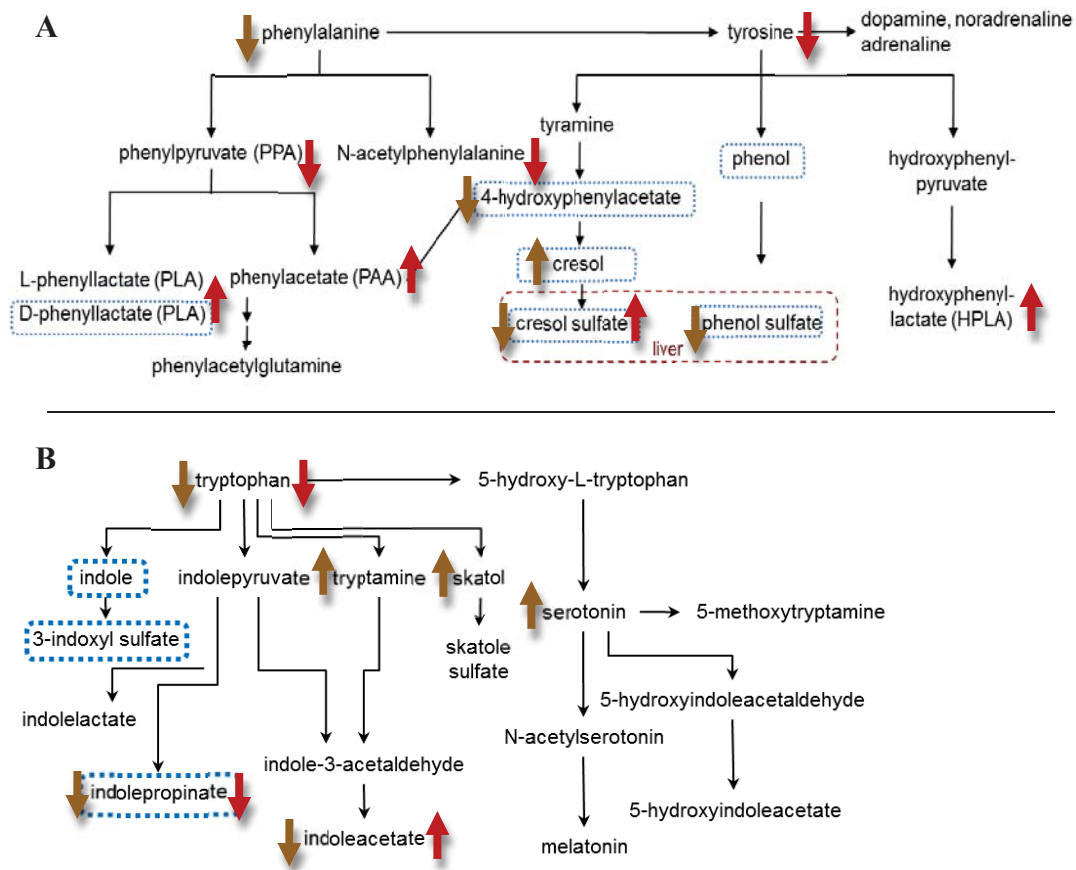


Figure 5

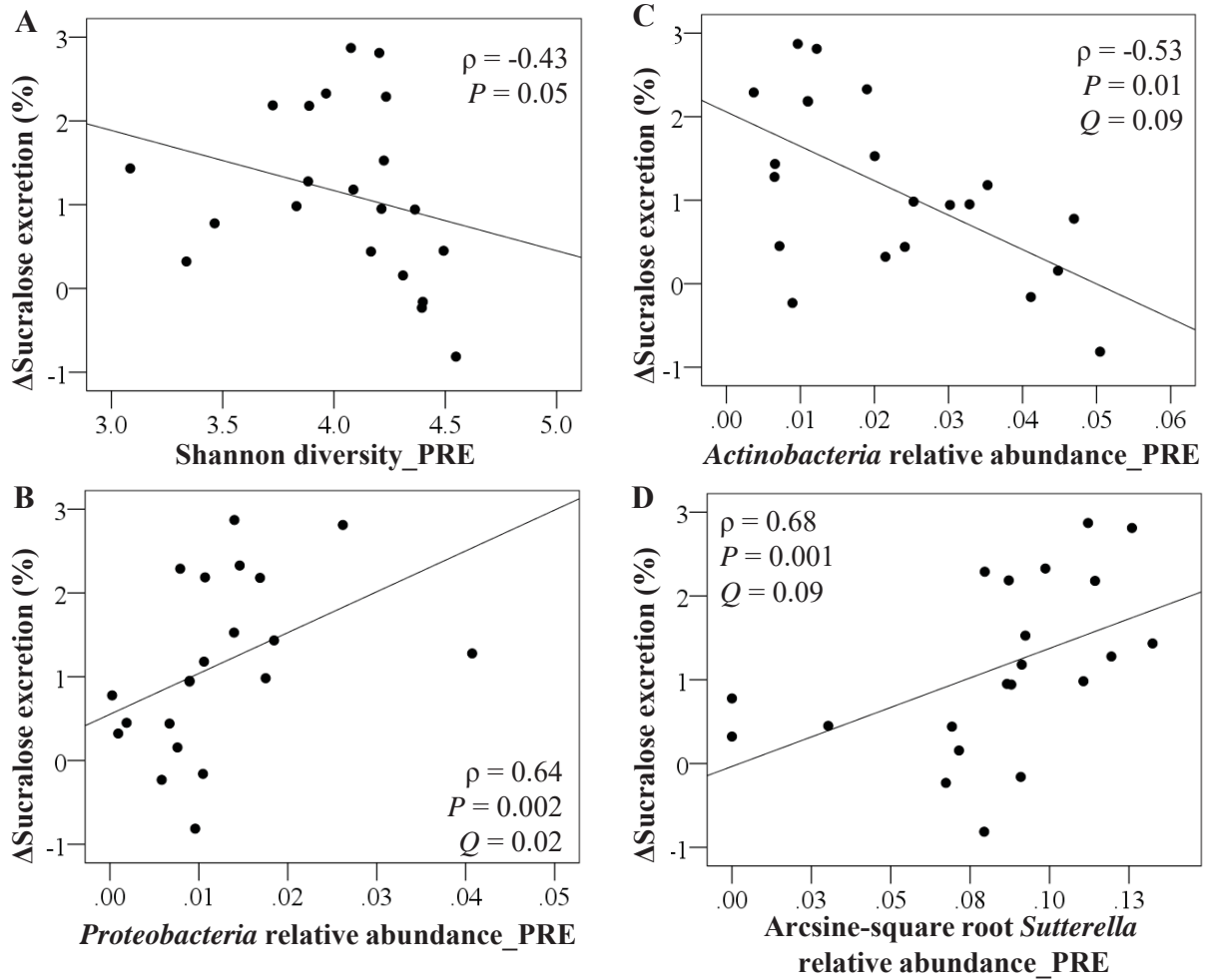


Figure 6

