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

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ABSTRACT

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

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

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

The intestinal microbiota and its metabolites are integral mediators of intestinal barrier function and IP, capable of both perturbing and enhancing intestinal barrier integrity by modulating immune responses, oxidative stress, inflammation, vagal signaling, and nutrient availability (40). Intestinal microbiota composition and activity are malleable, influenced by the availability of undigested dietary components (13, 40) and the intestinal environment (e.g., pH, motility, inflammation, immune activity) (48). Dietary ratios of fiber, carbohydrate, protein, and fat are also important as low fiber, high protein, and high fat diets reportedly increase intestinal inflammation and IP by altering ratios of microbes and metabolites that modulate inflammation (13, 14, 41). Severe physical stress (12, 15, 54), psychological stress (34), sleep deprivation and circadian disruption (17, 50), and environmental stressors (9, 24) have also been independently associated with altered intestinal microbiota composition and increased IP. However, current understanding of the intestinal microbiota’s role in mediating effects of physical, psychological,
and environmental stressors on the intestinal barrier is largely limited to information derived from animal models which may not fully represent the human condition (12, 34).

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

The present study used a physically demanding military training exercise as a model for elucidating the effects of physiologic and metabolic stress on IP and intestinal microbiota composition and activity, and to identify associations between dietary intake, IP, inflammation and the intestinal microbiota. The data were collected during a trial designed to determine to what extent dietary carbohydrate and protein supplementation spare whole-body protein and attenuate decrements in physiologic status during military training (32, 43). We hypothesized
that the multiple-stressor environment, which was expected to induce negative energy balance and body weight loss, would adversely affect intestinal microbiota composition (e.g., decrease diversity, increase abundance of pro-inflammatory taxa, and decrease abundance of putatively beneficial taxa), and increase IP. We further hypothesized, that supplemental protein would exacerbate these decrements by promoting the generation of potentially harmful bacterially-derived metabolites, whereas carbohydrate supplementation would attenuate these decrements by reducing the magnitude of negative energy balance.

METHODS

Participants and experimental design

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

Study staff block randomized volunteers by body weight to a control (CNTRL, n=18), protein-supplement (PRO, n=28), or carbohydrate supplement (CHO, n=27) group in a 1:3 (control:intervention) ratio. All volunteers were provided three Norwegian arctic rations/d to
consume during the 4-d training exercise. The PRO group was also provided four whey protein-based snack bars/d, while the CHO group was provided four carbohydrate-based snack bars/d. Bars were similar in appearance, taste and texture enabling investigators, study staff and volunteers to remain blind to the macronutrient composition. The training consisted of a 51 km cross-country ski-march during which volunteers skied in 50:10 min work-to-rest ratios while carrying a ~45 kg pack (STRESS). Stool samples were collected over the 2 d prior to STRESS, and the night of or day after completing STRESS in a self-selected subset of volunteers. 24-hr urine collections were completed the day prior to STRESS and on the 3rd day of STRESS. Blood samples were collected the morning before and the morning after STRESS. Primary study objectives were to determine the effects of macronutrient supplementation on whole body protein balance, body mass, and physiological status during military training, and are reported elsewhere (32, 43). This report details secondary study objectives of determining the impact of a multiple-stressor military training environment on IP, and intestinal microbiota composition and activity.

Volunteers began consuming provided rations 2 d prior to training and the intervention snack bars on day one of STRESS. Three Norwegian field rations provide 14.6 MJ, 141 g protein, 435 g carbohydrate, and 126 g fat. The four protein-based snack bars provided an additional 4.4 MJ kcal, 85 g whey protein, 102 g carbohydrate, 35 g fat, and <1 g fiber, while the four carbohydrate-based snack bars provided an additional 4.4 MJ, 11 g whey protein, 189 g carbohydrate, 29 g fat, and 1 g fiber. All snack bars were manufactured by a third party that did not participate in data collection (Combat Feeding Directorate, Natick Soldier Systems, Center, Natick, MA). Investigators, study staff, and volunteers were blind to the macronutrient composition of the bars. Volunteers were asked to consume the rations and bars as they normally would during training, and to consume only foods and caloric beverages provided to
them by the study team. All volunteers were provided with ration-specific food logs which were collected and reviewed daily by study staff, and used to calculate actual intakes (Table 1).

Intestinal permeability assay

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

Blood biochemistries

Blood was collected following an overnight fast by antecubital venipuncture, separated into serum or plasma, and immediately frozen. Samples were then shipped on dry ice to the U.S. Army Research Institute of Environmental Medicine where they were stored at -80°C until being
shipped to Pennington Biomedical Research Center (Baton Rouge, LA) or Metabolon, Inc. (Durham, NC) for analysis. Plasma LPS was measured by ELISA (Cusabio, College Park, MD), serum IL-6 by the Milliplex MAP (Millipore, Billerica, MA), serum high-sensitivity C-reactive protein (CRP) by a chemiluminescent immunometric assay (Siemens Immulite 2000; Siemens Inc., Malvern, PA), and serum creatine kinase (a marker of muscle damage) by an automated chemistry analyzer (Beckman Coulter DXC 600 Pro, Beckman Coulter, Brea, CA).

**Stool microbiota composition**

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

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

Samples were selected for DNA extraction in random order, and DNA was extracted using the PowerFecal DNA Isolation kit (MO BIO Laboratories, Inc., Qiagen, Carlsbad, CA). Primers designed to amplify the V3-V4 region of the 16S rRNA gene were employed for PCR amplification (22) according to the Illumina 16S Metagenomic Sequencing Library Preparation manual (Part # 15044223 Rev B; Illumina, Inc., San Diego, CA). A limited cycle PCR generated a single amplicon of ~460 bp to which Illumina sequencing adapters and dual-index barcodes were added.
were added. Paired 300 bp reads and MiSeq v.3 reagents were used to generate full-length reads of the V3 and V4 region in a single run on the Illumina MiSeq platform.

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

**Stool and plasma metabolomics**

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

Several recovery standards were added prior to the first step in the extraction process, and were analyzed with the experimental samples for quality control. All analysis methods utilized a Waters ACQUITY UPLC (Waters Corp., Milford, MA) and a Thermo Scientific Q-Exactive high resolution/accurate mass spectrometer interfaced with a heated ESI-II source and Orbitrap mass analyzer operated at 35,000 mass resolution. Sample extracts were dried and reconstituted in solvents compatible to each of the four methods. Each reconstitution solvent also contained a series of standards at fixed concentrations to ensure injection and chromatographic consistency.
One aliquot was analyzed using acidic positive ion conditions, chromatographically optimized for more hydrophilic compounds. In this method, the extract was gradient eluted from a C18 column (Waters UPLC BEH C18-2.1x100 mm, 1.7 µm) using water and methanol containing 0.05% perfluoropentanoic acid and 0.1% formic acid. Another aliquot was also analyzed using acidic positive ion conditions; however, it was chromatographically optimized for more hydrophobic compounds. In this method, the extract was gradient eluted from the same aforementioned C18 column using methanol, acetonitrile, water, 0.05% perfluoropentanoic acid, and 0.01% formic acid and was operated at an overall higher organic content. Another aliquot was analyzed using basic negative ion optimized conditions using a separate dedicated C18 column. The basic extracts were gradient eluted from the column using methanol and water, however with 6.5mM Ammonium Bicarbonate at pH 8. The fourth aliquot was analyzed via negative ionization following elution from a HILIC column (Waters UPLC BEH Amide 2.1x150 mm, 1.7 µm) using a gradient consisting of water and acetonitrile with 10mM Ammonium Formate, pH 10.8. The MS analysis alternated between MS and data-dependent MS^n scans using dynamic exclusion. The scan range varied slightly between methods but covered 70-1000 m/z.

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

**Bioinformatics**

Analyses were completed using R v.3.3.1, Multiexperiment Viewer v.4.9.0, SPSS v.21, and XLSTAT v.2015. An average of 140,762 ± 103,480 16S rDNA sequences per stool sample were obtained which clustered into 2,015 OTUs at 97% sequence identity. OTUs could be assigned to 12 phyla and 83 genera. Alpha-diversity (Shannon and Chao1 indices, and observed OTUs) was calculated using the *phyloseq* R bioconductor package, and β-diversity calculated using Bray-Curtis distances. Prior to statistical analysis of sequencing data, phylum, genus, and OTU-level relative abundances were calculated by dividing the number of reads for each taxa by the total number of reads in the sample. Ordination and cluster analyses were conducted on OTU-level relative abundances, whereas differential analyses were conducted on phylum and genus-level relative abundances. For differential analyses, any OTUs that could not be assigned to the genus level were grouped at the next lowest level of classification possible (e.g., family or order). Relative abundances were arcsine square-root transformed prior to differential analysis to stabilize variance and better approximate normality. Prior to analysis of stool and plasma metabolites, any missing values were imputed using the minimum observed value for each compound, normalized to set the median equal to 1, and log10-transformed.

Ordinations were conducted by principal coordinates analysis (PCoA) of the OTU Bray-Curtis dissimilarity matrix, principal components analysis (PCA) of metabolite data, and hierarchical complete-linkage clustering of Euclidean distances (OTU and metabolite data). Supervised classification of pre- and post-STRESS samples was conducted using Random Forest
analysis, and the mean decrease accuracy used to identify taxa driving classification. To examine associations between stool microbiota composition and global metabolite profiles, metabolite PCA ordinations were compared to OTU PCoA ordinations using Procrustes analysis implemented in the R package vegan.

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

**Statistical analysis**

Sample size calculations were based on primary study outcomes which have been previously reported (32, 43). Statistical analyses were completed using SPSS v.21 and R v.3.3.1. Data were assessed for normality prior to analysis and transformed if necessary to meet model
assumptions. When transformation was not successful, non-parametric tests were used.

Repeated measures ANOVA was used to test effects of STRESS and diet, and their interaction on study outcomes. Pairwise comparisons of pre- and post-STRESS genus relative abundances were conducted using the Wilcoxon-signed rank test, and between group comparisons of changes in genus relative abundances were conducted using the Kruskal-Wallis test. Spearman’s rank correlation ($\rho$), Pearson’s correlation ($r$), multiple linear regression, and linear mixed models were used to examine associations among variables. Relationships between surcalose excretion, LPS, IL-6, and CRP concentrations with ordinations of stool microbiota composition, and stool/plasma metabolites were also assessed using linear mixed models. All mixed models included subject as a random factor and time as a continuous covariate. Sucralose excretion, LPS, IL-6 or CRP were entered as dependent variables, and scores for the first three principal components of the ordinations were included as independent variables. Finally, backwards stepwise regression was used to identify the strongest predictors of changes in IP. Independent variables included in the regression model were those that were significantly correlated with changes in sucralose excretion, and included dietary parameters (protein intake), changes scores of inflammation markers (IL-6 and CRP), pre-STRESS stool microbiota characteristics (Shannon diversity, Actinobacteria and Proteobacteria relative abundances), and change scores for stool metabolites linked to changes in microbiota composition changes (cysteine and arginine).

Changes in Shannon diversity and pre-STRESS Sutterella relative abundance were also considered in place of pre-STRESS Shannon diversity and Proteobacteria relative abundance, respectively.

The false discovery rate for all tests including taxa or metabolite data was controlled by adjusting $P$-values using the Benjamini-Hochberg procedure. Adjusted $P$-values are presented as...
Q-values. Data are presented as mean ± SD unless otherwise noted. Statistical significance was set at $P \leq 0.05$ or $Q \leq 0.10$.

RESULTS

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

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

Intestinal permeability, plasma LPS, and inflammation

Sucralose excretion increased 62 ± 57% during STRESS independent of diet (main effect of time, $P < 0.001$; Figure 1A), suggesting increased IP, and was correlated with changes in creatine kinase ($r = 0.34$, $P = 0.02$), CRP ($\rho = 0.36$, $P = 0.01$), IL-6 (Figure 1B), and protein
intake ($\rho = -0.31$, $P = 0.03$). Mannitol excretion also increased during STRESS independent of diet (Pre: 28 ± 8% vs Post: 33 ± 13%; main effect of group, $P = 0.01$). Plasma LPS concentrations did not differ from pre- to post-STRESS ($P = 0.79$; Figure 1C). However, Soldiers with increased LPS concentrations demonstrated a trend to have greater increases in IL-6 relative to individuals with no change or a decrease in LPS concentrations (Figure 1D).

**Stool microbiota composition**

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

**Stool and plasma metabolites**

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

A total of 737 compounds were identified in plasma, of which 478 demonstrated statistically significant changes during STRESS ($Q < 0.10$). Changes primarily reflected increases in host energy metabolism, lipolysis, fatty acid oxidation, branched-chain amino acid catabolism, and steroid metabolism (data not shown). However, changes in plasma concentrations of several metabolites known to be partially or fully derived from microbial metabolism were also observed. Specifically, mean concentrations of phenylalanine and tyrosine metabolites including p-cresol sulfate (+48%), p-cresol-glucuronide (+79%), phenylacetate (+44%), phenyllactate (+42%), phenylacetylglutamine (+24%), and 3-(4-hydroxyphenyl)lactate (+40%) were increased (Figure 4). In contrast, mean concentrations of the benzoate metabolites 2-hydroxyhippurate (-22%), 3-hydroxyhippurate (-61%), and 4-hydroxyhippurate (-35%) were decreased ($Q < 0.10$). Mean concentrations of secondary bile acids in plasma demonstrated more variable responses as glycocolithocholate sulfate (+21%), glycohyocholate (+6%), taurolithocholate 3-sulfate (+89%), and taurocholenate sulfate (+56%) concentrations increased,
while deoxycholate (-66%), ursodeoxycholate (-63%), and isoursodeoxycholate (-51%) concentrations decreased ($Q < 0.10$).  

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

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

Procrustes analysis demonstrated a significant association between the ordinations of stool metabolites and stool microbiota composition ($M^2 = 0.76$, Monte Carlo $P = 0.001$; Figure 6A) indicating an association between stool metabolites and the stool microbiota. Additionally, prediction models linked changes in stool microbiota composition to 69 of the metabolites found to be altered in stool (Supplemental Table 3). These models were supported by Procrustes analysis on ordinations of the significantly altered taxa and these metabolites ($M^2 = 0.72$, Monte Carlo $P = 0.001$). Of the 69 metabolites, amino acid and nucleotide metabolites comprised the majority, and were generally lower post- relative to pre-STRESS ($Q < 0.10$). Changes in two, arginine and cysteine, were correlated with changes in sucralose excretion during STRESS.
Changes in the concentrations of another 14 metabolites were also inversely correlated with changes in sucralose excretion (Table 2). In accord, scores on the 1st principal component from the ordination of stool metabolite data were associated with sucralose excretion ($\beta \pm SE = -0.05 \pm 0.01$, $P = 0.01$) indicating that the effect of STRESS on stool microbiota was associated with IP.

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

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

Discussion

The magnitude, temporal dynamics, and physiologic effects of intestinal microbiome responses to stress are poorly characterized. Our findings demonstrate that a multiple-stressor environment characterized by high physical exertion, suboptimal energy intake, muscle damage,
and inflammation adversely effects intestinal barrier integrity concomitant to alterations in
intestinal microbiota composition and metabolism. Observed associations between increased IP,
the pre-stress microbiota, and stool metabolites associated with the microbiota suggest that
targeting the intestinal microbiota could provide novel strategies for maintaining intestinal
barrier integrity during physiologic stress.

The observed increase in IP in association with increased inflammation (Figure 1) is
consistent with the only other study to our knowledge that has assessed IP in military personnel
during training (29). In these environments, intense or prolonged exercise may reduce
splanchnic perfusion which can trigger intestinal hypoxia, inflammation, and oxidative stress that
collectively degrade intestinal barrier integrity and increase IP (15, 24, 54). Stress-induced
muscle damage may also contribute to inflammation, potentiating increases in IP by inducing
tight junction dysfunction (15). Ultimately, the increase in IP is thought to result in mild
endotoxemia and inflammation, and contribute to gastrointestinal distress in endurance athletes
(4, 15, 20, 24) and possibly military personnel (29). Although gastrointestinal symptoms were
not assessed in this study, Li et al. (29) reported that 70% of Soldiers participating in a 6-wk
combat training course reported gastrointestinal distress symptomology (i.e., abdominal pain,
diarrhea, constipation), those symptoms were more frequent in Soldiers with the largest increases
in IP, and symptoms were associated with psychological decrements. Gastrointestinal distress,
to include infectious diarrhea, is historically the leading non-battle injury encountered in
deployed military personnel, representing a significant burden to military health care and
operational readiness (45-47). Identifying mediators of intestinal barrier responses to severe
stress, and developing strategies to target those mediators may therefore have substantial benefit
for military personnel.
Our findings suggest that the intestinal microbiota may be one mediator of IP responses to severe physiologic stress, and that targeting the microbiota before stress exposure may be one strategy for maintaining IP. In particular, increasing microbiota diversity and *Actinobacteria* relative abundance, and decreasing *Proteobacteria* and *Sutterella* relative abundances before stress exposure may be effective in lieu of the observed associations with changes in IP during stress (Figure 5). Greater microbiota diversity is generally considered indicative of a healthy intestinal ecosystem, having been frequently associated with lower chronic disease risk (11, 19). Similarly, species within the *Actinobacteria* phyla including those belonging to the *Bifidobacterium* and *Collinsella* genera have favorable anti-inflammatory and immuno-modulatory effects which may protect the intestinal barrier during stress (2, 42). In support, *Bifidobacterium* strains are included in multi-strain probiotics that have demonstrated some efficacy, albeit weak, for favorably impacting IP in athletes (25, 49). Increasing *Bifidobacterium* relative abundance using prebiotics such as oligofructose has also been shown to promote intestinal barrier integrity in animal models (6). In contrast, *Proteobacteria* are endotoxin producers which have been linked to inflammatory bowel diseases and subclinical inflammation (19, 27). *Sutterella*, a genus within the *Proteobacteria* phyla, have been shown to promote inflammatory bowel disease by inhibiting immunoglobulin-A secretion (39). As such, although findings are correlative and the study design precludes determining causality, the observed associations between the pre-stressed microbiota and changes in IP during stress are plausible and provide potential targets for further study.

To our knowledge, this study is the first to examine intestinal microbiota responses during military training, and expands knowledge regarding the temporal effects of exercise and psychological stress on the microbiome, which is largely limited to animal studies at present (12,
Human studies have demonstrated that drastic changes in diet impact intestinal microbiota composition (13, 41) by altering the availability of metabolic substrates for intestinal microbes (23). Our findings contrast with those reports in demonstrating alterations in microbiota composition that most likely were not solely attributable to diet, and which were more pronounced than is commonly reported in human diet studies (Figure 2). Although potential mechanisms were not directly assessed, changes in immune activity, intestinal inflammation and oxidative stress, and altered hypothalamic-pituitary-adrenal axis and vagal signaling have all been postulated as mechanisms through which physical and psychological stress modulate the microbiome (12, 34).

The increase in Shannon α-diversity and the numerous genus-level changes in relative abundance demonstrated that changes in microbiota composition were broadly characterized by an increase in abundance of less dominant taxa at the expense of more dominant taxa such as Bacteroides (Figure 2). This included increased relative abundances of several potentially deleterious and infectious taxa (e.g., Peptostreptococcus, Staphylococcus, Peptoniphilus, Acidaminococcus, Fusobacterium), and decreased relative abundances of several taxa thought to deter pathogen invasion, reduce inflammation and promote immunity (e.g., Bacteroides, Faecalibacterium, Collinsella, Roseburia). As such, an increase in the ratio of less-abundant, potentially harmful taxa to beneficial taxa may explain the unexpected observation that greater increases in diversity during training were correlated with larger increases in IP. However, several alternative explanations exist. Individuals with the lowest pre-STRESS Shannon diversity also demonstrated the largest increases in diversity during STRESS (r = -0.60, P = 0.001). Therefore, the association between increased diversity and increased IP may attributable to lower pre-STRESS diversity. Alternately, higher stool microbiota diversity has been
correlated with longer intestinal transit time and higher urinary concentrations of potentially
harmful degradation products of bacterial protein metabolism (48). In this study, stool and
plasma concentrations of protein degradation products did not uniformly change, although they
were more commonly decreased in stool and increased in plasma (Figure 4, Supplemental Tables
2 and 3). Whether these observations reflect changes in transit time could not be determined
from the collected data. Nonetheless, no protein degradation metabolite was independently
associated with increased IP or inflammation. This observation contrasts with reports that
bacterial protein metabolites induce intestinal barrier damage and inflammation in vitro (56), and
suggests that the positive association between protein intake and increases in IP during training
was not mediated by bacterial metabolism of diet-derived amino acids.

Decreased concentrations of several stool metabolites were associated with increased IP
(Table 2). Metabolites included two amino acids, arginine and cysteine, which were predicted to
be associated with changes in microbiota composition, and which are plausible modulators of IP
based on known physiologic functions. Specifically, arginine is a precursor to polyamines
required for intestinal mucosal growth and repair, and for nitric oxide, a potent vasodilator that
may protect intestinal barrier integrity by improving splanchnic perfusion, deterring pathogen
invasion, and modulating inflammation (28, 54). In support, arginine supplementation has
preserved intestinal barrier integrity in various animal stress and intestinal injury models (2),
although the effects in humans are less clear (5). Cysteine is an essential component of
 glutathione, an antioxidant tripeptide critical to maintaining a favorable redox balance in the
intestine (10). Of note, Phua et al. (44) recently reported that increases in urinary concentrations
of a glutathione metabolite, possibly reflecting increased oxidative stress, were associated with
gastrointestinal symptomology during military training. Our findings also suggest that
interactions between the intestinal microbiota and dietary fat metabolism may impact IP (Table 2). 7-ketodeoxycholate and 12-dehydrocholate are secondary bile acids derived from bacterial metabolism of bile acids secreted in response to dietary fat intake. Secondary bile acids are recognized as important signaling molecules with functions that are thought to include promotion of gut barrier integrity (52). Collectively, these findings suggest that changes in intestinal microbiota composition and metabolism may impact IP during physiologic stress by modulating the availability of amino acid precursors critical to moderating inflammation and oxidative stress, and of secondary bile acids.

Study strengths include the provision of diets of known composition providing a range of macronutrient intakes, and the integration of physiologic, stool microbiota composition, and metabolomics data. However, results should be interpreted in the context of the study design and several limitations. The physically demanding environment coupled with the physiologic demands imposed by undereating may have masked some associations, and limited generalizability of the findings, but provides unique and novel insights into the temporal dynamics of host-microbiome interactions during prolonged physical stress. While psychological and sleep deprivation stress were likely also present, we did not quantify those responses. Study participants were predominantly young males, and findings may not be generalizable to older populations or females. Limitations include the correlative nature of associations between outcomes from which causality cannot be determined despite evidence of plausibility, and limited statistical power for some analyses, especially those including between-group comparisons, resulting from only a subset of the full cohort participating in stool collections. The method for measuring plasma LPS concentrations is also a limitation as it did not quantify endotoxin activity which is known to vary between LPS forms (18). Nonetheless,
the weak association between changes in plasma LPS and IL-6 are consistent with the well-established pro-inflammatory effects of the compound (18). Including metagenomic or transcriptomic analysis of stool samples would have strengthened findings and complemented the metabolomics analysis by allowing more accurate functional predictions of microbiota function. Reliance on stool for measurements of microbiota composition and metabolites is also a limitation as the composition of the stool may be more reflective of the distal colon than the entirety of the gastrointestinal tract. However, the addition of plasma metabolite measurements was included to better capture bacterial metabolism along the full gastrointestinal tract. Finally, logistical constraints prevented more frequent measurements which would have provided additional insight into temporal dynamics.

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

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Disclaimers

The opinions or assertions contained herein are the private views of the author(s) and are not to be construed as official or as reflecting the views of the Army or the Department of Defense. Citation of commercial organizations or trade names in this report does not constitute an official Department of the Army endorsement or approval of the products or services of these organizations. Opinions, interpretations, conclusions, and recommendations are those of the authors and are not necessarily endorsed by the US Army.

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Disclosures

No authors report a conflict of interest.


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

A) Intestinal permeability measured by 24 hr urine collection following ingestion of 2 g sucralse (n = 49), and C) plasma LPS concentrations (n = 67) before (PRE) and after (POST) military training. Boxes are median and interquartile range, whiskers are 1.5 times the interquartile range, or minimum and maximum if no observations within that range, and solid circles are data points >1.5 times the interquartile range. *Repeated measures ANOVA, main effect of time, \( P < 0.001 \).  

B) Changes in intestinal permeability are correlated with changes in serum interleukin (IL)-6 concentrations; Pearson’s correlation (n = 46).  

D) Soldiers experiencing increases in plasma LPS during training demonstrate a trend for larger increases in serum IL-6 relative to those experiencing a decrease or no change in plasma LPS; repeated measures ANOVA, time-by-ΔLPS interaction, \( P = 0.07 \), #significantly different from PRE, \( P < 0.05 \).  

CHO, carbohydrate-supplement group; CNTRL, control group (rations only); PRO, protein-supplement group.

Figure 2. Military training elicits changes in intestinal microbiota composition. A) Alpha-diversity before (PRE) and after (POST) military training. Boxes are median and interquartile range, whiskers are 1.5 times the interquartile range, or minimum and maximum if no observations within that range, and solid circles are data points >1.5 times the interquartile range. *Repeated measures ANOVA, main effect of time, \( P = 0.04 \).  

B) Principal coordinates analysis of Bray-Curtis dissimilarity matrix indicates that stool microbiota community composition was more strongly influenced by the training environment than by individual variability or diet group.  

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

Figure 3. Stool metabolomics before (PRE) and after (POST) military training. A) Principal components, B) hierarchical complete-linkage clustering of Euclidean distances; and C) random forest analyses of stool metabolites; n = 25. A) Individual data points represent the metabolite composition within a single individual. Points closer together are more similar. B) Columns are individuals and rows are metabolites shaded by abundance within sample. Branches (lines) within the same node (points where branches split) reflect similarity in metabolite composition. Stool metabolites did not demonstrate any distinct clustering pattern. C) Top 30 metabolites with the strongest influence on prediction accuracy of the random forest analysis are presented in order of importance (top to bottom). Random forest analysis used individual metabolite profiles to predict whether the samples were from PRE or POST training. Mean decrease in prediction accuracy is the mean decrease in the percentage of observations classified correctly when that
metabolite is assigned a random value. Arrows indicate direction of metabolite change from PRE to POST. CHO, carbohydrate-supplement group; CNTRL, control group (rations only); PRO, protein-supplement group.

Figure 4. Qualitative changes in phenylalanine and tyrosine (A) and tryptophan (B) metabolites in stool and plasma during military training. Arrows indicate direction of change in stool (brown) and plasma (red) from pre- to post-training (repeated measures ANOVA, main effect of time, $Q < 0.10$). Metabolites circled by dashed line are compounds known to be wholly or partially derived from microbial metabolism. Compounds without arrows were either unchanged ($Q > 0.10$) or not detected.

Figure 5. Factors associated with increased intestinal permeability during military training. Intestinal permeability measured by 24 hr urine collection following ingestion of 2 g sucralose. Spearman’s correlation ($\rho$) ($n = 21$). $P$-values for correlations with taxa adjusted using Benjamini-Hochberg correction ($Q$).

Figure 6. Stool microbiota composition is associated with stool metabolite, and plasma metabolite concentrations. Procrustes analysis of stool microbiota data ordinated using principal coordinates analysis of Bray-Curtis distances, and stool (A) and plasma (B) metabolite profiles ordinated using principal components analysis. The first three components of each ordination were extracted and analyzed using Procrustes rotation which attempts to rotate ordinations to maximal similarity. Open circles represent the stool microbiota community of a single individual before or after military training. Arrowheads represent the stool or plasma
metabolite profile of a single individual before or after military training. Vectors connect microbiota composition with metabolite profiles of the same individual for each time point. Longer vectors indicate greater intra-individual dissimilarity. The fit of each Procrustes rotation over the first three dimensions is reported as the $M^2$ value. P-values were calculated after 1000 permutations. Results indicate similar clustering patterns between stool microbiota composition and stool metabolites, and between stool microbiota composition and plasma metabolites. C) Venn diagram of stool and plasma metabolites that were significantly altered during military training ($Q \leq 0.10$). Diagram indicates that prediction models linked changes in stool microbiota composition to 69 of the metabolites found to be altered in stool, 30 of which were also significantly altered in plasma.
Table 1 Volunteer characteristics, energy expenditure, and dietary intake.

<table>
<thead>
<tr>
<th></th>
<th>CNTRL (n = 18)</th>
<th>CHO (n = 27)</th>
<th>PRO (n = 28)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>19 ± 2</td>
<td>20 ± 1</td>
<td>20 ± 1</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>23.6 ± 1.8</td>
<td>24.1 ± 2.3</td>
<td>23.3 ± 2.1</td>
</tr>
<tr>
<td>Energy expenditure (MJ/d)</td>
<td>25.5 ± 1.7</td>
<td>25.8 ± 2.1</td>
<td>25.8 ± 2.5</td>
</tr>
<tr>
<td>Energy intake (MJ/d)</td>
<td>10.5 ± 1.7</td>
<td>13.1 ± 2.6*</td>
<td>11.8 ± 2.5</td>
</tr>
<tr>
<td></td>
<td>(6.5 – 13.0)</td>
<td>(7.7 – 16.4)</td>
<td>(7.1 – 16.8)</td>
</tr>
<tr>
<td>Carbohydrate (g/d)</td>
<td>312 ± 47</td>
<td>434 ± 86*</td>
<td>321 ± 77†</td>
</tr>
<tr>
<td>Protein (g/d)</td>
<td>100 ± 15</td>
<td>98 ± 22</td>
<td>148 ± 25*†</td>
</tr>
<tr>
<td></td>
<td>(65 – 124)</td>
<td>(58 – 130)</td>
<td>(96 – 191)</td>
</tr>
<tr>
<td>Fat (g/d)</td>
<td>91 ± 20</td>
<td>107 ± 24</td>
<td>102 ± 23</td>
</tr>
<tr>
<td></td>
<td>(57 – 117)</td>
<td>(56 – 146)</td>
<td>(59 – 141)</td>
</tr>
<tr>
<td>Fiber (g/d)</td>
<td>25 ± 4</td>
<td>25 ± 6</td>
<td>22 ± 5†</td>
</tr>
<tr>
<td></td>
<td>(16 – 32)</td>
<td>(13 – 33)</td>
<td>(12 – 33)</td>
</tr>
</tbody>
</table>

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

*,†Means were compared by one-way ANOVA; *different from CNTRL (P < 0.05), †different from CHO (P < 0.05).
Table 2. Stool metabolites associated with changes in intestinal permeability during military training.

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

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

*Significantly increased from pre- to post-training. All other listed metabolites decreased (Q < 0.10; see also Supplemental Table 2).
Table 3. Model predicting changes in intestinal permeability during military training (STRESS).

<table>
<thead>
<tr>
<th></th>
<th>( \beta \pm SE )</th>
<th>Standardized ( \beta )</th>
<th>( P )-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actinobacteria relative abundance (pre-STRESS)</td>
<td>-45.0 ± 8.5</td>
<td>-0.59</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>( \Delta \log_{10} \text{IL-6 (pg/mL)} )</td>
<td>0.4 ± 0.6</td>
<td>0.43</td>
<td>0.003</td>
</tr>
<tr>
<td>( \Delta \log_{10} \text{Stool cysteine} )</td>
<td>-2.4 ± 0.6</td>
<td>-0.43</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Intercept</td>
<td>1.4 ± 0.3</td>
<td></td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

Adjusted \( R^2 = 0.84 \)

Dependent variable is change in sucralose excretion (post – pre) measured from 24 hr urine collection following ingestion of 2 g sucralose and expressed as percent of ingested dose (n = 15).
Figure 1

A. Sucralose excretion (%)

B. ΔLog_{10}(Sucralose excretion) vs ΔLog_{10}(IL-6 (pg/mL))

C. LPS (pg/mL)

D. IL-6 (pg/mL)

ΔLPS

ΔLog_{10}(Sucralose excretion)

ΔLog_{10}(IL-6 (pg/mL))

r = 0.34
P = 0.02

No change/ decrease (n = 35)
Increase (n = 29)
Figure 2

A

Shannon

B

PRE-CHO

PRE-CNTRL

PRE-PRO

POST-CHO

POST-CNTRL

POST-PRO

C

PC1 (41.0%)

PC2 (4.5%)

D

CNTRL

PRO

CHO

CNTRL

PRO

CHO

Relative abundance

Unclassified

Verrucomicrobia

TM7

Tenericutes

Spirochaetes

Proteobacteria

Lentisphaerae

Fusobacteria

Firmicutes

Euryarchaeota

Cyanobacteria

Bacteroidetes

Actinobacteria
PRE-CNTRL  |  POST-CNTRL  |  PRE-PRO  |  POST-PRO  |  PRE-CHO  |  POST-CHO

Overall prediction accuracy = 84%

Mean decrease in prediction accuracy

Study groups:
- CNTRL-PRE  
- CHO-PRE  
- PRO-PRE  
- CNTRL-POST  
- CHO-POST  
- PRO-POST

Super pathway:
- Amino Acid  
- Carbohydrate  
- Cofactors and Vitamins  
- Energy  
- Lipid  
- Nucleotide  
- Peptide  
- Xenobiotics
Figure 4

A

\begin{align*}
\text{phenylalanine} & \rightarrow \text{tyrosine} \\
\text{phenylpyruvate (PPA)} & \rightarrow \text{N-acetylphenylalanine} \\
\text{tyramine} & \rightarrow \text{phenol} \\
\text{4-hydroxyphenylacetate} & \rightarrow \text{cresol} \\
\text{L-phenyllactate (PLA)} & \rightarrow \text{phenylacetate (PAA)} \\
\text{D-phenyllactate (PLA)} & \rightarrow \text{phenylacetyleglutamine} \\
\text{hydroxyphenylpyruvate} & \rightarrow \text{hydroxyphenylacetate (HPLA)}
\end{align*}

B

\begin{align*}
\text{tryptophan} & \rightarrow 5\text{-hydroxy-L-trytophan} \\
\text{indole} & \rightarrow \text{indolepyruvate} \\
\text{tryptamine} & \rightarrow \text{skatol} \\
\text{skatoie sulfate} & \rightarrow \text{serotonin} \\
\text{5-hydroxyindoleacetaldehyde} & \rightarrow \text{N-acetylserotonin} \\
\text{5-hydroxyindoleacetate} & \rightarrow \text{melatonin}
\end{align*}
Figure 5

A. ΔSucralose excretion (%) vs. Shannon diversity

B. ΔSucralose excretion (%) vs. Proteobacteria relative abundance

C. ΔSucralose excretion (%) vs. Actinobacteria relative abundance

D. ΔSucralose excretion (%) vs. Arcsine-square root Sutterella relative abundance

\[ \rho = -0.43, P = 0.05 \]

\[ \rho = 0.64, P = 0.002, Q = 0.02 \]

\[ \rho = -0.53, P = 0.01, Q = 0.09 \]

\[ \rho = 0.68, P = 0.001, Q = 0.09 \]

\[ \rho = -0.43, P = 0.05 \]
Figure 6

A 微生物 metabolites

M² = 0.76
Monte Carlo P = 0.001

B 微生物 metabolites

M² = 0.49
Monte Carlo P = 0.001

C 便 Stool 血浆 Plasma

205 39 30 448

Predicted to be derived from intestinal microbiota.