Ultra -Endurance Triathlon Competition Shifts Fecal Metabolome Independent of Changes to Microbiome Composition

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ABSTRACT

Understanding changes to gut microbiota composition and metabolic output in response to acute exercise may be necessary for understanding variability in performance and long-term health benefits of exercise. We characterized acute changes in the fecal microbiome and metabolome following participation in an ultra-endurance (3.9km swim, 180.2km bike, 42.2km run) triathlon. Associations between athlete-specific factors (race performance [i.e., time to completion] and lifetime years of endurance training) with gut microbiota and metabolite profiles were also explored. Stool samples from 12 triathletes (9M/3F; 43±14 yrs, 23±2 kg/m^2) were collected ≤48 hours before and the first bowel movement following race completion. Intra- and inter-individual diversity of bacterial species were unaltered following race completion ($P>0.05$). However, significant reductions ($P<0.05$) in free and secondary bile acids (DCA, 12-ketoLCA) and short chain fatty acids (butyric and pivalic acids), paired with significant increases ($P<0.05$) in long chain fatty acids (oleic and palmitoleic acids) were observed. Our analysis revealed associations between bacterial taxa and fecal metabolites with athlete-specific factors, including correlations ($P<0.05$) between race performance with *Akkermanisa Muciniphila* (positive) and *Methanobrevibacter smithii* (negative), as well as lifetime history of endurance training with *Bacteroides spp.* (positive) and *Roseburia*_cp_CAG_471 (negative). Several fecal bile acids and fatty acids significantly differed ($P<0.05$) with race completion time and lifetime history of endurance training. These findings suggest that 1) acute ultra-endurance exercise shifts microbial metabolism independent of changes to community composition and 2) exercise-related adaptions in gut microbial ecology are linked to performance level and training history.

Keywords: microbiome, Ironman, endurance exercise, physical activity, triathlete

NEW & NOTEWORTHY: This is the first study to characterize acute changes in gut microbial ecology and metabolism following an ultra-endurance triathlon. We demonstrate changes in gut microbial community function, but not structure, as well as several associations between gut microbiome and fecal metabolome characteristics with race completion time and lifetime history of endurance training. These data add to a small but growing body of literature seeking to characterize the acute and chronic effects of exercise on the gut microbial ecosystem.

INTRODUCTION

Physical activity provides both immediate and long-term whole-body health benefits. However, the cellular and molecular mechanisms that mediate these health benefits have not been fully elucidated. In addition to the multiplicity of bodily systems that are affected by physical activity, it has become apparent that microorganisms in the gastrointestinal tract (i.e., gut microbiome) are also influenced by exercise with the potential for positive health effects (32).

Studies have demonstrated that athletes exhibit a higher gut microbial diversity (a proxy for health) compared to non-athletes (5) and that this diversity is correlated with cardiorespiratory fitness (i.e., VO_2max) (12, 14). Furthermore, it has been shown that 6-wk exercise training alters the gut microbiome, independent of diet (1), and that these microbiome adaptations differ as a function of training load and type (35). Contrariwise, we observed only very modest changes in the gut microbial profiles of sedentary individuals following 8-wk of exercise training, highlighting a lack of consensus as to the effects of short-to-medium term exercise training on the gut microbiome (7). In regards to an acute bout of exercise, a case study of a world-class ultramarathon runner from our group demonstrated rapid and profound shifts in gut microbiome composition following a 161-km ultramarathon race (17). Interestingly, alterations in the initial post-race time frame were greater than any observed following 20-wk of training leading into the race. While the physiological relevance of these acute exercise-induced microbial changes remains uncertain, they are likely mediated, at least in part, by changes in microbial metabolites (i.e., xenometabolites). Indeed, changes in microbiome composition alter the metabolome (46), and commensal bacteria produce many important chemicals, hormones, and vitamins that influence health (4). An improved understanding of acute and chronic changes in microbial community structure and metabolic output that occur as a result of exercise is necessary to complete our understanding of the molecular signals induced by exercise that contribute to health benefits.

To shed further light on the acute effects of exercise on the gut microbial ecosystem we compared gut microbial and metabolite profiles, from fecal samples, in triathletes before and after competing in an ultra-endurance (i.e., Ironman: 3.9 km swim, 180.2 km bike, 42.2 km run) triathlon. To

better understand the link between exercise and the gut microbial ecosystem we explored associations between athlete-specific factors (race performance [i.e., time to completion; a proxy for fitness], and lifetime years of endurance training) with gut microbiota and metabolites profiles. We choose bile acid and fatty acid analysis as differential functional readouts of the microbiome that are demonstrated to be responsive to exercise. We hypothesized that robust and congruent shifts in the gut microbiota and metabolites would be observed following the race, and that gut microbiota and metabolite characteristics would differ with race performance and lifetime years of training.

METHODS

Subject recruitment and sample collection

A total of 12 triathletes (9 male, 3 female) registered to participate in 2021 Ironman Indiana were recruited to participate in the study. Inclusion criteria included age between 18 to 65 years of age and body mass index < 30 kg/m^2. All procedures involving human subjects were approved by the Institutional Review Board at Georgia Southern University (H22279) and the study was performed in accordance with the ethical standards of the Declaration of Helsinki. All participants provided consent to participate.

Prior to the race all athletes completed an online questionnaire for determination of anthropometric characteristics, training and medical history, and usual dietary habits via a short-form food frequency questionnaire (SF-FFQ) (6). Stool samples were self-collected within, but not before, 48 hours of race start (Pre) and the first bowel movement following the race (Post; average time between race completion and sample collection = 15.5 $\pm$ 13.1 hours). Two samples were collected at each time-point and were stored in separate collection devices for subsequent microbiome (OMNIgene•GUT) and metabolomics (OMNImet•GUT) analyses. Fecal microbiome samples were stored at room temperature (18°C) until DNA extraction. Metabolomics samples were frozen at -20°C within 7 days of collection.

DNA extraction and shotgun preparation of samples

Bacterial DNA was extracted from 0.25g of stool using Power Fecal Pro Extraction kit from Qiagen (QIAGEN, United Kingdom). Extracted DNA was quantified using Qubit® 2.0 Flurometer (ThermoFisher Scientific, Ireland) and normalized to 0.2ng/µl of DNA. Normalized DNA was prepared for shotgun sequencing using the Nextera XT DNA protocol (Illumina, United States of America), as per the standard protocol guide. The prepared library was sequenced on the Illumina NextSeq Platform, following standard Teagasc protocols.

Bioinformatic processing of microbial metagenomics sequencing

Trimming and adapter removal of paired-end reads was performed using TrimGalore (v0.6.7), while human contamination removal was performed using BowTie2 (v2.3.4). MetaPhlAn3 (v3.0) was used to assign taxonomy.

Metabolomic Sample Preparation

For each sample, 1mL of fecal material was aliquoted and concentrated using a MiVac system. 10mg of dried sample was added individually into sterile screw capped tubes pre-filled with 1.4mm diameter ceramic beads (Roche Diagnostics, Switzerland) to facilitate bile acid extraction. Samples were spiked with deuterated (D) internal standards (D4 cholic acid, D4 chenodeoxycholic acid, D4 deoxycholic acid) and ice-cold 50% methanol was added to each. Samples was then subjected to three, 30 second rounds of beating (MagNA Lyser Instrument (Roche™) at 6,000 rpm before centrifugation at 10,000 x g for 10 minutes. Extracted mixtures were dried before metabolite extraction (**Supplemental Tables 1 & 2**) in ice-cold 95% acetonitrile (ACN) containing 5% formic acid. The dried extracts were reconstituted in 150µL of 50% methanol, transferred into glass vials (Waters Ltd) then applied to Ultra Performance Liquid Chromatography Mass Spectrometry (UPLC-MS).

Extraction of Fecal Short Chain Fatty Acids (SCFAs)

A tiered system of matrix dilutions (1:5 and 1:100) was applied to expand the limit of detection, according to Han *et al.* (18). Samples were homogenized as described above then extraction of matrix dilutions was performed in 50% acetonitrile (ACN) where supernatants were collected in advance of derivatisation. Briefly, in separate reactions, the extracted supernatant and standard curve solution was combined with 0.5 v/v 0.3M Nitrophenylhydrazine hydrochloride (3NPH) and 0.5 v/v 0.3M 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) derivatising reagents (prepared in 50% acetonitrile (ACN)). Mass spectrometry grade isotopic mixtures (deuterated or carbon 13 labelled Internal standard mixtures (13C acetic acid, D5 propionic acid, 13C lactic acid, D7 Butyric acid, D9 Pivalic acid, D9 Valeric acid, D9 Isovalaric acid, D3 Hexanoic acid, D7 Heptanoic acid and D15 Octanoic acid)) were treated similarly. Derivatisation was induced for 30 minutes at 40°C. Reactions were quenched by addition of 10% ACN. A 10-point standard curve was constructed as described **Supplemental Table 2** and utilized for semi quantitative measures.

Metabolomic Analysis

Ultra-performance liquid chromatography (UPLC) tandem mass spectrometry experiments were performed with samples injected in triplicate. All procedures were performed in accordance with Joyce *et al.* 2014 (22) and Pereira *et al.* 2014 (37). Briefly, 5µL of extracted bile acid were injected onto a 50 mm Acquity UPLC BEH C18 column (Waters Corp.) and were eluted using a 25-min gradient of 42% A to 68% B (A, water pH 4, 7.5 mM NH4 Acet.; B, 95%MeOH:5%ACN pH 4) at a flow rate of 300 µL/min and column temperature of 45°C. Samples were analyzed using XEVO-G2QTOF (Waters Ltd.) in negative electrospray mode with a scan range of 50–1,000 m/z, capillary voltage 2.5 KV, sampling cone 40V, desolvation temperature 450°C, source temperature 120°C and desolvation gas flow 800L/h. Each analyte was identified according to its mass and retention time. Standard curves were performed, for BA, MCFA, and LCFA from 1mg/mL stock solutions diluted to concentration ranges between 0.0064 -20 µg/mL, using known BA listed in **Supplemental Table 1**.

For straight chain and branched SCFA (and SCFA-OH): Post-derivatisation, samples and standard curve dilutions were each combined with Internal Standard and added to glass vials (Waters). For each sample or standard, 5µL were injected, in triplicate, onto an ACQUITY HSS T3 column (Waters) for UPLC-QTOF-MS (Xevo G2 Q-TOF) application and detection, at 55°C, run in negative ion mode at 2kV capillary and 50V sampling cone voltage. Derivatised samples/standards were eluted for 16 min using a staggered gradient (Buffer A (isopropanol with 10mM ammonium acetate) to Buffer B (water with 10mM ammonium acetate)) mixed at 85%B to15%A and at a flow rate of 0.35ml/min and desolvation temperature of 450°C.

Quantification and Statistical Analysis

Descriptive statistics were compiled as means and standard deviation (SD). Microbiome statistical analysis was carried out in RStudio v 4.1.1. The vegan package was used to calculate alpha and beta diversity of participant's pre- and post-race. Linear discriminant analysis (LDA) effect size (LEfSe) was used to determine if any discriminatory features were found between groups. A Jaccard similarity distance (vegan, R) was used to calculate the stability of the microbiome pre and post-race. A Kruskal-Wallis test was used to assess the variance between metadata and microbiome abundances. P-values were corrected for multiple comparisons using the Benjamin-Hochberg adjustment. Results were plotted using ggplot2. Microbiome abundance data and metabolome quantitative data were used to assess for possible correlations between bacterial taxa and metabolites. Spearman (corrplot, R) correlations were used to assess for any correlations between the datasets. The p-value was adjusted for false discovery rate (FDR) using the Bonferroni method. A Spearman's correlation of 0.6 was considered strong and a p value of 0.05 was considered significant.

BA metabolites were quantified according to the standard curve and normalized according to the deuterated internal standards. SCFA were semi-quantified according to relative derivatization matched to standard curves for each FA. MassLynx and TargetLynx ^{TM} applications by Waters^{R} were applied to sample data processing, quality and quantity determinations. Tabulated quantities were subjected to

Multivariate analysis, Principal Component Analysis (PCA) and Heatplots analysis using RStudio Version 1.2.5033 and using MetaboAnalyst version 5.0.

RESULTS

Participants

All 12 participants completed the race under the 17-hour (1020 min) cutoff (range: 538 – 849 min) and provided pre- and post-race specimens for metagenomics analysis. One participant did not provide a sample for pre-race metabolomics analysis. A summary of participant characteristics for the sample as a whole, and evenly split by lifetime years of endurance training into shorter (≤ 12 years) and longer (> 12 years of training) training duration histories is provided in Table 1. Compared to the athletes with ≤ 12 years of training, athletes with a longer history of training were significantly older ($P < 0.05$) and by design reported a longer history of lifetime endurance training ($P < 0.01$). No other significant group differences were detected and SF-FFQ scores were relatively homogenous (range: 7-12) and therefore not considered in gut microbiome analyses.

Metagenomic analysis reveals microbiome compositional stability in response to Ironman triathlon

Shotgun metagenomics sequencing analysis of gut microbiome composition is displayed in Figure 1. Intra-individual diversity (α-diversity; observed function, Shannon Index, and Simpson Index) of bacterial species was not different pre- versus post-race ($P<0.05$, **Figure 1A**). Likewise, assessment of inter-individual variability (β-diversity; Bray-Curtis dissimilarity) via Principal Coordinate Analysis revealed no significant separations or clustering pre- and post-race of bacterial species ($P = 0.999$; Figure 1B). Further supporting the stability of the microbiome following race participation, microbiome stability testing using Binary Jaccard matrix on paired samples revealed no statistically significant microbiome variability ($P > 0.05$).

Global decrease in fecal bile acids in response acute long-distance endurance exercise

Targeted fecal metabolomics analysis was used to assess for pre- vs. post-race differences in bile acids and fatty acids. Principal component analysis and heat plots of fecal bile acid concentrations revealed a separation pre vs. post-race (**Figure 2 A & B**) that included a significant reduction in both secondary ($P < 0.001$) and free ($P < 0.05$) bile acids. At an individual level, secondary BA-associated significant reductions in deoxycholic acid (DCA) and 12-keto-lithocholic acid (KLCA_12) were observed ($P<0.05$ respectively, **Figure 2C**). Meanwhile, pre- vs. post-race comparison of lithocholic acid (LCA) concentrations was inconclusive ($P = 0.054$).

Fecal long chain- and short chain- fatty acids are significantly altered by acute long-distance endurance exercise

Principal component analysis and heat plots revealed differences in long-chain (**Figure 3 A & B**) and short-chain fatty acids pre vs. post-race (**Figure 4 A & B**). Significant increases ($P < 0.05$) in the concentrations of the long-chain fatty acids oleic and palmitoleic acid were observed post- vs. pre-race (**Figure 3C**). Conversely, a significant reduction in the concentration of short chain fatty acids butyric ($P < 0.05$) and pivalic ($P < 0.01$) acids was detected post- vs. pre-race (**Figure 4C**). No significant correlations between bacterial taxa and metabolites were observed pre or post-race ($P > 0.05$).

Bacterial taxa and fecal metabolites are associated with race performance and training history

To better understand the link between gut microbiota and fecal metabolite profiles with exercise we examined associations between pre- and post-race levels of bacterial species and metabolites (bile acids and fatty acids) with race performance (**Figure 5**) and lifetime history of endurance training (**Supplemental Table 3**). Using spearman correlational analysis, we identified 71 bacterial species that were associated with race time ($P < 0.05$, **Supplemental Figure 1**). Of note, *Akkermanisa muciniphila* abundance was greater in athletes who took less time to complete the race ($P < 0.05$) while

Methanobrevibacter smithii was more abundant in athletes who took longer to complete the race ($P < 0.05$). Numerous significant inverse correlations ($P < 0.05$) were observed between bile acids and race performance (**Figure 5A**), including significant inverse associations with total and free bile acid levels. However, the pre-race ratio of bile acids AlloLCA/IsoAlloLCA, inflammatory dampening bile acids with implications for the adaptive immune response (19), was positively correlated with finish time ($P < 0.05$). The fatty acids palmitic acid, saturated fatty acids, and stearic acid pre- and post-race were all inversely correlated with finish time ($P < 0.05$). Similarly, pre-race concentrations of linoleic and oleic acid were also inversely correlated with finish time ($P < 0.05$), but the pre-race concentration of isovaleric acid was positively correlated with finish time ($P < 0.05$).

We also examined associations between the fecal microbiota taxa and metabolites with lifetime history of training. Adonis implementation of PERMANOVA demonstrated that years of training ($r^2 = 0.103$) and age ($r^2 = 0.100$) explained similar variance in microbial taxa ($P < 0.05$). Exploratory analysis of relations between individual bacterial species and years of training (**Supplemental Figure 2**) revealed that athletes who had trained for a longer duration (i.e., > 12 years) possessed a greater abundance of *Ruminococcus lactaris* and *Ruminococcus gnavus*, as well as *Bacteroides spp*. (*Bacteroides thetaiotaomicron, Bacteroides salyersiae, Bacteroides plebeius, Bacteroides faecis* and *Bacteroides coprocola*) ($P < 0.05$). Meanwhile, athletes who had trained ≤ 12 years posessed a greater abundance of *Ruminococcus torques* and *Roseburia*_cp_CAG_471 ($P < 0.05$). For bile acids and training history (**Supplemental Table 3**), pre-race concentrations of hyodeoxycholic acid (HDCA) and β-deoxycholic acid and post-race concentrations of 3-oxo-deoxycholic (3oxo-DCA) acid were greater ($P < 0.05$) in athletes with > 12 years training history. After adjusting for age, relations between training history with β-deoxycholic acid were conserved ($P < 0.05$), but not HDCA acid or 3oxo-DCA ($P > 0.05$). Long chain fatty acids were comparable between training groups pre- and post-race ($P > 0.05$), but propionic acid and hexanoic acid were greater ($P > 0.05$) in athletes with a longer training history (**Supplemental Table 3**), though significance was lost after adjusting for age ($P > 0.05$).

DISCUSSION

We used multi-omics analysis to characterize acute changes in the gut microbiome and metabolome associated with completing an ultra-endurance triathlon. We predicted the gut microbiome was based on individual characteristics of an athlete; thus, we explored associations between race performance and lifetime years of endurance training (i.e., athlete-specific factors) with gut microbiota composition and fecal metabolite profiles. Despite marked stability of gut microbiome composition at the post-race time-point, metabolomics analysis demonstrated significant changes in bile acid and fatty acids profiles. We also observed several associations between pre- and post-race gut microbiota and metabolite profiles with race performance and training history.

Shotgun metagenomics sequencing analysis demonstrated remarkable similarity of gut microbiome composition following race completion. This finding is contrary to our hypothesis, and in direct contrast to acute changes in the gut microbiome that have been previously observed following long-distance running from our group (17), and others (41). However, consistent with our observations, a recent study reported no significant changes in intra- or inter-individual diversity of intestinal microbiota in Japanese ultramarathon runners following participation in a ~100km footrace (40). Substantial inter-individual variability in gut microbiota and host responses to diet and exercise interventions have been observed (20, 29), and stability and resilience have been identified as essential ecological characteristics of the gut microbiome with relevance for human health (16, 48). Indeed, while the gut microbiota is relatively stable in young healthy individuals, the microbiome of older adults and clinical populations is notably less resilient (11, 28). In this context, it is perhaps unsurprising that the gut microbiome of the high-fit individuals in our study displayed marked stability despite such a robust physiological stimulus. Whether acute exercise might have a more pronounced impact on microbial structure in less-fit/sedentary individuals is uncertain, but worthy of future investigation.

Despite microbial community stability, fecal metabolomics revealed significant changes in bile acid and fatty acid profiles following race completion. Given the tremendous metabolic demand of competing in an Ironman event (~10,000 kcals) (24), these changes may be explained by alterations

in the functional activity of microbiota to support energy balance (23). Though data are sparse, our data align with previously reported changes in fecal metabolite profiles in cross-country runners following acute moderate-intensity exercise (45), as well as interventional exercise training studies in humans (1, 13) and rodents (9, 10) showing that exercise appears to favorably alter fecal bacterial metabolites. Bile acids are one such metabolite that appear to be responsive to exercise and are key players in dictating metabolism-associated responses, energy expenditure, gut barrier function, and immune activity. However, the relation between acute and chronic exercise and bile acids concentrations is unclear. We observed a significant reduction in free and secondary bile acids in the feces following race completion, a finding that is consistent with reductions in circulating bile acids following a 21.2 km run (8), as well as the lower fecal bile acid concentrations that are observed in more physically active individuals (44, 49). Meanwhile, a persistent (60-180 min) increase in the plasma primary bile acid TCA and the secondary bile acid LCA following both endurance and resistance exercise in healthy males has been observed (34). This discrepancy may be explained by the measurement of bile acid concentrations in different biospecimens (i.e., blood vs feces). Alternatively, it may be that systemic bile acid concentrations following exercise exhibit a phasic pattern (i.e., rise and then fall) attributable to bile acid receptor-mediated FXR activation of FGF19/21 (negative regulators of bile acid synthesis). Future investigations are needed to confirm this hypothesis, as well as the host significance of acute, exercise-mediated changes in bile acids levels.

In further support of acute alterations in gut microbiome function associated with competing in ultra-endurance triathlon, significant changes in fatty acid levels were observed. Interestingly, while we observed an increase in the long chain oleic and palmitoleic acids at the post-race time-point, decreases in the short chain butyric and pivalic acids were detected. To our knowledge, direct links between oleic and palmitoleic acid and physical exercise have not been previously reported. However, in cultured cells increases in oxygen consumption, fatty acid oxidation, and glucose utilization are demonstrated to associate with exposure to these substrates (27), and would seem appropriate during prolonged exercise. While the acute effect of exercise on fecal butyrate concentrations is also uncertain,

a positive correlation has been shown between cardiorespiratory fitness (VO_2max) and fecal butyrate concentrations (14), and chronic exercise training has been demonstrated to evoke significant increases in butyrate and other SCFAs (1, 13). Butyrate and other short-chain fatty acids are produced by the gut microbiota from fiber fermentation and will subsequently be either absorbed by the gut or excreted in feces. Thus, observed reductions in fecal butyrate levels are not necessarily indicative of systemic SCFA declines owing to reduced production rates, and may represent increased SCFA absorption. Increased SCFA absorption during exercise would be reflected by an increase in circulating levels, which we were unable to measure in the present study. However, given the direct (i.e., substrate metabolism) and indirect (i.e., molecular signaling) benefits of increased circulating SCFA levels during exercise (36), increased SCFA absorption rather than reduced production seems like the most likely scenario (25), though joint fecal and circulating metabolomics analysis are needed to confirm this postulate.

Consistent with our hypothesis, we observed numerous associations between gut microbiota and fecal metabolite profiles and race performance and lifetime years of training. The greater *Akkermanisa muciniphila* and the lower *Methanobrevibacter smithii* in participations who completed the race faster were of particular interest. *Akkermanisa muciniphila* is a mucin-degrading bacterium and numerous studies have linked a lack or decreased abundance of this microorganism to multiple disease sates (3, 15). Mechanistically, the health benefits of *Akkermanisa muciniphila* may be attributed to regulation of gut barrier function and metabolic homeostasis, which may have contributed to the faster finishing time in these individuals. Less expectedly, we observed a greater abundance of *Methanobrevibacter smithii* in slower finishers. Recently, a greater abundance of *Methanobrevibacter smithii* transcripts were identified to be a defining characteristic of professional vs. amateur cyclists (38). *Methanobrevibacter smithii,* the dominant archeon in the human gut ecosystem, is positively associated with diets high in carbohydrates (21) and beneficially affects energetic efficiency through its influence on polysaccharide fermentation (39). Thus, while its greater abundance in less competitive athletes may conflict with previous reports (38), it may that its enrichment in this group was

metabolically significant to make it through the longer race. Almost unanimously, we observed an inverse correlation between fecal bile acid and fatty acid concentrations and race performance. While an increased abundance of SCFAs has emerged as a hallmark characteristic of the athletic gut microbiota (31), greater bile acid levels among faster finishers in the present study was more unexpected given the lower fecal bile acids previously reported in active compared to sedentary individuals (44, 49). To date, our understanding of the physiological significance of bile acids has largely originated from clinical studies that have implicated bile acid involvement in obesity and diabetes. However, in addition to their known role as lipid detergents, emerging research has demonstrated that bile acids also function as gut hormones with potentially systemic significance (30). Thus, our findings, which demonstrate elevated bile acid levels in faster athletes, provide novel perspective regarding relations between bile acids, fitness, and health.

In athletes who reported a longer history of endurance training, enriched populations of *Bacteroides* spp., an important saccharolytic group in the colon that promotes branched-chain amino acid catabolism in brown fat and inhibits obesity (50), were observed. With advancing age, a reduction in the intestinal abundance of *Bacteroides* spp. has been implicated in contributing to weight gain (33). Therefore, the greater abundance of this genera in athletes who reported a longer history of endurance training, and were also older, alludes to the notion that a proliferation of *Bacteroides* spp. in older athletes may serve as a mechanism to protect against age-associated weight gain. Meanwhile, advancing age is also associated with reductions in butyrate-producing Roseburia (2), which we observed to be lower in athletes reporting a longer (older) vs. shorter (younger) duration training history. Thus, it is reasonable to speculate that long-term endurance training was able to protect against some, but not all, age-related changes in gut microbiome composition. However, no differences in butyrate levels were observed between groups, and the SCFA propionic acid was actually higher in athletes with a longer endurance training history. Of further interest, bile acids 3oxo-DCA and HDCA were elevated in athletes with a longer training history. Proliferation of 3oxo-DCA could be an important adaptation to exercise since this intermediate promotes Treg and pro-inflammatory TH17 cells

expression to modulate inflammation (19, 26, 43). HDCA is anti-obesogenic associated and though not classically represented among human bile acids is positively correlated with Lachnospiracease (42) and with Rumminococcae in humans (47). Notably however, associations between training history and 3oxo-DCA and HDCA were lost after adjusting for age, bringing into question the exact stimulus behind these relations.

Findings from the present study should be interpreted in the context of its strengths and limitations. First, post-race samples were compared to only a single pre-race time-point, which may not be wholly representative of an athlete's 'standard' microbiome composition. However, as these samples were collected within a relatively short window of race start (i.e., ≤ 48 hours), they are more likely to be representative of gut microbial ecology during race participation. Second, our study lacked a non-exercising control to account for temporal variability in quantitative human gut microbiome profiles. Third, while extremely unique, our relatively small sample size left us unable to definitively control for potentially relevant biological variables such as age and gender. Lastly, we were unable to account for race day diet as participant self-reports lacked sufficient quantitative description. Major strengths include that we are the first to characterize acute changes in gut microbial ecology during an Ironman triathlon, complimentary metagenomic and metabolomic measures, and that we were able to study athletes from all ends of the competitive spectrum, including two age-group winners who qualified for the World Championships.

In conclusion, we demonstrate acute changes in fecal bile acid and fatty acid profiles despite marked stability of gut microbiome composition following participation in an ultra-endurance triathlon. We also observed several associations between gut microbiome and fecal metabolome characteristics with race performance and lifetime history of endurance training. Collectively, these findings indicate that 1) acute ultra-endurance exercise affects microbial community function, but not structure, and 2) exercise-related adaptations in the gut microbial ecology are influenced by athlete-specific factors. These data add to a small but growing body of literature seeking to characterize the acute and chronic effects of exercise on the gut microbial ecosystem.

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AUTHOR CONTRIBUTIONS

This work was conceived and designed by GJG and JP. The experimental work was performed by GJG, JP,JA, JC collection and co-ordination LW & OOS (Microbial) KQ & DM (MS) and BGP (bacteria). All authors analysed the data and wrote and edited the manuscript.

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FIGURE LEGEND

Figure 1. Shotgun metagenomic sequencing analysis of gut microbiome composition pre- and post-race. A) Boxplots of observed functions, and Shannon's and Simpson's diversity index. B) Beta-diversity based PCA analysis of fecal microbiota pre- and post-race. Numbers indicate individual pre- and post-race data.

Figure 2. Fecal bile acid concentrations. PCA and Heatplot clustered by row of individual BA concentrations pre- and post-race (A+C). (B) Boxplots of significant and near significant BA post IRONMAN race. Statistical Analysis carried out using paired or unpaired t-Test (* $P \leq 0.05$, ** $P \leq 0.01$, , *** $P \leq 0.001$) or paired and unpaired wilcoxon (# $P \leq 0.05$, ## $P \leq 0.01$, , ### $P \leq 0.001$) depending on normality

Figure 3. Fecal LCFA Concentrations. PCA and Heatplot clustered by row of individual LCFA concentrations pre- and post-race (A+C). (B) Boxplots of significant LCFA post IRONMAN race. Statistical Analysis carried out using paired or unpaired t-Test (* $P \leq 0.05$, ** $P \leq 0.01$, , *** $P \leq 0.001$) or paired and unpaired wilcoxon (# $P \leq 0.05$, ## $P \leq 0.01$, , ### $P \leq 0.001$) depending on normality

Figure 4. Fecal SCFA Concentrations. PCA and Heatplot clustered by row, of individual SCFA concentrations pre and post race (A+C). (B) Boxplots of significant SCFA post IRONMAN race. Statistical Analysis carried out using paired or unpaired t-Test (* $P \leq 0.05$, ** $P \leq 0.01$, , *** $P \leq 0.001$) or paired and unpaired wilcoxon (# $P \leq 0.05$, ## $P \leq 0.01$, , ### $P \leq 0.001$) depending on normality

Figure 5. Correlational analysis of A) BA and B) LCFA and SCFA with respect to race time.

Table 1. Athlete characteristics

	All athletes (n=12)	Athletes with ≤ 12 yrs of training (n=6)	Athletes with > 12 yrs of training (n=6)
Gender (M/F)	9/3	4/2	5/1
Age (yrs)	43.2 ± 14.1	35.3 ± 11.6	51.0 ± 12.4*
Height (m)	1.8 ± 0.1	1.8 ± 0.1	1.8 ± 0.1
Weight (kg)	72.6 ± 10.0	72.1 ± 9.5	73.1 ± 1.4
BMI (kg/m^2)	23.1 ± 1.8	22.6 ± 1.0	23.6 ± 2.4
Short-Form FFQ Score	9.8 ± 1.6	9.2 ± 1.5	10.3 ± 1.6
Lifetime training (yrs)	16.5 ± 12.2	7.5 ± 2.8	25.5 ± 11.1*
Finish time (min)	695 ± 100	681 ± 126	710 ± 75

*P < 0.05 vs. athletes with ≤ 12 yrs of training

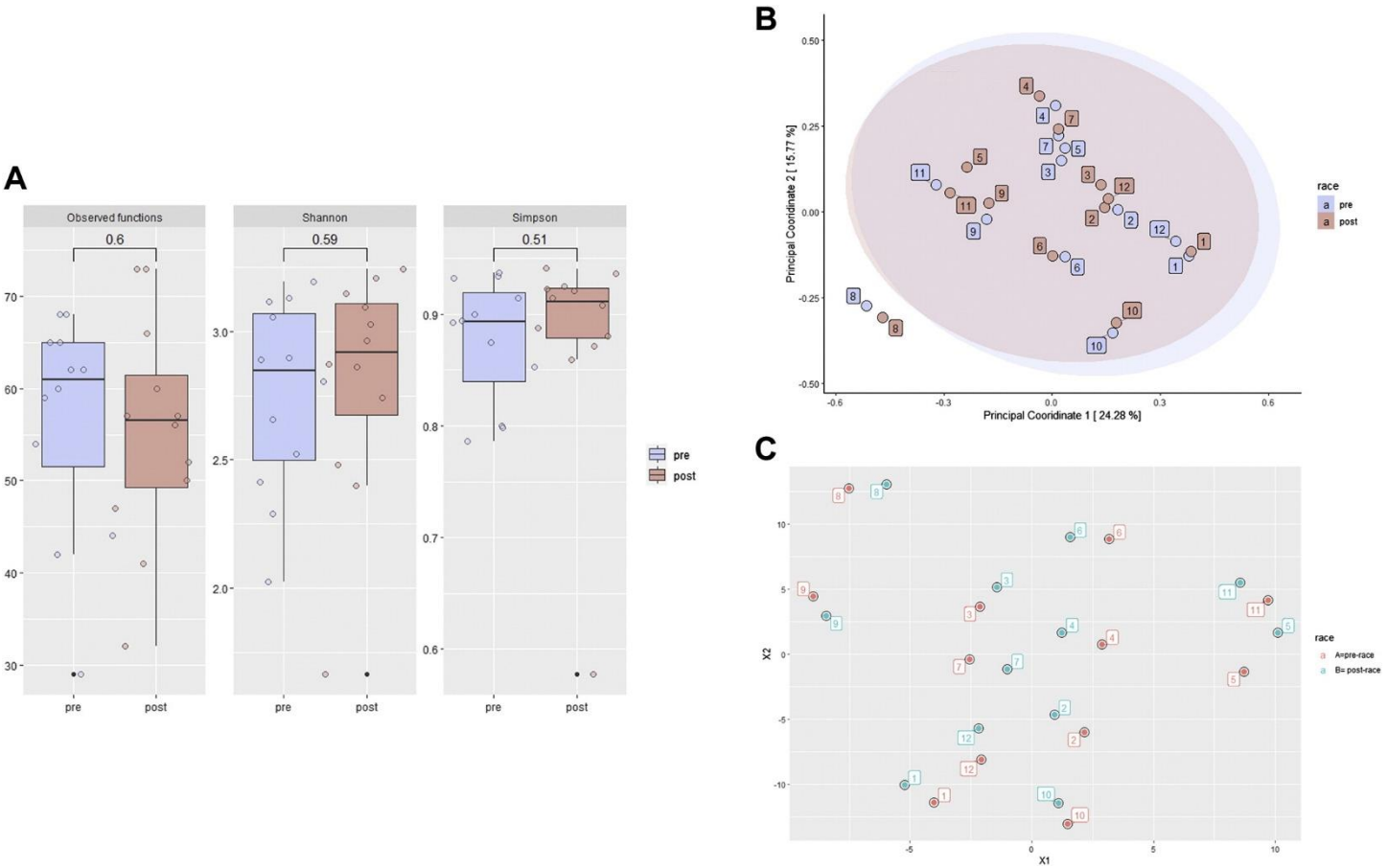

Figure 1. Shotgun metagenomic sequencing analysis of gut microbiome composition pre- and post-race. *A*: boxplots of observed functions, and Shannon's and Simpson's diversity index. Visualization of β-diversity (Bray–Curtis) via principal coordinate analysis (*B*) and t-distributed stochastic neighborhood embedding (t-SNE) (*C*) of fecal microbiota pre- and post-race.

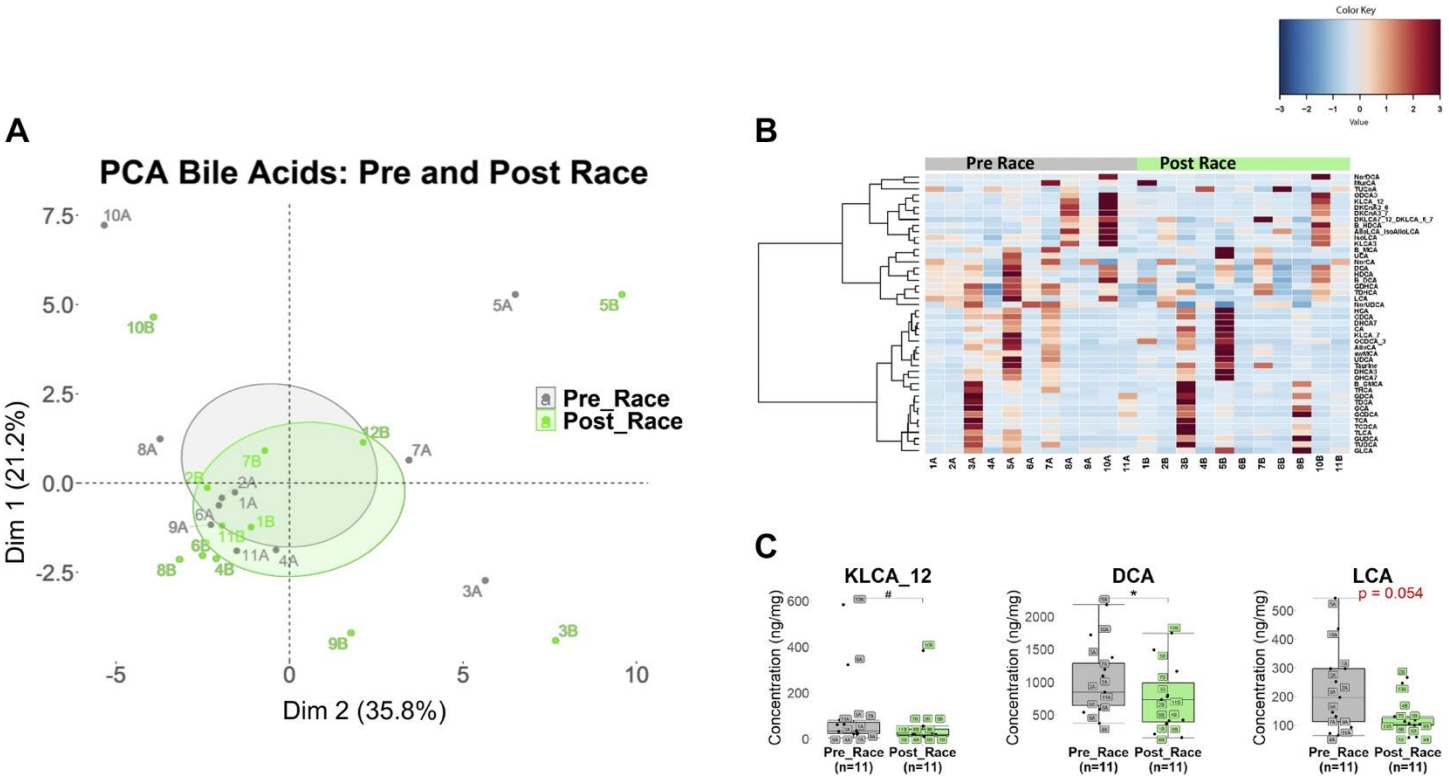

Figure 2. Fecal bile acid concentrations. Principal component analysis (PCA) and heat plot clustered by row of individual BA concentrations pre- and post-race (*A* + *B*). *C*: boxplots of significant and near significant BA post IRONMAN race. Statistical analyses carried out using paired *t* test (*$P \leq 0.05$) or Wilcoxon signed-rank test (#$P \leq 0.05$), depending on normality. DCA, deoxycholic acid; KLCA, keto-lithocholic acid; LCA, lithocholic acid.

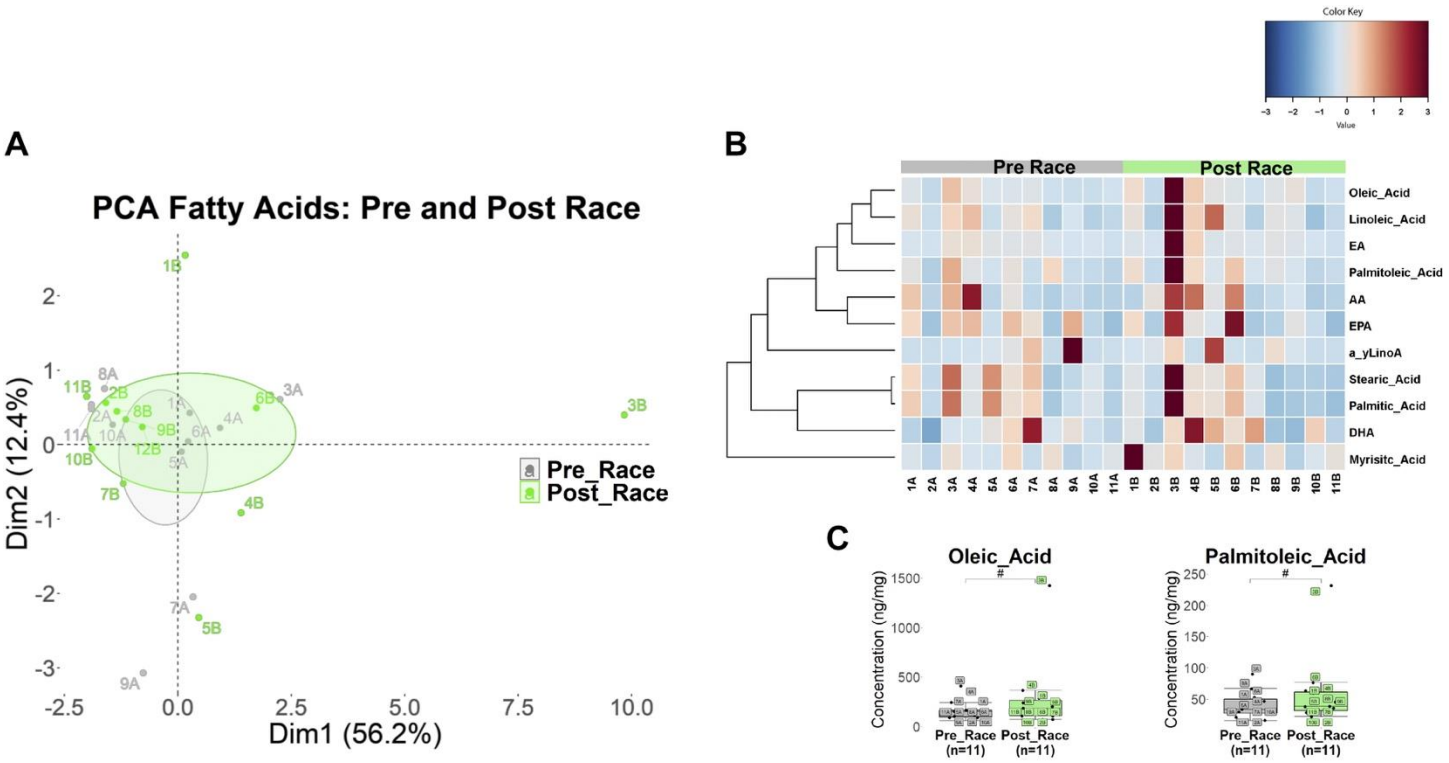

Figure 3. Fecal LCFA concentrations. Principal component analysis (PCA) and heat plot clustered by row of individual LCFA concentrations pre- and post-race (*A* + *B*). *C*: boxplots of significant LCFA post IRONMAN race. Statistical analyses carried out using paired t-test or Wilcoxon signed-rank test (#$P \leq 0.05$), depending on normality.

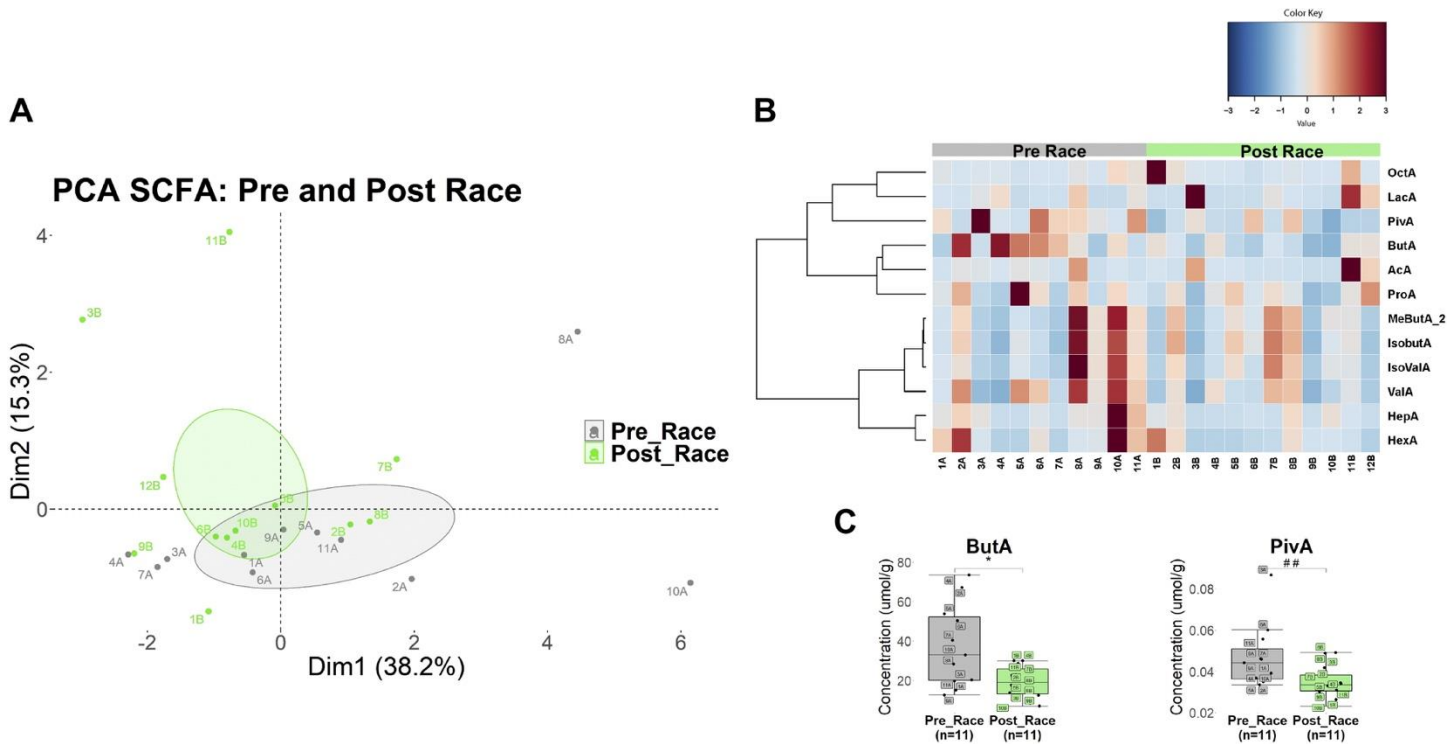

Figure 4. Fecal short-chain fatty acid (SCFA) concentrations. Principal component analysis (PCA) and heat plot clustered by row, of individual SCFA concentrations pre- and post-race (*A* + *B*). *C*: boxplots of significant SCFA post IRONMAN race. Statistical analyses carried out using paired *t* test (*$P \leq 0.05$) or Wilcoxon signed-rank test (##$P \leq 0.01$), depending on normality.

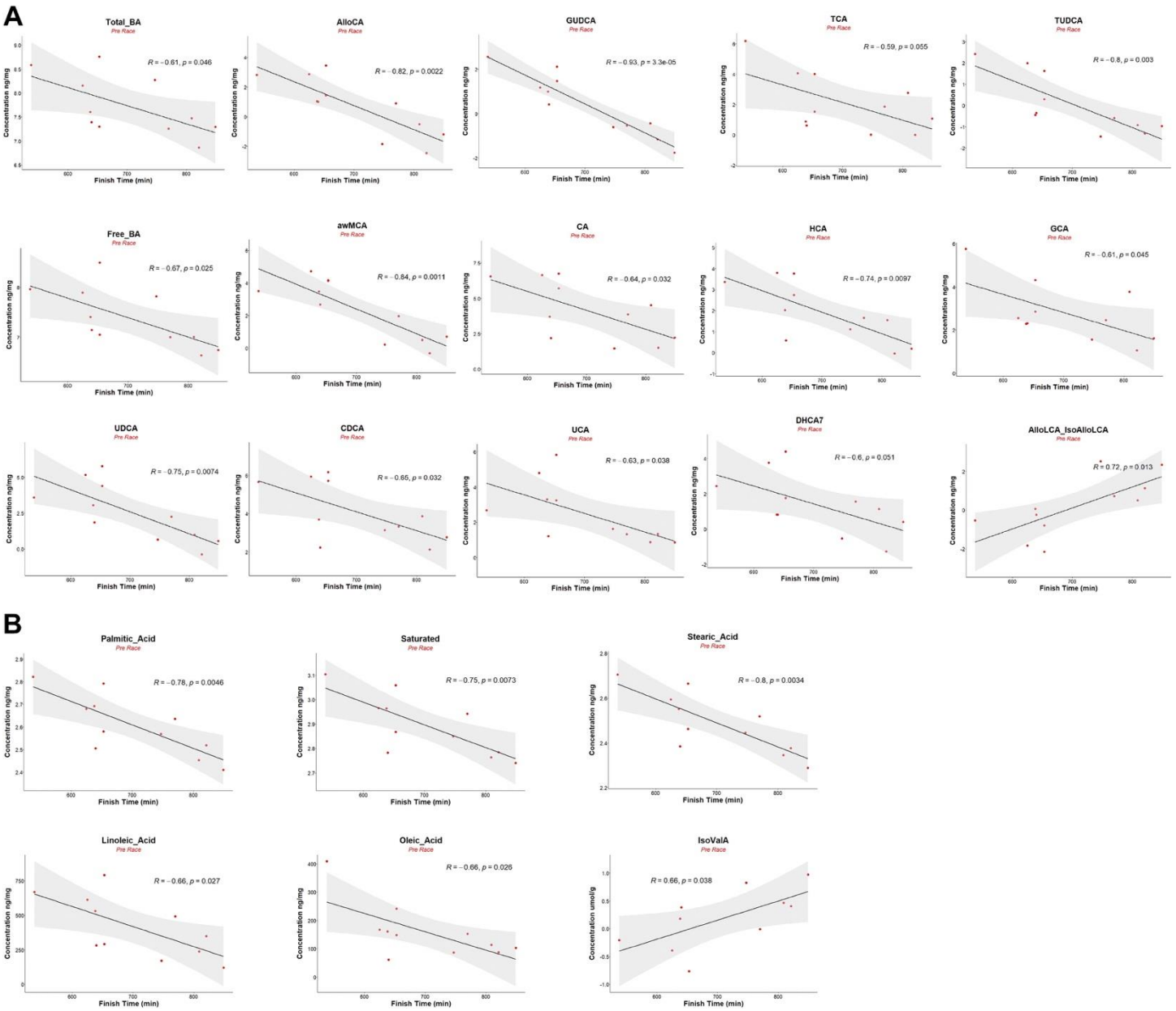

Figure 5. Correlational analysis of pre-race concentrations of BA (*A*) and LCFA and short-chain fatty acid (SCFA) (*B*) with respect to race time.