TITLE:

Reliability of Gastrointestinal Barrier Integrity and Microbial Translocation Biomarkers at Rest and Following Exertional Heat Stress

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SUBJECT AREA

Environmental and Exercise Physiology

NEW FINDINGS:

What is the central question(s) of this study?

To assess the reliability of gastrointestinal barrier integrity and microbial translocation biomarkers both at rest and in response to exertional-heat stress.

What is the main finding and its importance?

Acceptable and defined-levels of reliability are presented for the serum Dual-Sugar Absorption (lactulose/$_{L}$-rhamnose) Test, Intestinal Fatty-Acid Binding Protein, Claudin-3, Lipopolysaccharide Binding Protein, total 16s DNA, but not the *Bacteroides*/total 16s DNA ratio at both measurement time-points.

ABSTRACT

Purpose: Exertional-heat stress adversely distrupts (GI) barrier integrity and, through subsequent microbial translocation (MT), negativly impacts health. Despite widespread application, the temporal reliability of popular GI barrier integity and MT biomarkers is poorly characterised. **Method:** Fourteen males completed two 80-minute exertional-heat stress tests (EHST) separated by 7-14 days. Venous blood was drawn pre, immediately- and 1-hour post both EHSTs. GI barrier integrity was assessed using the serum Dual-Sugar Absorption Test (DSAT), Intestinal Fatty-Acid Binding Protein (I-FABP) and Claudin-3 (CLDN-3). MT was assessed using plasma Lipopolysaccharide Binding Protein (LBP), total 16S bacterial DNA and *Bacteroides* DNA. **Results:** No GI barrier integrity or MT biomarker, except absolute *Bacteroides* concentration, displayed systematic trial order bias ($p \geq 0.05$). I-FABP (trial 1 = Δ 0.834 ± 0.445 $ng \cdot ml^{-1}$; trial 2 = Δ 0.776 ± 0.489 $ng \cdot ml^{-1}$) and CLDN-3 (trial 1 = Δ 0.317 ± 0.586 $ng \cdot ml^{-1}$; trial 2 = Δ 0.371 ± 0.508 $ng \cdot ml^{-1}$) were increased post-EHST ($p \leq 0.01$). All MT biomarkers were unchanged post-EHST. Coefficient of variation and typical error of measurement post-EHST were: 11.5% and 0.004 (ratio) for the DSAT 90-minutes post probe ingestion; 12.2% and 0.004 (ratio) at 150-minutes post probe ingestion; 12.1% and 0.376 $ng \cdot ml^{-1}$ for I-FABP; 4.9% and 0.342 $ng \cdot ml^{-1}$ for CLDN-3; 9.2% and 0.420 $\mu g \cdot ml^{-1}$ for LBP; 9.5% and 0.15 $pg \cdot \mu l^{-1}$ for total 16S DNA; and 54.7% and 0.032 for Bacteroides/total 16S DNA ratio. **Conclusion:** Each GI barrier integrity and MT translocation biomarker, except *Bacteroides*/total 16S ratio, had acceptable reliability at rest and post exertional-heat stress.

ABBREVIATIONS

ANOVA	Analysis of variance
B-A	Bland-Altman Limits of Agreement
Bact.	*Bacteroides*
BactDNA	Bacterial DNA
CLDN-3	Claudin-3
CV	Coefficient of Variation
DSAT	Dual Sugar Absorption Test
EDTA	Ethylenediaminetetraacetic acid
EHST	Exertional Heat Stress Test
ELISA	Enzyme Linked Immunosorbent Assay
GI	Gastrointestinal
HPLC	High Performance Liquid Chromatography
HR	Heart Rate
I-FABP	Intestinal Fatty-Acid Binding Protein
ISAK	International Society for the Advancement of Anthropometric Kinanthropometry
LBP	Lipopolysaccharide Binding Protein
L/R	Lactulose-to-Rhamnose
MT	Microbial Translocation
PCR	Polymerase Chain Reaction
RH	Relative Humidity
RPE	Rate of Perceived Exertion
SD	Standard Deviation
T_{core}	Core Body Temperature
T_{body}	Mean Body Temperature
TEM	Typical Error of Measurement
T_{skin}	Mean Skin Temperature
TS	Thermal Sensation
$\dot{V}O_{2max}$	Maximal Oxygen Uptake

INTRODUCTION

The gastrointestinal (GI) microbiota is a complex microbial ecosystem, which performs numerous functions symbiotic to human health (Cani, 2018). However, to prevent immune activation the microbiota must remain contained within the GI lumen, a process that is tightly regulated by the multi-layered GI barrier (Wells et al., 2017). Exertional heat stress is one stimulus that adversely disrupts GI barrier integrity, and in a linear manner to the severity of splanchnic hypoperfusion (van Wijck et al., 2011) and core body temperature (Pires et al., 2017). In severe cases, luminal microbial products are capable of transversion into the systemic circulation, a response now considered to underlie multiple common athletic health conditions (Costa et al., 2017). Specifically, the most concerning of these health conditions include exercise-induced anaphylaxis (Christensen et al., 2019) and exertional heatstroke (Lim, 2018). In non-exercise settings, research presently links GI microbial translocation (MT) within the pathophysiology of numerous chronic illnesses, including GI disease (Camilleri et al., 2012), cardiovascular disease (Neves et al., 2013) and degenerative disorders of the central nervous system (Mulak and Bonaz, 2015). Thus, reliable biomarkers of GI barrier integrity and/or MT appear important in the surveillance, diagnosis and treatment of these conditions. To date, there is little evidence documenting the reliability of most commonplace assessment biomarkers, which limits interpretation of their application in both laboratory and field settings.

GI barrier integrity can be assessed *in vivo* using several biomarkers of intestinal permeability, epithelial injury and tight junction integrity (Wells et al., 2017). The Dual-Sugar Absorption Test (DSAT) is the gold-standard GI permeability technique (Bischoff et al., 2014). The traditional endpoint of the DSAT is the 5-hour urinary recovery of pre-ingested lactulose-to-$_{L}$-rhamnose (L/R; Bischoff et al., 2014) and offers good test-retest reliability when applied at rest (Marchbank et al., 2011). Analytical improvements have recently validated a serum DSAT over a reduced (i.e. 1-3 hours) time course (van Wijck et al., 2013) and with improved diagnostic sensitivity (JanssenDuijghuijsen et al., 2016; Pugh et al., 2017a). However, given the transient appearance of sugar probes within the blood (Fleming et al, 1996), potentially due to the wide heterogeneity in gastric emptying rates following exercise (Costa et al., 2017), the reliability of this technique requires verification. Intestinal Fatty Acid-Binding Protein (I-

FABP) is a cytosolic protein expressed exclusively within enterocytes of the duodenum/jejunum, and has a half-life of 11 minutes in the systemic circulation following epithelial injury (van de Poll et al. 2007). These characteristics have popularised I-FABP as a prominent biomarker of small GI epithelial injury (Wells et al., 2017), with serum concentrations strongly predictive of small GI histological injury (Schellekens et al., 2014). The temporal reliability of I-FABP has never been directly assessed and requires interrogation given its high sensitivity to sub-clinical small GI injury. Claudin-3 (CLDN-3) is a conserved GI epithelial transmembrane protein, which performs an integral role in GI paracellular homeostasis (Zeissig et al., 2007). As a biomarker of GI tight junction (TJ) integrity, preliminary research has shown a strong relationship between urinary CLDN-3 concentration and histological GI CLDN-3 breakdown (Thuijls et al., 2010a, 2010b). Similar to I-FABP, the temporal reliability of plasma CLDN-3 is currently unknown.

GI MT can be assessed *in vivo* through several indirect biomarkers considered to be indicative of systemic microbial exposure (Wells et al., 2017). Endotoxin, a form of lipopolysaccharide located on the outer membrane of gram-negative bacteria, has traditionally been utilised for this purpose (Costa et al., 2017). However, the search for improved GI MT biomarkers is ongoing, given endotoxin analysis is susceptible to both false-positive (e.g. from exogenous contamination) and false-negative (e.g. from rapid hepatic clearance) results (Dullah and Ongkudon, 2017). Lipopolysaccharide binding protein (LBP) is a type-1 acute phase protein, secreted hepatically following systemic exposure to numerous microbial-associated molecular patterns (Schumann, 2011). However, as an acute-phase protein, its temporal reliability is likely highly subject to influence from numerous co-variates (e.g. infection) (Citronberg et al., 2016). Bacterial DNA (bactDNA), through conserved 16S gene sequencing, is an emerging biomarker of GI MT (Paisse et al., 2016). In comparison with alternative MT measures, one major advantage of bactDNA is an apparent independence of hepatic clearance (Mortensen et al., 2013). One innovative study recently proposed a bactDNA methodology aimed to improve analytical specificity and reliability through targeting a predominant GI bacterial genus (*Bacteroides*) and correcting for total 16S DNA concentration (March et al., 2019).

158 The aim of the present study was to determine the reliability of biomarkers of GI
159 barrier integrity (DSAT, I-FABP, CLDN-3) and microbial translocation (LBP, total 16s bacterial
160 DNA, *Bacteroides* DNA) at rest and following exertional-heat stress. These data should inform
161 prospective study design, including biomarker selection and statistical power.

162 **METHODS**

163 **Participants and Ethical Approval**

164 Fourteen healthy males (Table 1) volunteered to participate in the present study. All
165 participants were non-smokers, habitually active, non-endurance trained (>4 $h \cdot week^{-1}$) and
166 unacclimated to hot environments. A general medical questionnaire was used to screen for
167 previous histories of gastrointestinal, cardiorespiratory and metabolic illnesses. No
168 participant took pharmacological medications (e.g. laxatives, antibiotics) or reported
169 suffering from an acute illness within 14 days prior to data collection. Informed consent was
170 obtained for each participant following a full written and oral explanation of the experimental
171 procedures. The study protocol was approved by Plymouth MARJON University Research
172 Ethics Committee (Approval Code: EP040) and was conducted in accordance with the
173 principles outlined in the Declaration of Helsinki, except for trial registration within a
174 database.

175 [Table 1 – Insert Here]

176 **Experimental Overview**

177 Participants visited the laboratory on three occasions. During the first visit, baseline
178 anthropometrics and maximal oxygen uptake ($\dot{V}O_{2max}$) were assessed. The second and third
179 visits were the main experimental trials. These were separated by 7-14 days to negate the
180 influence of prior exertional-heat stress on thermoregulatory (Barnett and Maughan, 1993)
181 and GI barrier integrity (Snipe et al., 2017) responses. During both main experimental trials,
182 participants completed an intermittent exertional-heat stress test (EHST), consisting of two
183 bouts of 40 minutes fixed-intensity treadmill walking (6 $km \cdot h^{-1}$ and 7% gradient) in the heat
184 (35°C and 30% relative humidity; RH). The exercise bouts were separated by 20-minutes
185 seated recovery, including 4-minutes forearm cold water immersion. This protocol is
186 consistent with general military guidance on work/rest schedules for sustained physical

activity in the heat (Military Headquarters of the Surgeon General, 2017) and unpublished pilot data from our laboratory showing a ~ 2-fold elevation in DSAT responses relative to rest (n= 6; DSAT 90-minute post probe ingestion; [rest] = 0.014 ± 0.006, [post EHST] = 0.028 ± 0.005; p = 0.02). Data collection coincided with non-summer months in Plymouth, United Kingdom, where daily mean ambient temperature at a local meteorological station (Camborne, United Kingdom; latitude: 50.218 ° N) remained below 20°C (Met Office, 2019). A schematic illustration of the protocol is shown in Figure 1.

[Figure 1 – Insert Here]

Dietary and Lifestyle Controls

Dietary supplementation (e.g. glutamine, probiotics, bovine colostrum) and prolonged thermal exposures (e.g. saunas, sunbeds) were prohibited from 14 days before until the end of data collection (Costa et al., 2017). Alcohol, caffeine, strenuous physical activity and non-steroidal anti-inflammatory drugs (e.g. ibuprofen) were all abstained for 48 hours before main experimental visits (Costa et al., 2017). Participants adhered to a ≥ 10 hour overnight fast and consumed 500 ml of plain water two hours prior to main experimental visits. Conformity with all pre-trial controls was assessed in writing upon laboratory arrival using a pre-trial control questionnaire. Participants remained fasted throughout all main experimental trials (Edinburgh et al., 2018), but were permitted a 12 ml·kg^{-1} bolus of ambient temperature water (28-30°C) to drink over 20 minutes following both 40-minute EHST bouts.

Anthropometric Measurements

Participants height, weight and body fat were measured following ISAK guidelines (Marfell-Jones et al. 2006). Height was measured barefoot using a stadiometer to the nearest 0.1 cm (Marsden HM-200, Rotherham, UK), whilst body mass was measured on an electronic scale to the nearest 0.05 kg (Tanita MC 180 MA, Tokyo, Japan). Skinfold thicknesses were taken in duplicate by the same researcher at the bicep, tricep, subscapular and suprailliac using skinfold callipers to the nearest 0.1 cm (Harpenden, Holtain Ltd, Crymych, UK). Predictions of body density were calculated using age and gender related equations (Durnin

and Womersley, 1974).

Maximal Oxygen Uptake

Maximal oxygen uptake ($\dot{V}O_{2max}$) was determined using an incremental treadmill test (Desmo HP, Woodway GmbH, Weil am Rhein, Germany) to volitional exhaustion. The test was undertaken in normothermic laboratory conditions (18-22°C, 40-60% RH). The test began at a speed of 10 $km \cdot h^{-1}$ on a fixed 1% inclination. The treadmill speed was then increased at 1 $km \cdot h^{-1}$ increments every three minutes until reaching 13 $km \cdot h^{-1}$, when inclination was then increased by 2% every two minutes. Expired metabolic gases were measured continuously using a breath-by-breath metabolic cart (Metalyser 3B, Cortex, Leipzig, Germany). Heart rate (HR; Polar FT1, Polar Electro OY, Kempele, Finland) and rating of perceived exertion (RPE; Borg, 1970) were measuring during the final ten seconds of each stage. The highest 30 second average $\dot{V}O_2$ was taken to be $\dot{V}O_{2max}$.

Exertional-Heat Stress Test

EHSTs commenced in the morning (08:30 ± 1 hour) to avoid the influence of circadian variation (Waterhouse et al., 2005). Upon laboratory arrival, participants provided a capillary blood sample into a K_2EDTA microtube (Microvette®, Sarstedt, Numbrecht, Germany) for duplicate hydration assessment via plasma osmolality using freeze-point depression (Osmomat 3000, Gonotec, Berlin, Germany). Participants then measured their own nude body mass (Tanita MC 180 MA, Tokyo, Japan). They then self-inserted a single use rectal thermistor (T_{core}; Phillips 21090A, Guildford, UK) 12 cm beyond the anal sphincter and a HR monitor was positioned around their chest (EQ02, Equivital™, Cambridge UK). Next they dressed in standard summer military clothing (i.e. jacket [zipped, sleeves extended], trousers, boxer briefs, socks, trainers) and entered the environmental chamber that was regulated at ~35°C (Trial 1: 35.2 ± 0.3°C; Trial 2: 35.4 ± 0.4°C; p= 0.15) and ~30% RH (Trial 1: 28 ± 4%; Trial 2: 28 ± 2%; p= 0.25). Skin thermistors (EUS-UU-VL3-O, Grant Instruments, Cambridge, UK) were then affixed on the participant using one layer (5 x 5 cm) of cotton tape (KT Tape®, KT Health, UT, USA) and mean skin temperature (T_{skin}) was calculated using standard equations (Ramanathan, 1964).

Participants then undertook the pre-defined EHST. Throughout, T_{core} and T_{skin} were

recorded using a temperature logger (Squirrel SQ2010, Grant Instruments, Cambridge, UK) and HR using a Sensor Electronics Module (SEM) unit (EQ02, Equivital™, Cambridge UK). Mean whole body temperature (T_{body}) was calculated from simultaneous T_{core} and T_{skin} measurements (Jay and Kenny, 2007). All data, including RPE (Borg et al., 1970) and thermal sensation (TS; Toner et al., 1986) were reported at 20 minute intervals. Between the two walking bouts, participants immersed their forearms in a ~15°C cold-water bath (Trial 1: 15.4 ± 0.8°C, Trial 2: 15.3 ± 0.7°C; p = 0.39). Upon EHST termination, participants were removed from heat and their post-EHST nude body mass was recorded. Absolute sweat losses were calculated from the change in dry nude body mass from pre-to-post EHST after correction for fluid intake and blood withdrawal.

Blood Collection and Analysis

Venous blood samples (12 ml) were drawn immediately pre, post and one-hour post EHST. Participants stood upright for a minimum of 20 minutes before collection to allow capillary filtration pressure to stabilise (Shirreffs and Maughan, 1994). Blood was drawn from a forearm antecubital vein under minimal stasis (<30 seconds). Samples were collected proportionally into serum-separator (SST II) and K_2 EDTA tubes (Becton Dickinson and Company, Plymouth, UK). The SST II tube was allowed to clot for 30-40 minutes at room temperature. A 0.5ml aliquot of K_2EDTA blood was removed for immediate haematological analysis. Samples were centrifuged at 1300*g* for 15 minutes at 4°C to separate serum and plasma. Aliquots were frozen at -80°C until analyses. All blood handling was performed with sterile (pyrogen, DNA free) pipette tips and microtubes.

Haematology

Haemoglobin was measured in duplicate using a portable photometric analyser (Hemocue® Hb 201+, EFK Diagnostics, Madeburg, Germany; Duplicate) and haematocrit in duplicate using the microcapillary technique following centrifugation at 14,000*g* for 4 minutes at room temperature (Haematospin 1400, Hawksley and Sons Ltd, Lancing, England). Plasma volume was estimated using standard equations (Dill and Costill, 1974). Post-exercise analyte concentrations were left uncorrected for acute plasma volume shifts, given the similarity of responses between trials and the low molecular weights of quantified analytes.

Dual-Sugar Absorption Test

Participants orally ingested a standard sugar probe solution containing 5 g Lactulose (Lactulose Oral Solution, Sandoz, Holzkirchen, Germany) and 2 g $_{L}$-Rhamnose ($_{L}$-rhamnose FG, 99% pure, Sigma Aldrich, Missouri, USA) dissolved within 50 ml of plain water (osmolality = ~750 mOsm·kg^{-1}) ten minutes into the EHST. Probe concentrations were determined from serum samples collected immediately pre, 90 minutes (i.e. post-EHST) and 150 minutes (i.e. 1-hour post-EHST) post probe ingestion following a previously described high performance liquid chromatography protocol (Fleming et al., 1996). The recovery of both sugars was determined per litre serum (mg·l^{-1}). Calculation of the L/R ratio was made corrected relative (%) to the concentration of sugar consumed. The limit of detection was 0.1 mg·l^{-1} and the laboratory reference coefficient of variation was 1.8-8.5% for both probes (Fleming et al., 1996).

Enzyme Linked Immunosorbent Assays

I-FABP ([1:2 serum dilution]; ELH-FABP2, Raybiotech®, Norcross, USA), CLDN-3 ([undiluted plasma]; EH1342, Wuhan Fine Biotech, Wuhan, China) and LBP ([1:250 plasma dilution]; RK01764, ABcloncal, Wuburn, USA) were measured in duplicate immediately pre and post EHST using a solid-phase sandwich ELISA. Optical density was measured at 450 nm using a microplate reader and sample concentrations were determined from a logarithmic standard curve. The intra-assay coefficients of variation were 5.0% (I-FABP), 1.5% (CLDN-3) and 2.6% (LBP).

Quantitative Real-Time Polymerase Chain Reaction

BactDNA was measured in duplicate plasma samples collected immediately pre- and post EHST using a quantitative real-time polymerase chain reaction assay (qPCR) on a LightCycler 96 instrument (LightCycler 96, Roche, Basel, Switzerland). Cell free DNA was isolated from plasma using a Quick-DNA Mini Prep Plus kit (D4068, Zymo Research, Irvine, CA, USA) following manufacturer's instructions. Total 16S bacterial DNA was quantified according to March et al. (2019) using a universal library probe (ULP, Roche, Basel, Switzerland), with standards (E2006-2, Zymo Research, Irvine, CA, USA) and primers (Eurogentec, Liège, Belgium) specific to a 16S region (limit of detection 0.1 pg·ul^{-1}). *Bacteroides* species DNA (*Bact.* DNA) were quantified using a double-dye probe/primer kit (Path-Bacteroides-spp,

Genesig, Primerdesign Ltd, Chandler's Ford, UK). Negative controls (PCR grade water) for the entire extraction process were below the limit of detection for both measures. Ratio data are presented as *Bacteroides*/total bacterial DNA (*Bact./*16S). The intra-assay coefficients of variation were 6.3% (total 16S) and 17.5% (*Bacteroides*).

Statistics

All statistical analyses were performed using Prism Graphpad software (Prism V.8, La Jolla, California, USA). Comparisons were made after determining normal distribution using a Shapiro-Wilk test ($p \geq 0.05$). A two-way analysis of variance (ANOVA) with repeated measures (time x trial) was used to identify differences between the two trials for whole-body physiological, GI barrier integrity and MT data. If Mauchly's test for sphericity was violated, Greenhouse Geiser corrections were applied for epsilon <0.75, while the Huynh-Feldt correction was used for less severe asphericity. When there was only a single comparison, a paired t-test or non-parametric Wilcoxon signed-ranks test was used to determine between-trial differences. Statistical significance was accepted at the alpha level of $p \leq 0.05$. Data are presented as mean ± standard deviation (SD).

A composite *a priori* battery of statistical tests was conducted to determine inter-trial reliability (Atkinson and Nevill, 1998). The DSAT was compared at each 90- and 150-minutes following sugar-probe ingestion, whilst each GI biomarker was compared at rest, post EHST and the delta (Δ). Systematic bias was assessed using a paired t-test or non-parametric Wilcoxon signed-ranks test. Meaningful differences were evaluated using Cohen's *d* (Lakens, 2013). Effect sizes were categorised as trivial (≤0.19), small (0.20-0.49), medium (0.50-0.79) and large (≥0.8). Relative reliability was assessed using a Pearson's product-moment correlation coefficient or non-parametric Spearman's rank correlation coefficient. Correlations were classified as small (≤0.69), moderate (0.70-0.89) and high (≥0.90) (Vincent and Weir, 1995). Absolute reliability was assessed using each the: coefficient of variation ([SD/mean]*100), typical error of the measurement (TEM; SD of difference between scores/√2) and Bland-Altman (B-A) plots with mean difference (bias) and 95 % Limits of Agreement (LoA; Bland and Altman, 1986). CVs were classified as very good (≤ 10%) and acceptable (≤ 20%). Relationships between biomarkers were compared using a Pearson's product-moment correlation coefficient or non-parametric Spearman's rank correlation

coefficient. Heteroscedasticity was examined from the non-parametric correlational coefficient between absolute differences and individual means presented on B-A plots. Outliers were defined as ± 2.4 SD units (normally distributed) or ± 4.0 SD units (non-normally distributed) outside of the mean and were removed from subsequent analysis (Aguinis et al., 2013).

Power Analysis

Given the novelty of the dependent variables being evaluated and statistical approach to undertake a battery of reliability statistical tests, it was determined infeasible to perform an *a priori* sample size calculation. Instead, general guidance on appropriate sample sizes (*n* = 12) for pilot studies were followed, whilst accounting for a ~20% anticipated participant drop-out rate (Julious, 2005).

RESULTS

Thermoregulatory, Cardiovascular and Perceptual Strain

T_{core} (Figure 2A; time x trial $p = 0.63$), T_{skin} (Figure 2B; time x trial $p = 0.13$) and T_{body} (Figure 2C; time x trial $p = 0.43$) all increased over time to a similar extent between trial one and two. The reliability of peak, mean and Δ in T_{core}, T_{skin} and T_{body} were all good (Table 2). Pre-trial plasma osmolality (trial one: 293 ± 7 mOsmol·kg^{-1}, trial two: 294 ± 7 mOsmol·kg^{-1}; $p = 0.67$), Δ plasma volume (trial 1: -0.61 ± 5.15%, trial 2: -0.02 ± 3.69%; $p = 0.67$), mean sweat rate (trial 1: 1.53 ± 0.38, trial 2: 1.56 ± 0.45 l·h^{-1}; $p = 0.61$) and percentage body mass loss (trial 1: 1.15 ± 0.48; trial 2: 1.21 ± 0.52%; $p = 0.31$) were all similar between trial one and two. HR (Figure 2D; time x trial $p = 0.11$), RPE (Figure 2E; time x trial $p = 0.38$) and TS (Figure 2F; time x trial $p = 0.56$) all increased over time to a similar extent between trial one and two. The reliability of peak, mean and Δ HR, RPE and TS were all good (Table 2).

[Figure 2 – Insert Here]

[Table 2 – Insert Here]

Dual-Sugar Absorption Test

Lactulose and L-rhamnose were both undetectable in all participants' basal sample prior to probe ingestion. Inter-trial DSAT responses displayed no systematic bias between trials at both 90- (Figure 3A) and 150-minutes (Figure 3C). There was moderate relative reliability and acceptable absolute reliability at both the 90- and 150-minute time-points. B-A plots displayed bias for both the 90- (Figure 3B) and 150-minute (Figure 3D) time-points. Individual Lactulose and L-rhamnose concentrations had worse reliability than the combined L/R ratio (Table 3). Heteroscedasticity was not present for any analyses.

Intestinal Fatty Acid Binding Protein

I-FABP displayed no trial order systematic bias at either rest, post- or the Δ time-point (Figure 3E). Following EHSTs, I-FABP was elevated above rest (trial 1: Δ = 0.834 ± 0.445 ng·ml^{-1} [56 ± 31%]; trial 2: Δ = 0.776 ± 0.489 ng·ml^{-1} [46 ± 26%]; $p \leq 0.01$; Figure 3E). At all time-points, I-FABP displayed moderate relative and acceptable absolute reliability (Table 3). B-A plots are presented to illustrate bias for post EHST concentrations (Figure 3F). Heteroscedasticity was not present for any analyses. One participant was excluded as an outlier. Two participants' I-FABP responses displayed unexplainably poor reliability both at rest and post EHSTs. These data were retained given where verbal adherence to pre-trial controls was verbally confirmed. However, removal of these data would have notably improved the reliability of I-FABP both at rest (r = 0.97; CV = 6.1%; TEM = 0.200 ng·ml^{-1}; B-A ± LoA = -0.046 ± 0.308 ng·ml^{-1}) and post the EHST (r = 0.97; CV = 7.2%; TEM = 0.221 ng·ml^{-1}; B-A ± LoA = 0.078 ± 0.467 ng·ml^{-1}).

Claudin-3

CLDN-3 displayed no trial order systematic bias at either rest, post- or the Δ time-point (Figure 3G). Following EHSTs CLDN-3 was elevated above rest (trial 1: Δ = 0.317 ± 0.586 ng·ml^{-1} [11 ± 17%]; trial 2: Δ = 0.371 ± 0.508 ng·ml^{-1} [9 ± 13%]; $p \leq 0.01$; Figure 3G). At all time-points, CLDN-3 displayed high relative and very good absolute reliability (Table 3). B-A plots are

presented to illustrate bias for post EHST concentrations (Figure 3H). Heteroscedasticity was not present for any analyses.

[Table 3 – Insert Here]

[Figure 3 – Insert Here]

Lipopolysaccharide Binding Protein

LBP displayed no trial order systematic bias at either rest, post EHSTs or the Δ time-point (Figure 4A). There was no influence of the EHST on LBP concentration (p = 0.41). At all-time-points, LBP displayed moderate relative and very good absolute reliability (Table 4). B-A plots are presented to illustrate bias for post EHST concentrations (Figure 4B). Heteroscedasticity was not present for any analyses.

Bacterial DNA

Total 16s (Figure 4C) and *Bact.*/16S (Figure 4G) displayed no systematic bias at either rest, post EHSTs or the Δ. *Bacteroides* concentrations (Figure 4E) were systematically lower in trial 2 versus trial 1 (p = 0.04). At rest, total 16s displayed moderate relative and very good absolute reliability, whereas *Bacteroides* displayed poor relative and absolute reliability. The combined *Bact.*/16S ratio subsequently showed poor relative and absolute reliability at rest (Table 4). There was no influence of the EHST on either total 16s (p = 0.39), *Bacteroides* (p = 0.33) or *Bact.*/16S (p = 0.18) responses. B-A plots are presented to illustrate bias for post EHST

concentrations (Figure 4D, 4F and 4H). Heteroscedasticity was not present for any analyses. One participant was excluded from all bactDNA analysis as an outlier.

[Table 4 – Insert Here]

[Figure 4 – Insert Here]

Association between Biomarkers

Validation of the DSAT at the 90 and 150- minutes time-points across both trial one and trial two (n=28), found responses to be systematically greater at 150- (0.034 ± 0.015) compared with 90-minutes (0.027 ± 0.013; p = 0.05, ES 0.50). There was poor relative (r = 0.08) and absolute (CV = 31.8%, TEM = 0.014) reliability between the sample time-points, suggestive of inter-individual variability in sugar probe kinetics. Few statistically significant correlations were reported when comparing GI barrier integrity and MT biomarkers. Small positive correlations were reported between absolute post EHST concentrations for: I-FABP and CLDN-3 (r = 0.41, p = 0.04), LBP and total 16s DNA (r = 0.48, p = 0.02), LBP and *Bacteroides* (r = 0.38; p = 0.05), *Bacteroides* and total 16s DNA (r = 0.40, p = 0.04). When displayed as pre-to-post DELTA, small positive correlations were reported between: LBP and DSAT at 150 minutes (r = 0.54; $p < 0.01$).

DISCUSSION

The aim of this study was to determine the short-term (one-two weeks) temporal reliability of several empirical biomarkers of GI barrier integrity (DSAT, I-FABP, CLDN-3) and MT (LBP, total 16s bacterial DNA, *Bacteroides* DNA) following exertional-heat stress. The main findings of this study were that the serum DSAT, I-FABP, CLDN-3, LBP and total 16s bacterial DNA all displayed moderate-to-strong relative and acceptable absolute reliability between

repeat EHSTs. In comparison, absolute *Bacteroides* DNA and *Bact./*total 16s DNA ratio displayed weak relative and unacceptable absolute reliability between repeat EHSTs.

The serum DSAT is a valid alternative of the traditional urine DSAT (Fleming et al, 1996; van Wijck et al., 2011), which offering improved sensitivity to detect transient losses in GI barrier integrity following exercise (JanssenDuijghuijsen et al., 2016; Pugh et al., 2017a). Despite this, the temporal reliability of the serum DSAT has never been previously assessed. Potential sources of variability with the serum DSAT might relate to both the transient time course of sugar probes in the blood and low absolute lactulose concentrations that challenge the detection limits of common analytical techniques (Fleming et al., 1996; van Wijck et al., 2013). In this study, we show for the first time that the serum DSAT can be utilised with acceptable reliability, which is comparative to that previously reported with the urine DSAT over both a three-day (van Elburg et al. 1995) and two-week period (Marchbank et al., 2011). The optimal time-point for blood collection with the serum DSAT is an unresolved issue that concerns the methodological implementation of this measure. Herein, blood was collected at both 90-minutes post probe ingestion as this provides the most valid estimate of the urine DSAT in basal conditions (Fleming et al., 1996), and at 150-minutes post as this is where peak responses arose following similar exercise stress (van Wijck et al., 2011). Remarkedly, the temporal reliability of both time-points assessed was almost identical, though given large inter-individual variation in probe kinetics, the magnitude of responses at the two time-points had poor validity. Together, these findings advocate the use of the serum DSAT at either 90- or 150- minutes following probe ingestion (where logistically most convenient) as a reliable alternative to the urine DSAT. There appears little requirement to correct for basal sugar probe concentrations (pre-probe ingestion) following a $\geq$ 10 hour overnight fast given that all participants samples were returned negative.

I-FABP is the principal biomarker of GI epithelial injury (Wells et al., 2017). Despite growing popularity, the temporal reliability of circulating I-FABP has never been previously assessed. In the present study, resting I-FABP concentrations were consistently at the upper end of the general healthy reference range for studies utilising an human ELISA kit (0.1-2.0 $ng \cdot ml^{-1}$; Treskes et al., 2017). These concentrations must be considered when evaluating the absolute reliability thresholds reported herein. The rationale for large between-study

discrepancies in absolute I-FABP concentrations are poorly understood, though are more likely attributable to analytical discrepancies (e.g. ELISA antibody, ELISA wash procedure, sample storage), than participant demographic (Treskes et al., 2017). The reliability of I-FABP at rest displayed moderate relative and acceptable absolute reliability. Following both EHSTs, I-FABP increased by approximately 50% or 0.800 ng·ml^{-1}. This response is comparable to numerous similar duration/intensity exercise protocols, such as: 45-to-60 minutes of ~70% watt$_{max}$ normothermic cycling (van Wijck et al., 2011, [61%, Δ 0.306 ng·ml^{-1}] 2012, [61%; Δ 0.179 ng·ml^{-1}] and 20-30 minutes of ~80% VO_{2max} running (Barberio et al., 2015 [46%, Δ 0.297 ng·ml^{-1}]; March et al., 2017 [72%; Δ 0.350 ng·ml^{-1}]). In comparison, far greater elevations in I-FABP have been shown following 90-120 minutes of moderate-intensity running performed in the heat (30°C; Morrison et al., 2014 [663%; Δ 0.203-0.806 ng·ml^{-1}]; Snipe et al., 2017 [288%, Δ 0.897 ng·ml^{-1}]; 2018 [432%, Δ 1.230 ng·ml^{-1}]. Given the high sensitivity of I-FABP to even minor GI injury, it is vital that known extraneous variables (e.g. prandial/hydration status, prior exercise) are tightly controlled prior to investigation. Whilst participants in the present study provided written conformity to all pre-trial controls, two participants' resting I-FABP concentrations appeared suspect to prior GI injury in one trial, which interestingly was unable to be detected by any other analyte.

CLDN-3 is the principle biomarker of GI TJ integrity (Wells et al., 2017). Despite introduction as a TJ biomarker almost a decade ago, the biological relevance of elevated circulating CLDN-3 is still poorly understood. This includes the assessment of temporal reliability, which is currently unknown. In the present study, resting CLDN-3 concentrations were consistent with previous evidence (0.5-15 ng·ml^{-1}) in healthy populations (Yeh et al., 2013; Typpo et al., 2015). At rest, large inter-individual variation in CLDN-3 concentration was evident, meaning that relative reliability was almost uniform. Following both EHSTs, plasma CLDN-3 consistently increased by approximately 8-10%. This finding compares well to the only previous exercise study, where concentrations increased directly following a one hour moderate-intensity (70% VO_{2max}) run in both temperate (22°C; 6.7 > 7.6 ng·ml^{-1}) and hot (33°C; 6.6 > 8.2 ng·ml^{-1}) ambient environments (Yeh et al., 2013). The clinical relevance of this small, transient increase in CLDN-3 following exercise is poorly understood, though is modest in comparison with the magnitude of increase (4-20 fold) shown acutely following major non-abdominal surgery (Typpo et al., 2015; Habes et al., 2017). Promisingly, of all the GI barrier

integrity biomarkers compared, CLDN-3 displayed the strongest relative and absolute reliability.

LBP is a type-1 acute phase protein that responds to a wide-variety of microbial-associated molecular patterns and is widely considered a stable indirect biomarker of bacterial endotoxin exposure (Dullah and Ongkudon, 2017). In the present study, resting LBP concentrations displayed showed moderate relative and good absolute reliability. These results are in support of one previous study, which found short-term (≤ 7 day) basal LBP responses to display moderate relative reliability (intraclass correlation coefficient = 0.61; Citronberg et al., 2016). In comparison, direct assessment of endotoxin appears to have weak basal temporal reliability, with an intra-individual CV of 22% reported over a similar 7-day period in basal conditions (Guy et al., 2017). Following the EHST, LBP was unchanged in both trials, with concentrations offering comparable levels of reliability compared to rest. Whilst the evidence is sparse regarding LBP responses to exercise, previous evidence has shown a minor elevation in LBP of 10-15% immediately following a fatiguing treadmill walk (4.5 $km \cdot h^{-1}$) in the heat (40°C; 106 minutes; Selkirk et al., 2008), and 1 hour of moderate intensity (70% VO_{2max}) treadmill running (Jonvik et al., 2019). A potential explanation for these discrepant findings likely relate to greater thermoregulatory/cardiovascular strain in previous studies.

BactDNA is an emerging GI MT biomarker, given the recent characterisation of the blood microbiome and improvements in 16S PCR sensitivity (Paisse et al., 2016). In the present study, resting total 16S DNA concentrations displayed moderate relative and good absolute reliability. This finding is promising, given previous concerns that plasma bactDNA concentrations are susceptible to background sample contamination (Glassing et al., 2016). Quantification of total plasma 16S bacterial DNA in exercise settings has never been previously examined, though consistent with other MT biomarkers, the present results show total 16S bactDNA to be stable following moderate intensity exertional-heat stress. One criticism of total 16S bactDNA assessment, particularly in exercise settings, is a lack of GI specificity, with total concentrations influenced by factors including: DNase concentration (Velders et al., 2014) and 16S DNA contamination from other body/blood compartments (Paisse et al., 2016). To account for this error, one hypothetically improved method involves targeting a highly abundant GI genus such as *Bacteroides* (~30% of GI microbiota) and

correcting for total 16S concentration (March et al., 2019). This method is particularly favourable given that the phyla *Firmicutes* and *Bacteroidetes* comprise >90% of the GI microbiome (*Bacteroides*; Huttenhower et al., 2012) and <5% of the plasma microbiome (Paisse et al., 2016). Utilising this hypothesis, March *et al* (2019) reported that the plasma *Bacteroides*/16S DNA ratio tended to increase (~25%; p = 0.07) following a one-hour moderate intensity (70% VO_{2max}) run in the heat (30°C), though large inter-individual variability in responses were evident. In the present study, the *Bacteroides*/16S DNA ratio was unchanged following the EHST and appeared to be systematically lower post the EHST in trial two (but not the Δ). This systematic bias was unexpected given the uniformity of all other analytes assessed and the poor analytical reliability of this biomarker (e.g. mean duplicate CV = 17.5%). It is presently unclear whether the poor reliability of this measure has obscured a true effect of the EHST and/or the meaningfulness of this variability during more severe MT.

Evidence directly comparing correlations between GI barrier integrity and/or MT biomarkers in exercise settings has been limited to date. Given general logistical constraints of the urine/plasma DSAT, the majority of relevant evidence has attempted to validate (correlate) this method against more practical GI barrier integrity biomarkers. These studies have generally shown significant, though weak correlations (r = 0.4-0.6) between basal corrected (Δ) DSAT (urine 5 hour) and I-FABP responses directly following minor exercise-induced GI barrier integrity loss (van Wijck et al., 2011, 2012; March et al., 2017). In the present study, the DSAT did not correlate with any other GI integrity biomarkers. A potential explanation for this null finding might result from the lack of basal DSAT correction or the low overall severity of GI barrier integrity loss. In comparison, a small positive correlation was reported between I-FABP and CLDN-3. This finding is supportive of previous evidence showing urinary I-FABP and CLDN-3 to weakly correlate (r = 0.38) in patients with major non-abdominal surgery (Habes et al., 2017). The expression of CLDN-3 across multiple tissues might partially explain why this correlation was not stronger (Thuijls et al., 2010a). In general, no GI barrier integrity and MT biomarkers, except DSAT 150 and Δ LBP, were found to correlate. Previous exercise gastroenterology research has shown various combinations of these biomarkers to weakly correlate (r = 0.1-0.6; Yeh et al., 2013; Sessions et al., 2016; March et al., 2019) or not correlate (Karhu et al., 2017; Snipe et al., 2018). Several physiological (e.g. hepatic/immune microbial clearance, transcellular microbial translocation, GI microbial density) and analytical

(e.g. exogenous sample contamination, inconsistent biomarker kinetics, DSAT/I-FABP limited to small GI integrity) factors all likely weaken this association (Wells et al, 2017).

LIMITATIONS

Despite implementation of a tightly controlled methodological design, which accounted for the majority of extraneous variables, the presented results were not without some limitations. First, the EHST was only able to evoke moderate GI barrier integrity loss and did not influence MT, potentially limiting the application of these findings in severe situations of GI barrier integrity loss. A previous systematic review has suggested an exercise induced hyperthermia threshold of 38.6°C T_{core} for GI barrier integrity loss (DSAT, I-FABP and endotoxin) to be commonplace (>50% incidence) and of 39.0°C for GI barrier integrity loss to be universal (100% incidence; Pires et al., 2017). Consistently, previous research supports the notion that MT biomarkers (endotoxin) are less responsive to subtle alterations in GI barrier integrity that were otherwise detected by the DSAT or I-FABP following exercise (Snipe et al., 2017, 2018; March et al., 2019). Positively, no GI barrier integrity or MT biomarker displayed statistical heteroscedasticity in this EHST model, suggestive that absolute reliability was not dependent upon the magnitude of biomarker response. Next, biomarker analysis was limited to a single time-point after the EHST (at termination), though this can be justified in that peak responses have been consistently shown to occur at this instance in comparable exertional-heat stress interventions (e.g. *I-FABP*, Snipe et al., 2017; *CLDN-3*, Yeh et al., 2013; *LBP*, Moncada-Jimenez et al., 2010; *Bact./16S*, March et al., 2019). Third, there was statistically significant systematic bias for peak T_{core} and T_{body} responses, which were lower (0.17°C and 0.18°C) following implementation of trial two. This result was not anticipated, given numerous previous studies showing a one week washout period to be sufficient in preventing carry-over (heat acclimation) effects following exertional-heat stress exposure of comparable severity (Barrett and Maughan, 1993; Willmott et al., 2015). The meaningfulness of this systematic bias did not appear to statistically influence any GI barrier integrity of MT biomarker. Finally, given neither a basal DSAT or urinary DSAT were performed, it was not possible to directly determine either than impact of the EHST on DSAT results or make comparisons between DSAT responses between biofluids. This decision was made to minimise participant time burden.

CONCLUSION

This is the first study to comprehensively assess the reliability of GI barrier integrity and/or microbial translocation biomarkers both at rest and following exertional (-heat) stress. Quantifying biomarker reliability is a vital step required to inform marker selection for application in laboratory and field settings. Each of the GI barrier integrity biomarkers assessed displayed moderate-to-good relative and acceptable absolute reliability both at rest and post the EHST. Serum DSAT responses had comparable reliability at two-separate time-points following sugar-probe ingestion (90- and 150-minutes), though response kinetics displayed inconsistent time courses. I-FABP and CLDN-3 both increased following the EHST and their responses were found to weakly correlate. None of the selected MT biomarkers were elevated following the EHST, suggestive that a greater severity of GI barrier integrity loss is required for MT. LBP and total 16S DNA bothl demonstrated moderate-to-good relative and acceptable absolute reliability at both time-points. There was a weak correlation between LBP and total 16S post-EHST responses. Despite offering superior methodological rationale, *Bacteroides* DNA had unacceptable reliability. The findings of the present study have direct relevance for evaluating the efficacy of interventions to attenuate the rise in GI barrier integrity/MT when exercising in the heat. Such interventions might include exercise training, heat acclimatisation and nutritional supplementation. The findings of this study might also have value to the pharmaceutical industry, to quantify the efficacy of drugs to maintain GI barrier integrity, or to evaluate improvements in drugs that traditionally resulted in GI integrity loss.

COMPETING INTERESTS

No competing interests

AUTHOR CONTRIBUTION

HO, JF, RC, SD, CW and JL concepted and designed the research; HO, AM, CW performed the experiments; HO, GD, SF and RE acquired data; HO, JF, RC, GD, SD and JL interpreted the results; HO wrote the manuscript; HO, JF, RC, GD, SF, RE, AM, CW, AM and JL edited, revised and agree to accountability of the accuracy and integrity of the manuscript. Data were collected at School of Sport, Health and Wellbeing, Plymouth MARJON University. All persons designated as authors qualify for authorship, and all those who quality for authorship are listed.

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629 **REFERENCES**

630 Aguinis, H., Gottfredson, R.K. and Joo, H. (2013). Best-practice recommendations for defining,
631 identifying, and handling outliers. *Organizational Research Methods*, 16(2), pp.270-301.

632 Armed Forces Surveillance Branch. (2018). Update: Heat illness, active component, US Armed
633 Forces, 2017. *Medical Surveillance Monthly Report*, 25(4), pp.6.

634 Atkinson, G. and Nevill, A.M. (1998). Statistical methods for assessing measurement error
635 (reliability) in variables relevant to sports medicine. *Sports Medicine*, 26(4), pp.217-238.

636 Barberio, M.D., Elmer, D.J., Laird, R.H., Lee, K.A., Gladden, B. and Pascoe, D.D. (2015).
637 Systemic LPS and inflammatory response during consecutive days of exercise in
638 heat. *International Journal of Sports Medicine*, 36(03), pp.262-270.

639 Barnett, A. and Maughan, R.J. (1993). Response of unacclimatized males to repeated weekly
640 bouts of exercise in the heat. *British Journal of Sports Medicine*, 27(1), pp.39-44.

641 Bischoff, S.C., Barbara, G., Buurman, W., Ockhuizen, T., Schulzke, J.D., Serino, M., Tilg, H.,
642 Watson, A. and Wells, J.M. (2014). Intestinal permeability–a new target for disease
643 prevention and therapy. *BMC Gastroenterology*, 14(1), p.189.

644 Bland, J.M. and Altman, D. (1986). Statistical methods for assessing agreement between two
645 methods of clinical measurement. *The Lancet*, 327(8476), pp.307-310.

646 Borg, G. (1970). Perceived exertion as an indicator of somatic stress. *Scandinavian Journal of*
647 *Rehabilitation Medicine*, 2, pp.92-98.

648 Camilleri, M., Madsen, K., Spiller, R., Van Meerveld, B.G. and Verne, G.N. (2012). Intestinal
649 barrier function in health and gastrointestinal disease. *Neurogastroenterology &*
650 *Motility*, *24*(6), pp.503-512.

651 Cani, P.D. (2018). Human gut microbiome: hopes, threats and promises. *Gut*, *67*(9), pp.1716-
652 1725.

Christensen, M.J., Eller, E., Kjaer, H.F., Broesby-Olsen, S., Mortz, C.G. and Bindslev-Jensen, C. (2019). Exercise-induced anaphylaxis: causes, consequences, and management recommendations. *Expert Review of Clinical Immunology*, 15(3), pp.265-273.

Citronberg, J.S., Wilkens, L.R., Lim, U., Hullar, M.A., White, E., Newcomb, P.A., Le Marchand, L. and Lampe, J.W. (2016). Reliability of plasma lipopolysaccharide-binding protein (LBP) from repeated measures in healthy adults. *Cancer Causes and Control*, 27(9), pp.1163-1166.

Costa, R.J.S., Snipe, R.M.J., Kitic, C.M. and Gibson, P.R. (2017). Systematic review: exercise-induced gastrointestinal syndrome—implications for health and intestinal disease. *Alimentary Pharmacology and Therapeutics*, 46(3), pp.246-265.

Dill, D.B. and Costill, D.L. (1974). Calculation of percentage changes in volumes of blood, plasma, and red cells in dehydration. *Journal of Applied Physiology*, 37(2), pp.247-248.

Dullah, E.C. and Ongkudon, C.M. (2017). Current trends in endotoxin detection and analysis of endotoxin–protein interactions. *Critical Reviews in Biotechnology*, 37(2), pp.251-261.

Durnin, J.V. and Womersley, J.V.G.A. (1974). Body fat assessed from total body density and its estimation from skinfold thickness: measurements on 481 men and women aged from 16 to 72 years. *British Journal of Nutrition*, 32(1), pp.77-97.

Edinburgh, R.M., Hengist, A., Smith, H.A., Travers, R.L., Koumanov, F., Betts, J.A., Thompson, D., Walhin, J.P., Wallis, G.A., Hamilton, D.L. and Stevenson, E.J. (2018). Preexercise breakfast ingestion versus extended overnight fasting increases postprandial glucose flux after exercise in healthy men. *American Journal of Physiology-Endocrinology and Metabolism*, 315(5), pp.1062-1074.

Fleming, S.C., Duncan, A., Russell, R.I. and Laker, M.F. (1996). Measurement of sugar probes in serum: an alternative to urine measurement in intestinal permeability testing. *Clinical Chemistry*, 42(3), pp.445-448.

677 Glassing, A., Dowd, S.E., Galandiuk, S., Davis, B. and Chiodini, R.J. (2016). Inherent bacterial
678 DNA contamination of extraction and sequencing reagents may affect interpretation of
679 microbiota in low bacterial biomass samples. *Gut Pathogens*, 8, pp.24-24.

680 Guy, J.H., Edwards, A.M., Miller, C.M., Deakin, G.B. and Pyne, D.B. (2017). Short-term
681 reliability of inflammatory mediators and response to exercise in the heat. *Journal of Sports*
682 *Sciences*, *35*(16), pp.1622-1628.

683 Habes, Q.L., Linssen, V., Nooijen, S., Kiers, D., Gerretsen, J., Pickkers, P., Scheffer, G.J. and
684 Kox, M. (2017). Markers of intestinal damage and their relation to cytokine levels in cardiac
685 surgery patients. *Shock*, 47(6), pp.709-714.

686 Huttenhower, C., Gevers, D., Knight, R., Abubucker, S., Badger, J.H., Chinwalla, A.T., Creasy,
687 H.H., Earl, A.M., FitzGerald, M.G., Fulton, R.S. and Giglio, M.G. (2012). Structure, function
688 and diversity of the healthy human microbiome. *Nature*, 486(7402), p.207.

689 JanssenDuijghuijsen, L.M., Mensink, M., Lenaerts, K., Fiedorowicz, E., Protégé Study Group,
690 van Dartel, D.A., Mes, J.J., Luiking, Y.C., Keijer, J., Wichers, H.J. and Witkamp, R.F. (2016).
691 The effect of endurance exercise on intestinal integrity in well-trained healthy
692 men. *Physiological Reports*, 4(20), p.e12994.

693 Jay, O. and Kenny, G.P. (2007). The determination of changes in body heat content during
694 exercise using calorimetry and thermometry. *Journal of the Human-Environment*
695 *System*, 10(1), pp.19-29.

696 Julious, S. A. (2005). Sample size of 12 per group rule of thumb for a pilot study.
697 *Pharmaceutical Statistics*, 4 (4), pp. 287–291.

698 Jonvik, K.L., Lenaerts, K., Smeets, J.S., Kolkman, J.J., Van Loon, L.J. and Verdijk, L.B. (2019).
699 Sucrose but Not Nitrate Ingestion Reduces Strenuous Cycling-induced Intestinal
700 Injury. *Medicine and Science in Sports and Exercise*, 51, pp.436-444.

Karhu, E., Forsgård, R.A., Alanko, L., Alfthan, H., Pussinen, P., Hämäläinen, E. and Korpela, R. (2017). Exercise and gastrointestinal symptoms: running-induced changes in intestinal permeability and markers of gastrointestinal function in asymptomatic and symptomatic runners. *European Journal of Applied Physiology*, *117*(12), pp.2519-2526.

Lim, C. (2018). Heat sepsis precedes heat toxicity in the pathophysiology of heat stroke—a new paradigm on an ancient disease. *Antioxidants*, 7(11), p.149.

March, D.S., Jones, A.W., Thatcher, R. and Davison, G. (2019). The effect of bovine colostrum supplementation on intestinal injury and circulating intestinal bacterial DNA following exercise in the heat. *European Journal of Nutrition*, 58(4), pp.1441-1451.

March, D.S., Marchbank, T., Playford, R.J., Jones, A.W., Thatcher, R. and Davison, G. (2017). Intestinal fatty acid-binding protein and gut permeability responses to exercise. *European Journal of Applied Physiology*, 117(5), pp.931-941.

Marchbank, T., Davison, G., Oakes, J.R., Ghatei, M.A., Patterson, M., Moyer, M.P. and Playford, R.J. (2010). The nutriceutical bovine colostrum truncates the increase in gut permeability caused by heavy exercise in athletes. *American Journal of Physiology-Gastrointestinal and Liver Physiology*, 300(3), pp.477-G484.

Marfell-Jones, M., Olds, T., Stewart, A. and Carter, L. (2006). *ISAK manual: international standards for anthropometric assessment*. South Africa: Potchefstroom.

Met Office. (2019). Cambourne. [Online]. Available at: https://www.metoffice.gov.uk/pub/data/weather/uk/climate/stationdata/cambornedata.txt [Accessed 14/10/2019]

Military Headquarters of the Surgeon General. (2017). [Online]. Available at: https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/793094/JSP_539_Part_2_V3.1__Updated_04-19_.pdf [Accessed 14/10/2019]

725 Moncada-Jiménez, J., Plaisance, E.P., Araya-Ramírez, F., Taylor, J.K., Ratcliff, L., Mestek, M.L.,
726 Grandjean, P.W. and AragonVargas, L.F. (2010). Acute hepatic response to diet modification
727 and exercise-induced endotoxemia during a laboratory-based duathlon. *Biology of*
728 *Sport*, 27(2), pp. 111-118.

729 Morrison, S.A., Cheung, S.S. and Cotter, J.D. (2014). Bovine colostrum, training status, and
730 gastrointestinal permeability during exercise in the heat: a placebo-controlled double-blind
731 study. *Applied Physiology, Nutrition, and Metabolism*, 39(9), pp.1070-1082.

732 Mortensen, C., Karlsen, S., Grønbæk, H., Nielsen, D.T., Frevert, S., Clemmesen, J.O., Møller,
733 S., Jensen, J.S. and Bendtsen, F. (2013). No difference in portal and hepatic venous bacterial
734 DNA in patients with cirrhosis undergoing transjugular intrahepatic portosystemic shunt
735 insertion. *Liver International*, 33(9), pp.1309-1315.

736 Mulak, A. and Bonaz, B. (2015). Brain-gut-microbiota axis in Parkinson's disease. *World*
737 *Journal of Gastroenterology*, *21*(37), p.10609.

738 Neves, A.L., Coelho, J., Couto, L., Leite-Moreira, A. and Roncon-Albuquerque, J.R., 2013.
739 Metabolic endotoxemia: a molecular link between obesity and cardiovascular risk. *Journal of*
740 *molecular endocrinology*, *51*(2), pp.R51-64.

741 Païssé, S., Valle, C., Servant, F., Courtney, M., Burcelin, R., Amar, J. and Lelouvier, B. (2016).
742 Comprehensive description of blood microbiome from healthy donors assessed by 16 S
743 targeted metagenomic sequencing. *Transfusion*, 56(5), pp.1138-1147.

744 Pires, W., Veneroso, C.E., Wanner, S.P., Pacheco, D.A., Vaz, G.C., Amorim, F.T., Tonoli, C.,
745 Soares, D.D. and Coimbra, C.C. (2017). Association between exercise-induced hyperthermia
746 and intestinal permeability: a systematic review. *Sports Medicine*, 47(7), pp.1389-1403.

747 Pugh, J.N., Impey, S.G., Doran, D.A., Fleming, S.C., Morton, J.P. and Close, G.L. (2017a).
748 Acute high-intensity interval running increases markers of gastrointestinal damage and
749 permeability but not gastrointestinal symptoms. *Applied Physiology, Nutrition, and*
750 *Metabolism*, 42(9), pp.941-947.

751 Ramanathan, N.L. (1964). A new weighting system for mean surface temperature of the
752 human body. *Journal of Applied Physiology*, 19(3), pp.531-533.

753 Schellekens, D.H., Grootjans, J., Dello, S.A., van Bijnen, A.A., van Dam, R.M., Dejong, C.H.,
754 Derikx, J.P. and Buurman, W.A. (2014). Plasma intestinal fatty acid–binding protein levels
755 correlate with morphologic epithelial intestinal damage in a human translational ischemia-
756 reperfusion model. *Journal of Clinical Gastroenterology*, 48(3), pp.253-260.

757 Schumann, R.R. (2011). Old and new findings on lipopolysaccharide-binding protein: a
758 soluble pattern-recognition molecule. *Biochemical Society Transactions*, *39*(4), pp.989-993.

759 Selkirk, G.A., McLellan, T.M., Wright, H.E. and Rhind, S.G. (2008). Mild endotoxemia, NF-κB
760 translocation, and cytokine increase during exertional heat stress in trained and untrained
761 individuals. *American Journal of Physiology-Regulatory, Integrative and Comparative*
762 *Physiology*, 295(2), pp.611-623.

763 Sessions, J., Bourbeau, K., Rosinski, M., Szczygiel, T., Nelson, R., Sharma, N. and Zuhl, M.
764 (2016). Carbohydrate gel ingestion during running in the heat on markers of gastrointestinal
765 distress. *European Journal of Sport Science*, *16*(8), 1064-1072.

766 Shirreffs, S.M. and Maughan, R.J. (1994). The effect of posture change on blood volume,
767 serum potassium and whole body electrical impedance. *European Journal of Applied*
768 *Physiology and Occupational Physiology*, 69(5), pp.461-463.

769 Snipe, R.M., Khoo, A., Kitic, C.M., Gibson, P.R. and Costa, R.J. (2017). Carbohydrate and
770 protein intake during exertional heat stress ameliorates intestinal epithelial injury and small
771 intestine permeability. *Applied Physiology, Nutrition, and Metabolism*, 42(12), pp.1283-
772 1292.

773 Snipe, R.M., Khoo, A., Kitic, C.M., Gibson, P.R. and Costa, R.J. (2018). The impact of
774 exertional-heat stress on gastrointestinal integrity, gastrointestinal symptoms, systemic
775 endotoxin and cytokine profile. *European Journal of Applied Physiology*, 118(2), pp.389-400.

776 Thuijls, G., Derikx, J.P., de Haan, J.J., Grootjans, J., de Bruïne, A., Masclee, A.A., Heineman, E.
777 and Buurman, W.A. (2010a). Urine-based detection of intestinal tight junction loss. *Journal*
778 *of Clinical Gastroenterology*, 44(1), pp.e14-e19.

779 Thuijls, G., Derikx, J.P., van Wijck, K., Zimmermann, L.J., Degraeuwe, P.L., Mulder, T.L., Van
780 der Zee, D.C., Brouwers, H.A., Verhoeven, B.H., van Heurn, L.E. and Kramer, B.W. (2010b).
781 Non-invasive markers for early diagnosis and determination of the severity of necrotizing
782 enterocolitis. *Annals of Surgery*, 251(6), pp.1174-1180.

783 Toner, M.M., Drolet, L.L. and Pandolf, K.B. (1986). Perceptual and physiological responses
784 during exercise in cool and cold water. *Perceptual and Motor Skills*, 62(1), pp.211-220.

785 Treskes, N., Persoon, A.M. and van Zanten, A.R. (2017). Diagnostic accuracy of novel
786 serological biomarkers to detect acute mesenteric ischemia: a systematic review and meta-
787 analysis. *Internal and Emergency Medicine*, 12(6), pp.821-836.

788 Typpo, K.V., Larmonier, C.B., Deschenes, J., Redford, D.T., Kiela, P.R. and Ghishan, F.K.
789 (2015). Clinical characteristics associated with postoperative intestinal epithelial barrier
790 dysfunction in children with congenital heart disease. *Pediatric Critical Care Medicine*, 16(1),
791 pp.37-44.

792 Van Elburg, R.M., Uil, J.J., Kokke, F.T.M., Mulder, A.M., Van De Broek, W.G.M., Mulder, C.J.J.
793 and Heymans, H.S.A. (1995). Repeatability of the sugar-absorption test, using lactulose and
794 mannitol, for measuring intestinal permeability for sugars. *Journal of Pediatric*
795 *Gastroenterology and Nutrition*, 20(2), pp.184-188.

796 Van Wijck, K., Lenaerts, K., Van Bijnen, A.A., Boonen, B., Van Loon, L.J., Dejong, C.H. and
797 Buurman, W.A. (2012). Aggravation of exercise-induced intestinal injury by Ibuprofen in
798 athletes. *Medicine and Science in Sports and Exercise*, 44(12), pp.2257-2262.

799 Van Wijck, K., Lenaerts, K., Van Loon, L.J., Peters, W.H., Buurman, W.A. and Dejong, C.H.,
800 (2011). Exercise-induced splanchnic hypoperfusion results in gut dysfunction in healthy
801 men. *PloS One*, 6(7), pp.e22366.

802 Van Wijck, K., Verlinden, T.J., van Eijk, H.M., Dekker, J., Buurman, W.A., Dejong, C.H. and
803 Lenaerts, K. (2013). Novel multi-sugar assay for site-specific gastrointestinal permeability
804 analysis: a randomized controlled crossover trial. *Clinical Nutrition*, 32(2), pp.245-251.

805 Velders, M., Treff, G., Machus, K., Bosnyák, E., Steinacker, J. and Schumann, U. (2014).
806 Exercise is a potent stimulus for enhancing circulating DNase activity. *Clinical*
807 *Biochemistry*, 47(6), pp.471-474.

808 Vincent, W.J. and Weir, J.P. (1995). *Quantifying reliability*. In Statistics in Kinesiology (5th
809 Ed.). Champaign, IL, USA: Human Kinetics , pp.214-228.

810 Waterhouse, J., Drust, B., Weinert, D., Edwards, B., Gregson, W., Atkinson, G., Kao, S.,
811 Aizawa, S. and Reilly, T. (2005). The circadian rhythm of core temperature: origin and some
812 implications for exercise performance. *Chronobiology International*, 22(2), pp.207-225.

813 Wells, J.M., Brummer, R.J., Derrien, M., MacDonald, T.T., Troost, F., Cani, P.D., Theodorou,
814 V., Dekker, J., Méheust, A., De Vos, W.M. and Mercenier, A. (2017). Homeostasis of the gut
815 barrier and potential biomarkers. *American Journal of Physiology-Gastrointestinal and Liver*
816 *Physiology*, 312(3), pp.171-193.

817 Yeh, Y.J., Law, L.Y.L. and Lim, C.L., 2013. Gastrointestinal response and endotoxemia during
818 intense exercise in hot and cool environments. European Journal of Applied
819 Physiology, 113(6), pp.1575-1583.

820 Zeissig, S., Bürgel, N., Günzel, D., Richter, J., Mankertz, J., Wahnschaffe, U., Kroesen, A.J.,
821 Zeitz, M., Fromm, M. and Schulzke, J.D. (2007). Changes in expression and distribution of
822 claudin 2, 5 and 8 lead to discontinuous tight junctions and barrier dysfunction in active
823 Crohn's disease. *Gut*, 56(1), pp.61-72.

824 **Table 1.** Participant demographic characteristics

Measure	**Mean ± SD**
Age (years)	26 ± 5

Height (m)	1.78 ± 0.06
Body Mass (kg)	83.4 ± 12.6
Physical Activity (h·week^{-1})	6 ± 3
Body Fat (%)	16.1 ± 4.0
$\dot{V}O_{2max}$ (ml·kg^{-1}·min^{-1})	49 ± 4

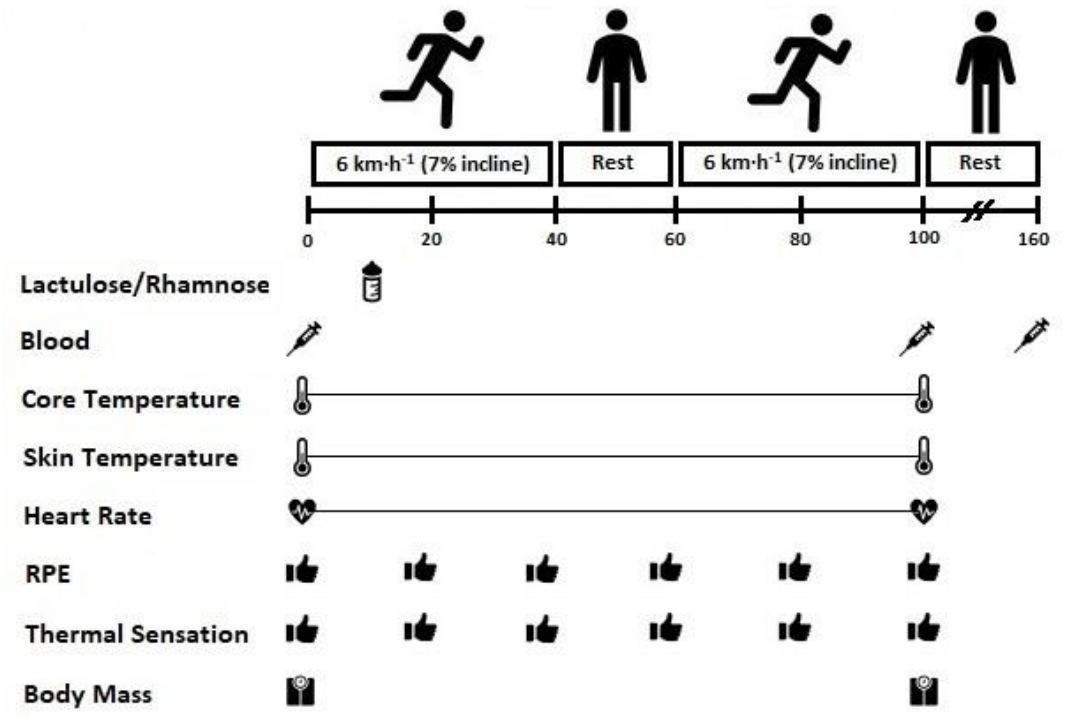
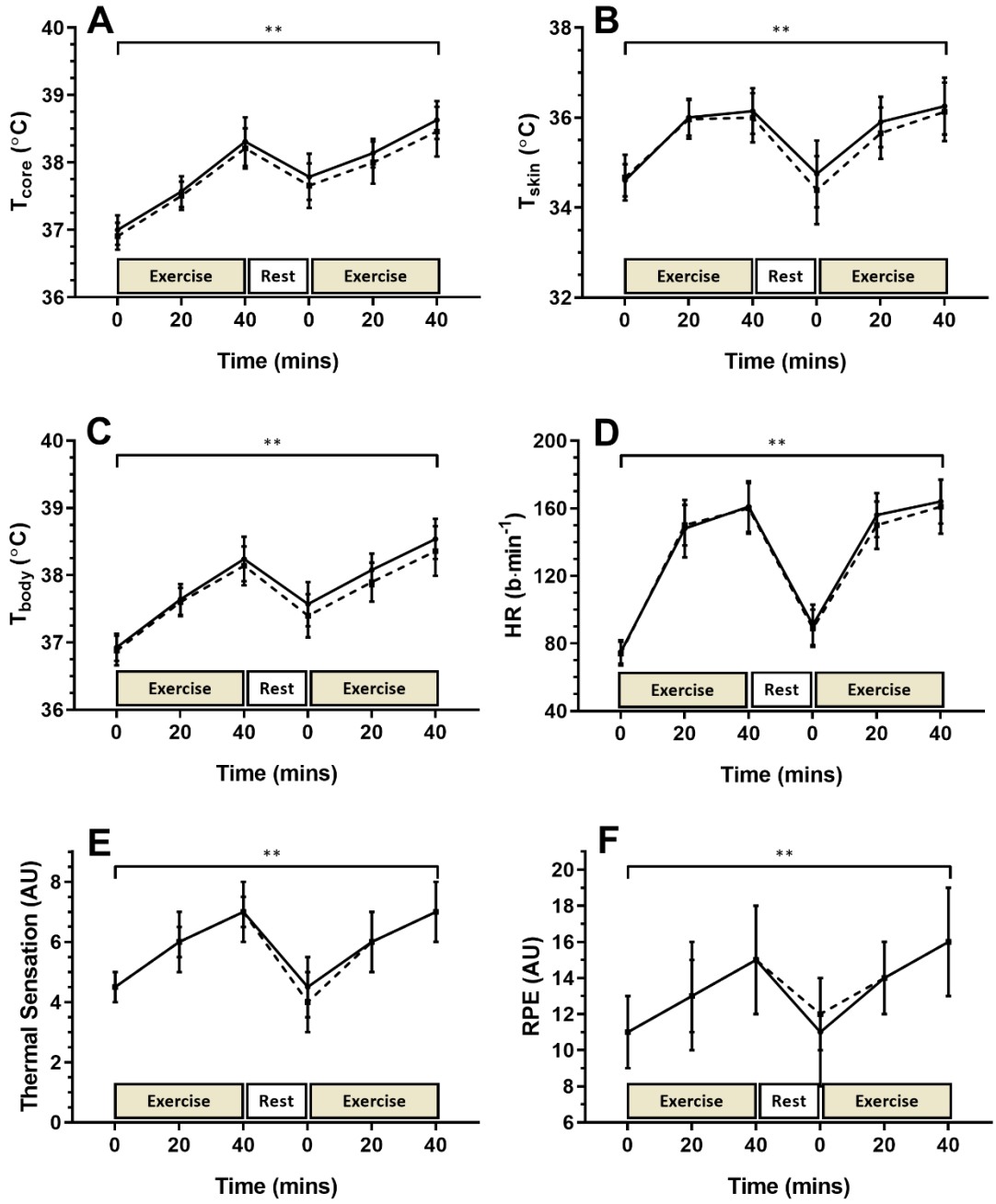


Figure 1. Schematic illustration of the experimental measurement timings



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832 **Figure 2.** Whole-body physiological responses to repeated EHSTs: (A) = core temperature;
833 (B) = mean skin temperature (n=13); (C) = mean body temperature (n=13); (D) = heart rate;
834 (E) = thermal sensation; and (F) = rate of perceived exertion. Solid line = trial 1, broken line =
835 trial 2. Significant overall effect of time (*$p \leq 0.05$; ** $p \leq 0.01$).

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838 **Table 2.** Relative and absolute reliability of whole-body physiological responses

	Trial 1 (SD)	Trial 2 (SD)	*p*	*d*	*r*	CV	TEM	Bias (LoA)
T_{core} (°C) Peak	38.63 ± 0.28	38.46 ± 0.37	0.02	0.49	0.78**	0.5	0.14	0.17 ± 0.46
T_{core} (°C) Mean	37.87 ± 0.19	37.78 ± 0.23	0.09	0.43	0.68**	0.3	0.13	0.09 ± 0.43
T_{core} (°C) Δ	1.62 ± 0.29	1.55 ± 0.44	0.59	0.19	0.48	-	0.26	0.07 ± 0.77
T_{skin} (°C) Peak	36.27 ± 0.63	36.13 ± 0.65	0.23	0.22	0.83**	0.6	0.27	0.13 ± 0.74
T_{skin} (°C) Mean	35.75 ± 0.44	35.64 ± 0.49	0.13	0.24	0.83**	0.4	0.17	0.11 ± 0.46
T_{skin} (°C) Δ	1.65 ± 0.63	1.46 ± 0.65	0.18	0.30	0.71**	-	0.35	0.20 ± 0.97
T_{body} (°C) Peak	38.54 ± 0.30	38.36 ± 0.37	0.01	0.53	0.79**	0.4	0.16	0.18 ± 0.44
T_{body} (°C) Mean	37.83 ± 0.20	37.74 ± 0.23	0.08	0.42	0.70**	0.3	0.12	0.09 ± 0.33
T_{body} (°C) Δ	1.62 ± 0.30	1.49 ± 0.40	0.18	0.37	0.60*	-	0.23	0.13 ± 0.63
HR ($b \cdot min^{-1}$) Peak	164 ± 13	162 ± 14	0.19	0.15	0.93**	2.0	4	2 ± 11
HR ($b \cdot min^{-1}$) Mean	150 ± 14	148 ± 13	0.29	0.14	0.84**	2.8	5	2 ± 15
HR ($b \cdot min^{-1}$) Δ	90 ± 11	90 ± 13	0.99	0.00	0.88**	-	4	0 ± 12
RPE (AU) Peak	16 ± 3	16 ± 3	0.99	0.00	0.92**	4.1	1	0 ± 3
RPE (AU) Mean	13 ± 2	13 ± 2	0.88	0.00	0.80**	5.2	1	0 ± 2
RPE (AU) Δ	6 ± 5	5 ± 3	0.95	0.24	0.72**	-	2	0 ± 4
TS (AU) Peak	7.0 ± 0.5	7.0 ± 0.5	0.99	0.00	0.84**	4.8	0.5	0.0 ± 1.0
TS (AU) Mean	6.0 ± 0.5	5.5 ± 0.5	0.02	0.00	0.88**	3.2	0.0	0.5 ± 0.5
TS (AU) Δ	2.5 ± 1.0	2.5 ± 0.5	0.12	0.00	0.61*	-	0.5	-0.5 ± 1.0

839 * = significant correlation ($p \leq 0.05$); ** = significant correlation ($p \leq 0.01$)

840 **Table 3.** Relative and absolute reliability of all GI barrier integrity biomarkers

	Trial 1 (SD)	Trial 2 (SD)	*p*	*d*	*r*	CV	TEM	Bias (LoA)
Lactulose ($mg \cdot l^{-1}$) 90	1.06 ± 0.38	0.90 ± 0.43	0.33	0.38	0.60*	21.3	0.263	0.151 ± 0.730
$_{L}$-Rhamnose ($mg \cdot l^{-1}$) 90	15.89 ± 3.91	15.85 ± 3.13	0.29	0.29	0.53	12.9	2.601	1.036 ± 6.930
DSAT (L/R) 90	0.028 ± 0.012	0.025 ±0.014	0.17	0.23	0.77**	11.5	0.004	-0.003 ± 0.011
Lactulose ($mg \cdot l^{-1}$) 150	0.97 ± 0.48	0.95 ± 0.52	0.53	0.05	0.71**	13.0	0.132	0.023 ± 0.364
$_{L}$-Rhamnose ($mg \cdot l^{-1}$) 150	12.01 ± 2.95	11.24 ± 2.96	0.09	0.27	0.86**	7.6	1.149	0.772 ± 3.060
DSAT (L/R) 150	0.033 ± 0.015	0.034 ± 0.016	0.37	0.06	0.69**	12.2	0.004	0.001 ± 0.011
I-FABP ($ng \cdot ml^{-1}$) Rest	1.560 ± 0.506	1.691 ± 0.555	0.11	0.25	0.75**	11.1	0.304	-0.180 ± 0.746
I-FABP ($ng \cdot ml^{-1}$) Post	2.394 ± 0.731	2.467 ± 0.875	0.63	0.09	0.80**	12.1	0.376	-0.073 ± 1.040
I-FABP ($ng \cdot ml^{-1}$) Δ	0.834 ± 0.445	0.776 ± 0.489	0.65	0.12	0.65**	-	0.278	0.058 ± 0.772
CLDN-3 ($ng \cdot ml^{-1}$) Rest	6.205 ± 4.382	5.971 ± 4.062	0.17	0.06	0.99**	6.8	0.423	0.233 ± 1.172
CLDN-3 ($ng \cdot ml^{-1}$) Post	6.592 ± 4.770	6.323 ± 4.270	0.34	0.06	0.99**	4.9	0.485	0.181 ± 1.341
CLDN-3 ($ng \cdot ml^{-1}$) Δ	0.317 ± 0.586	0.371 ± 0.508	0.68	0.10	0.62*	-	0.342	-0.055 ± 0.948

841 * = significant correlation ($p \leq 0.05$); ** = significant correlation ($p \leq 0.01$)

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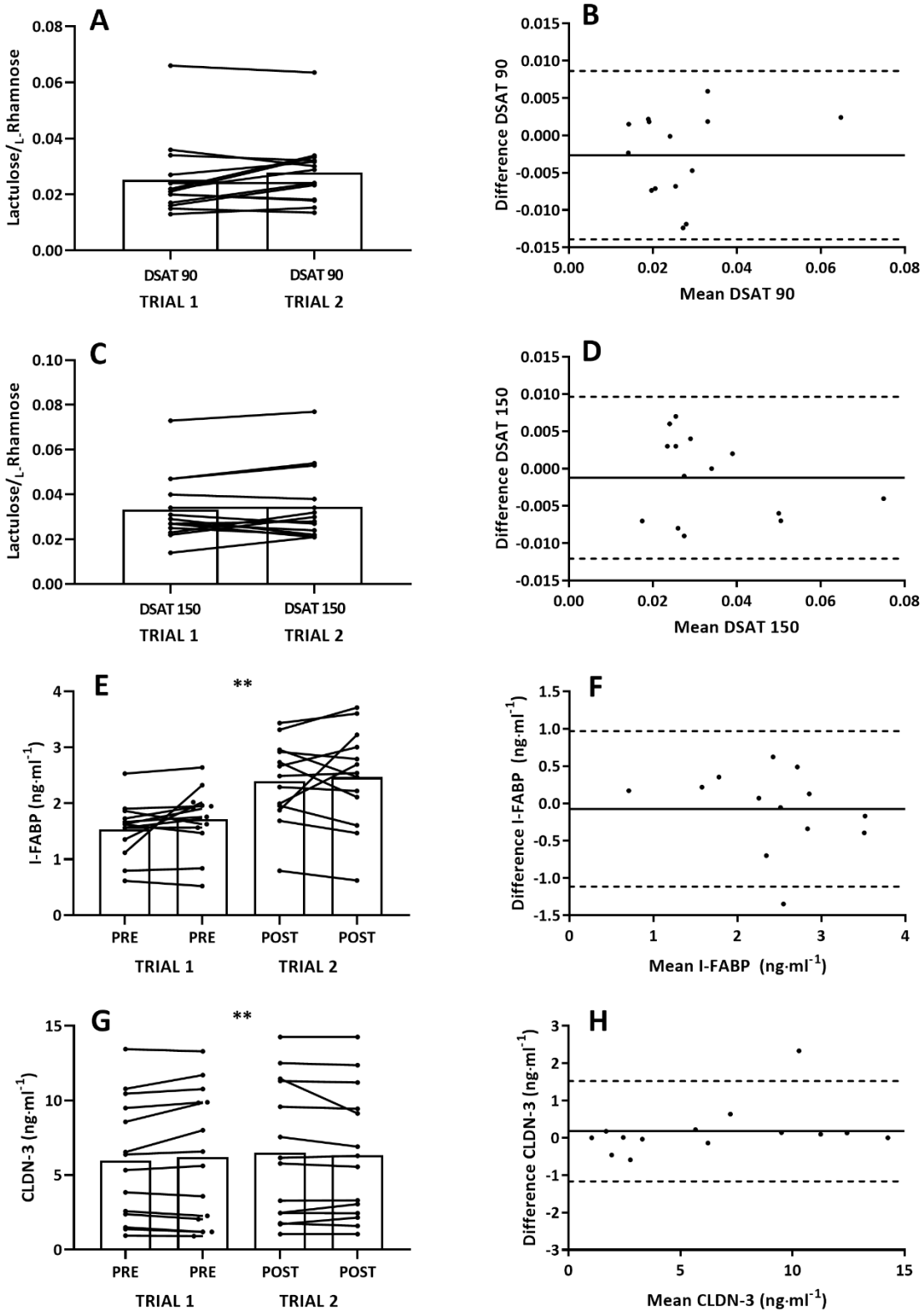

Figure 3. GI barrier integrity responses to EHST trial 1 and trial 2: (A) = L/R ratio at 90 minutes; (C) = L/R ratio at 150 minutes; (E) I-FABP (n=13); and (G) = CLDN-3. Significant overall effect of time (*$p \leq 0.05$; ** $p \leq 0.01$). Bland-Altman mean bias and 95% LoA between post EHST trial 1 and trial 2: (B) = L/R ratio at 90- and (D) 150- minutes; (F) I-FABP (n=13); and (H) = CLDN-3

Table 4. Relative and absolute reliability of all GI barrier integrity biomarkers

	Trial 1 (SD)	**Trial 2 (SD)**	***p***	***d***	***r***	**CV**	**TEM**	**Bias (LoA)**
LBP ($\mu g \cdot ml^{-1}$) Rest	2.625 ± 0.993	2.564 ± 0.871	0.79	0.07	0.85**	10.0	0.378	0.061 ± 1.048
LBP ($\mu g \cdot ml^{-1}$) Post	2.617 ± 1.080	2.650 ± 0.885	0.59	0.03	0.85**	9.2	0.420	-0.033 ± 1.166
LBP ($\mu g \cdot ml^{-1}$) Δ	-0.008 ± 0.250	0.086 ± 0.232	0.38	0.39	-0.16	-	0.260	-0.094 ± 0.721
16S DNA ($pg \cdot \mu l^{-1}$) Rest	1.43 ± 0.60	1.44 ± 0.65	0.79	0.02	0.87**	8.1	0.19	-0.02 ± 0.49
16S DNA ($pg \cdot \mu l^{-1}$) Post	1.47 ± 0.70	1.52 ± 0.73	0.45	0.07	0.82**	9.5	0.15	-0.05 ± 0.40
16S DNA ($pg \cdot \mu l^{-1}$) Δ	0.043 ± 0.28	0.08 ± 0.40	0.72	0.10	0.56*	-	0.24	-0.03 ± 0.65
Bact. DNA (copies·μl^{-1}) Rest	0.142 ± 0.116	0.102 ± 0.089	0.19	0.39	0.14	55.0	0.067	0.040 ± 0.186
Bact. DNA (copies·μl^{-1}) Post	0.202 ± 0.132	0.115 ± 0.106	0.04*	0.73	0.14	56.3	0.104	0.087 ± 0.287
Bact. DNA (copies·μl^{-1}) Δ	0.059 ± 0.149	0.013 ± 0.140	0.22	0.32	0.61*	-	0.091	0.047 ± 0.250
Bact./16S DNA Rest	96.08 ± 59.31	74.10 ± 52.82	0.31	0.39	0.13	60.2	52.49	-0.019 ± 0.348
Bact./16S DNA Post	143.41 ± 90.73	91.83± 124.76	0.07	0.47	0.20	54.7	88.18	0.032 ± 0.347
Bact./16S DNA Δ	47.32 ± 95.74	17.74 ± 138.78	0.35	0.25	0.69*	-	76.88	0.052 ± 0.378

* = significant correlation ($p \leq 0.05$); ** = significant correlation ($p \leq 0.01$)

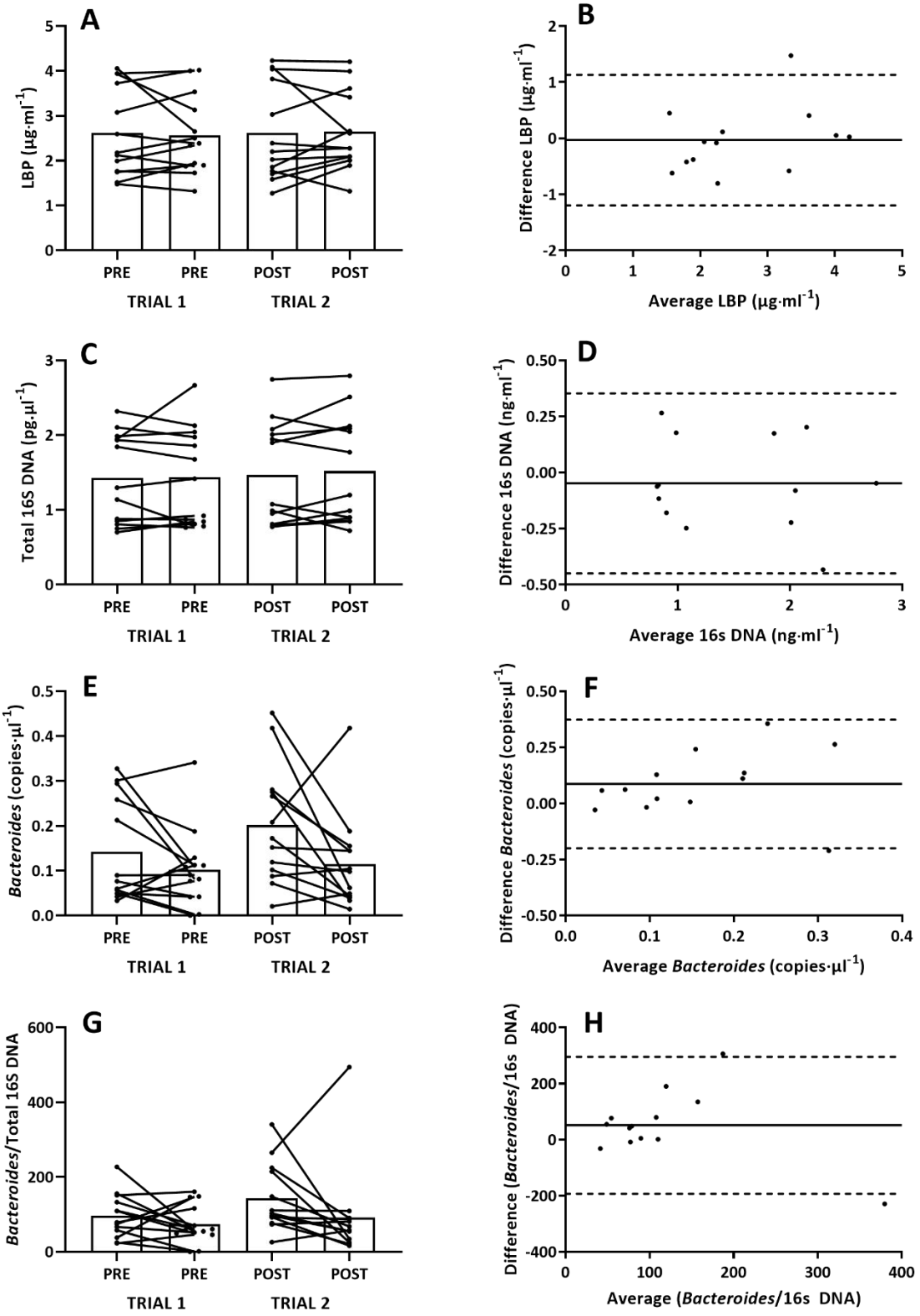

Figure 4. GI MT responses to EHST trial 1 and trial 2: (A) = LBP; (C) total 16S bacterial DNA (n=13); (E) = *Bacteroides* DNA; and (G) = *Bacteroides*/total 16s bacterial DNA (n=13). Significant overall effect of trial (+ $p \leq 0.05$). Bland-Altman mean bias and 95% LoA between post EHST trial 1 and trial 2: (B) = LBP; (D) = total 16S bacterial DNA (n=13); (F) *Bacteroides* DNA (n=13); and (H) = *Bacteroides*/total 16s bacterial DNA (n=13).

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