Anthocyanin-Rich Blackcurrant Extract Preserves Gastrointestinal Barrier Permeability and Reduces Enterocyte Damage but Has No Effect on Microbial Translocation and Inflammation After Exertional Heat Stress

Lee, B. J., Flood, T. R., Hiles, A. M., Walker, E. F., Wheeler, L. E. V., Ashdown, K. M., Willems, M. E. T., Costello, R., Greisler, L. D., Romano, P. A., Hill, G. W. & Kuennen, M. R

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Title: Anthocyanin-rich blackcurrant extract preserves gastrointestinal barrier permeability and reduces enterocyte damage but has no effect on microbial translocation and inflammation after exertional heat stress

Authors: Ben J Lee[1], Tessa R Flood[2], Ania M Hiles[2†], Ella F Walker[2], Lucy EV Wheeler[2], Kimberly M Ashdown[2], Mark ET Willems[2], Rianne Costello[3], Luke D Greisler[4], Phebe A Romano[4], Garrett W Hill[4], Matthew R Kuennen[4]

Institutions:

[1]Centre for Sport, Exercise and Life Sciences, Coventry University, Coventry, UK

[2]Institute of Sport, Nursing and Allied Health, University of Chichester, Chichester, UK.

[3]Centre for Nutrition and Health, Oxford Brookes University, UK

[4]Department of Exercise Science, High Point University, High Point, USA

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Corresponding author:
Ben J Lee Ph. D
Centre for Sport, Exercise and Life Sciences,
Occupational and Environmental Physiology Group,
Coventry University
Priory Street,
Coventry,
CV1 5FB
Email: AC2389@Coventry.ac.uk

Contact Information for Other Authors:

Tessa R Flood	**Email:** T.Flood@chi.ac.uk
Ella F Walker	**Email:** EFWalker@mail.dstl.gov.uk
Lucy EV Wheeler	**Email:** LucyWheeler@hotmail.co.uk
Kimberly M Ashdown	**Email:** K.Ashdown@chi.ac.uk
Mark ET Willems	**Email:** M.Willems@chi.ac.uk
Rianne Costello	**Email:** RCostello@brookes.ac.uk
Luke D Greisler	**Email:** Lgreisle@highpoint.edu
Phebe A Romano	**Email:** Promano@highpoint.edu
Garrett W Hill	**Email:** Ghill1@highpoint.edu
Matthew R Kuennen	**Email:** Mkuennen@highpoint.edu

† Miss Ania M Hiles sadly passed away during the preparation of this manuscript.

ABSTRACT

This study investigated the effects of 7 days of 600 mg/day anthocyanin-rich blackcurrant extract intake on small intestinal permeability, enterocyte damage, microbial translocation and inflammation following exertional heat stress. Twelve recreationally active men (maximal aerobic capacity = 55.6 ± 6.0 $mL \cdot kg^{-1} \cdot min^{-1}$) ran (70% VO_2max) for 60 minutes in an environmental chamber (34°C, 40% relative humidity) on two occasions (Placebo/Blackcurrant, randomized double-blind cross over). Permeability was assessed from a 4-hour urinary excretion of lactulose (L) and rhamnose (R) and expressed as a ratio of L/R. Venous blood samples were taken at rest and 20, 60 and 240 min after exercise to measure enterocyte damage (intestinal fatty acid binding protein, I-FABP), microbial translocation (sCD14; lipopolysaccharide binding protein, LBP), and interleukins 6 (IL-6), 10 (IL-10) and 1 receptor antagonist (IL-1RA). Exercise increased rectal temperature (by ~2.8 °C) and heart rate (by ~123 $beats \cdot min^{-1}$) in each condition. Blackcurrant supplementation led to a) ~12% reduction in L/R ratio ($p<0.0034$) and enterocyte damage (~40% reduction in I-FABP area under the curve, AUC; $p<0.0001$) relative to placebo. No between condition differences were observed immediately after exercise for LBP (+80%, +61 to +99%; mean, 95% confidence interval), sCD14 (+37%, +22 to +51%), IL-6 (+494%, +394 to +690%), IL-10 (+288%, +105 to +470%) or IL-1RA (+47%, +13 to +80; all time main effects). No between-condition differences for these markers were observed after 60 or 240 min of recovery. Blackcurrant extract preserves the GI barrier, however at sub-clinical levels this had no effect on microbial translocation and downstream inflammatory processes.

Keywords: Exercise, Hyperthermia, Anthocyanins, Inflammation, Small intestinal permeability,

INTRODUCTION

Arduous physical activity performed in hot environments increases the metabolic and cutaneous demands for blood flow (González-Alonso et al., 2008). Competition for the available cardiac output is, in part, met by renal and splanchnic vasoconstriction, which creates ischemic/hypoxic stress at the gastrointestinal (GI) mucosa (Costa et al., 2017; Rowell, 1974). Splanchnic hypoperfusion and ischemia, alongside localised intestinal hyperthermia, disrupt enterocyte structure and alters the phosphorylation status of intestinal tight junction proteins, increasing small bowel permeability (Dokladny et al., 2006; Zuhl et al., 2014). As a consequence, immunomodulatory microbial products (e.g. lipopolysaccharide, bacterial DNA, flagellin) translocate into the systemic circulation and bind with toll-like receptors (TLR) located on the surface of cell membranes (Fukui, 2016). TLR activation can initiate nuclear factor kappa B (NF-κB) mediated pro-inflammatory responses that contribute to further body temperature rise, disseminated intravascular coagulation, and multiple organ failure (Asakura et al., 2003; Bouchama et al., 1991). For this reason, the GI-exertional heat stroke (EHS) paradigm has been hypothesised to play an important role in the pathology of exertional heat stroke when deep body temperature remains below the critical threshold for heat toxicity (42-44 °C; Lim 2018).

Given that the gastrointestinal tract plays an important role in pathophysiology of EHS, it is not surprising that there is significant interest in finding nutritional countermeasures which could modulate the key cellular pathways involved in GI barrier integrity loss and intestinal epithelial injury (for comprehensive reviews see Costa et al. 2020; Ogden et al. 2020). Supplementation with polyphenols, bioactive metabolites found in plants, has become increasingly popular in athletic populations (Knapik et al., 2018. Polyphenols can be further classified into flavonoids; phenolic acids; stilbenes; and lignans (Manach et al., 2004). Flavonoids can be sub-classified into flavonols, flavones, isoflavones, flavanones, flavanols, and anthocyanidins (Manach et al., 2004). Growing evidence from cell (Medda et al., 2015), animal (Akiyama et al., 2012; Murakami et al., 2015) and human pre-clinical trials (Biedermann et al., 2013; Roth et al.,

2016) suggest that anthocyanins attenuate NF-κB mediated inflammatory responses via inhibitory actions on TLR4 expression (Nair et al., 2014), and are protective against intestinal inflammation in diseases whose pathology are strongly associated with GI barrier dysfunction (Li et al., 2019). Supplementation with anthocyanin-rich berry extracts could therefore be a viable strategy to mitigate against exertional heat stress induced GI barrier damage.

Blackcurrants (*Ribes nigrum L.*) contain appreciable amounts of anthocyanins (~585 mg/100g), including primarily cyanidin-3-O-glucoside, cyanidin-3-O-rutinoside, delphinidin-3-O-glucoside and delphinidin-3-O-rutinoside (Nakamura et al., 2010). The ingestion of blackcurrant extract (~240 mg anthocyanins) prior to exercise was shown to alleviate oxidative stress, and enhanced ex-vivo immune responsiveness of peripheral blood mononuclear cells challenged with LPS (Lyall et al., 2009). However, no study has examined whether blackcurrant supplementation is effective at reducing hyperthermia-mediated GI dysfunction, microbial translocation, and subsequent inflammation *in-vivo*. The current study examined the effects of 7-day blackcurrant extract supplementation (210 mg anthocyanins/d) on small intestinal permeability (dual sugar absorption test), plasma intestinal epithelial injury (serum I-FABP), microbial translocation [serum lipopolysaccharide binding protein (LBP); soluble CD14 (sCD14)] in conjunction with associated systemic cytokine responses following an acute bout of exertional heat stress.

METHOD

Participants

Twelve healthy recreationally active men (age: 28 ± 6 years, stature: 1.81 ± 0.07 m, mass: 80.5 ± 9.8 kg, body surface area: 2.01 ± 0.15 m^2, body fat percentage: 12.0 ± 1.6 %, maximal aerobic capacity: 55.6 ± 6.0 $mL \cdot kg^{-1} \cdot min^{-1}$) volunteered to participate. The study was approved by the Human Research Ethics Committee at the University of Chichester in accordance with the Declaration of Helsinki, and all participants provided written informed consent prior to taking part. All participants completed a pre-study health screening questionnaire, and were

non-smokers, deemed healthy, injury-free, and absent of cardiovascular, pulmonary, or metabolic disease as defined by the American College of Sports Medicine (Riebe et al., 2015). Testing was completed between November and January, and participants were not heat acclimated and did not self-report regular sauna or hot tub use.

Experimental Design

Participants completed a preliminary testing session during which body composition and maximal aerobic capacity were assessed as previously described (Hiles et al., 2020). Participants then completed one thermally neutral habituation session (18°C, 40% relative humidity), and two exertional heat stress trials (34 °C, 40% RH) in a randomized, double-blind, crossover design separated by a 14-day washout period. In the 7-day lead up to each exertional heat stress trial, participants were supplemented with either 2 x Blackcurrant extract, or 2 x Placebo capsules per day. Trial order was determined using a free online tool (https://www. randomizer.org) and seven participants received blackcurrant extract as the first condition. Concealment was not broken until after sample and statistical analysis was completed. Blinding success was determined via an exit questionnaire administered after the final visit (Betts et al., 2020).

Dietary Supplementation and pre-trial standardization

The New Zealand Blackcurrant extract supplement and corresponding Placebo were donated by CurraNZ^{TM}, Health Currancy Ltd., Surrey, UK. Each 300 mg blackcurrant capsule contained 105 mg of anthocyanins, (i.e. 35–50% delphinidin-3-O-rutinoside, 5–20% delphinidin-3-O-glucoside, 30–45% cyanidin-3-O-rutinoside, 3–10% cyanidin-3-O-glucoside per capsule). Participants were required to take 2 x capsules (~210 mg anthocyanins per day) or 2 x Placebo capsules per day for the 7-days before experimental visits (Cook et al., 2017). Placebo capsules were visually identical and contained microcrystalline cellulose M102. Both the

Blackcurrant extract and Placebo supplements were taken upon waking, and at a consistent time within a participant.

Participants were not taking any medications (e.g., nonsteroidal anti-inflammatory drugs, antidepressants, or diuretics) or nutritional supplements (bovine colostrum, curcumin, dietary nitrate, glutamine, l-citrulline, l-arginine, probiotics, or quercetin) that might influence GI barrier function while enrolled in the study. Diet and exercise were recorded for 2 days before the first experimental trial and participants were asked to replicate their pre-trial diet and exercise before the remaining visit. For 24 h before each visit, participants refrained from strenuous exercise and alcohol. Macronutrient intake has been shown to alter GI barrier permeability (Etxebarria et al., 2021) and post exercise I-FABP responses (Snipe et al., 2017a), therefore participants attended the laboratory after an ~10 h overnight fast. On the morning of each experimental visit participants were instructed to drink 500 mL of water and take their final 2 capsules 2 hours before attending.

Exercise protocol and measurements

All exercise trials were performed between 06:00 and 08:00, with time of trial kept consistent within a participant. Upon arrival to the laboratory, a urine sample was taken for assessment of urine osmolality and specific gravity (ATAGO 2791, ATAGO, Tokyo, Japan) to examine hydration status ($mOsmol^{-1} \leq 600$; USG ≤ 1.020; (Sawka et al., 2007). Nude body mass was recorded, and participants inserted a polyethylene rectal thermistor (Edale Instruments, Cambridge, UK) 10 cm past the anal sphincter. A heart rate (HR) monitor strap was worn around the chest and skin thermistors (Edale Instruments, Cambridge, UK) attached to the mid-belly of the pectoralis major, triceps brachii, rectus femoris, and gastrocnemius. Mean skin and mean body temperature were calculated using standard equations (Kenny, 1998.; Ramanathan, 1964). Physiological strain index was calculated using a modified version of the PSI equation (Moran et al., 1998).

Following instrumentation, participants underwent a 20 min rest period. Physiological measurements were noted, and participants moved into an environmental chamber (TISS Services UK, Mestead, Hampshire, UK) where they rested for five minutes before beginning exercise. Participants ran at 70% $\dot{V}O_{2max}$ for 60 min at 1% incline within the environmental chamber that was controlled at 34.1 $\pm$ 0.1 °C and 40.8 $\pm$ 0.2 % relative humidity (RH). Expired gas fractions were collected into 200 L Douglas bags every 10 min, analysed immediately, and corrected for the inspired gas fraction measured concurrently within the climatic chamber. Heart rate, rectal temperature and skin temperatures were recorded following each Douglas bag collection. Twenty minutes into the exercise trial, a 50-ml sugar probe solution (5 g lactulose, 2 g rhamnose) was consumed for measurement of intestinal permeability. Chamber temperature bottled water (~34 °C) was available to participants during exercise *ad libitum* during the exercise trial and the volume drank recorded. On completion of exercise, participants' towel dried, and nude body mass was reassessed. Difference in pre to post exercise body mass was used to calculate whole body sweat rate, which was corrected for water ingestion and urine output, but not for respiratory water losses. Participants rested for 60 min post exercise, with physiological and thermoregulatory measurements collected 20 and 60 min after exercise. Participants then could leave the laboratory before returning to provide a final blood sample and return the urine collected throughout the 240 minutes post-exercise period. During this time the volume eaten and drank was recorded, and a photocopy was provided so that participants could replicate food and fluid consumption during subsequent visits. Participants self-reported adherence to their food and fluid intake after each trial.

Blood collection and analysis

Posture-controlled venous blood (~20 mL) was collected without stasis before exercise; and 20 min, 60 min and 240 min after exercise. Samples were drawn into sterile syringes and immediately transferred to chilled citrate (5 mL, Sarstedt, Leicester, UK), ethylenediaminetetraacetic acid (EDTA, 5mL; Sarsetdt, Leicester, UK) or pre-warmed (37°C)

EDTA tubes (10 mL). Haematocrit was determined from microcapillary tubes that were loaded in triplicate, and haemoglobin was assayed using microcuvettes (Hb 201, Hemocue®, Äbgelholm, Sweden) and a Hemocue photometer (Hb201+, Hemocue®, Äbgelholm, Sweden). Plasma volume changes were calculated from hematocrit and haemoglobin (Dill & Costill, 1974), and circulating measures of IFABP, LBP, sCD14, IL6, IL-10, and IL-1RA adjusted accordingly. I-FABP concentrations were measured as a marker of intestinal damage using a commercially available ELISA (Hycult Biotech, USA). LBP, sCD14, IL-6, IL-10 and IL-1RA were analysed in duplicate using ELISAs from R&D Systems. Inter and intra assay coefficient of variations were below 5%, except for the inter-assay CV for IL-6 (6.1%). All samples were assayed in duplicate.

Small intestinal permeability

Urine samples were assayed in duplicate and Lactulose was quantified with an EIA (K-LACT, Megazyme, Dublin, Ireland), with some deviations from the manufacturer's instructions, as described previously (Flood et al., 2020). Rhamnose was quantified using a colorimetric assay (K-Rhamnose, Megazyme, Dublin, Ireland) according to the manufacturer instructions. The recovery of both sugars was determined per litre urine ($mg \cdot l^{-1}$), where the lactulose/l-rhamnose (L/R) ratio was then corrected relative (%) to the concentration consumed.

Statistical analysis

Statistical analysis was performed using IBM SPSS for Windows (Version 23, SPSS, Chicago, Illinois). Differences in dietary intake, anthocyanin intake, ambient conditions, urine specific gravity, urine osmolality, fluid intake and sweat rate were determined using two-tailed paired t-tests. Differences in cardiovascular and thermoregulatory variables were determined using mixed linear models, where condition and time served as fixed effects. Statistical analysis of plasma I-FABP, LBP, sCD14, IL-6, IL-10, IL-1RA data was conducted on the absolute concentrations, after correction for plasma volume change. Interaction effects ($p<0.05$) were explored using paired t-tests with Tukey HSD post hoc procedure used to control for multiple

comparisons. Where no interaction effect was identified, simple main effects for time or condition are reported. Data in text and tables are presented as mean (SD) or mean (95% confidence interval, CI). To maintain clarity, where interaction effects are apparent, only the differences between the Placebo and Blackcurrant conditions are annotated as these are the results of greatest interest. The Time Series Response Analyser (Narang et al., 2020) was used to calculate the incremental area under the curve (AUC) summary statistic, and data displayed as mean ± 95% CI alongside all individual paired responses. Precise p-values are reported, and Cohen's *d* (paired t-test data) effect sizes are presented to indicate the magnitude of observed effects, which were considered 'trivial' (d<0.2), 'small' (d=0.2-0.49), 'moderate' (d=0.5-0.79) and 'large' (d ≥ 0.8), respectively.

RESULTS

Equality of study conditions and blinding success

Dietary intake, pre-trial body mass and hydration status, chamber conditions, running speed, exercise intensity and water consumption were no different between the Placebo and Blackcurrant conditions (**Table 1**). A difference between conditions was noticed by 3/12 participants, with 2/3 believing they could identify the treatment allocation. Only 1 of these 2 participants correctly identified treatment allocation. Taken together these data indicate successful blinding of the experimental supplements, which were provided as visually identical pill capsules.

Physiological responses

Our exertional heat stress protocol produced substantial increases in HR, T_{skin}, $T_{rec,}$ and physiological strain (**Table 1**). Changes over time are provided in **Figure 1.** Rectal temperature was ~ 0.1°C lower throughout the Blackcurrant trial (main effect for condition, F=9.035, p=0.003). However, delta change in rectal temperature was not different between conditions (Placebo: +2.86 ºC, Blackcurrant: +2.81 ºC; main effect for condition, F=0.760, p=0.783), and

the ~0.1 °C difference falls within accepted measurement error for this variable. No other differences in cardiovascular or thermoregulatory variables were observed.

Small intestinal permeability, intestinal damage, and microbial translocation

To provide an indication that our exertional heat stress model led to an increase in small intestinal permeability relative to less thermally challenging conditions, a subset of samples from the thermoneutral familiarisation trial were analysed for lactulose and rhamnose (n=8). Exertional heat stress induced a ~2-fold increase in L/R ratio relative to thermoneutral familiarisation session. The L/R ratio was 12% (-18 to -6%) lower following supplementation with Blackcurrant compared to Placebo [by -0.0065, -0.0104 to -0.0026, p=0.0034, d=0.84, *large effect;* **Figure 2**].

Absolute concentrations of plasma I-FABP were lower 20 min after exercise in Blackcurrant compared to Placebo (by 584 $pg \cdot mL^{-1}$, 255 to 914 $pg \cdot mL^{-1}$, mean, 95% confidence interval; p=0.0031, d=0.83, *large effect*), lower 60 minutes after exercise (by 633 $pg \cdot mL^{-1}$, 304 to 963 $pg \cdot mL^{-1}$, p=0.002, d=1.37, *large effect*), and lower 240 minutes after exercise (by 470 $pg \cdot mL^{-1}$, 140 to 799 $pg \cdot mL^{-1}$, p=0.0029, d=1.65, *large effect*; condition x time interaction, F = 3.98, p = 0.016, nP2 = 0.26; **Figure 3A**). The resulting plasma I-FABP AUC was reduced by ~40% following Blackcurrant supplementation compared to Placebo (p<0.0001; **Figure 3B;** d=-1.2, *large effect*).

Main effects of time were observed for sCD14 and LBP (<0.0001), however there was no condition effect and no condition x time interaction for either marker of microbial translocation (**Figure 3C, D & 3E, F**). Post hoc analysis of time main effects show sCD14 (+37%, +22 to +51%, p<0.0001) and LBP (+80%, +61% to +99%, p<0.0001) increased 20 min after exercise. Concentrations remained elevated 60 min after exercise for sCD14 (+32%, +17% to +48%) and LBP (+65%, +48% to +81%, p<0.0001), regressing towards baseline values after 240 min of recovery sCD14 (+17%, -7% to +34%, p=0.28), LBP (+14%, -3% to +30%, p=0.097).

Plasma cytokines

Circulating concentrations of IL-6, IL-10, and IL-1RA are depicted in **Figure 4**. Main effects of time were observed for IL-6, IL-10 and IL-1RA (all $p<0.0001$), however no differences in AUC were identified, and no main effects for condition or condition x time interactions were observed for IL-6 and IL-10. A condition x time interaction effect was observed for IL-1RA, however after adjustment for multiple comparisons, no between-condition differences were found. Post hoc analysis of time main effects show IL-6 (+494%, +347 to +640%, $p<0.0001$), IL-10 (+288%, +105 to +470%, $p<0.0001$), and IL-1RA (+47%, +13 to +80%, $p=0.098$) increased 20 min after exercise. Concentrations remained elevated 60 min after exercise for IL-6 (+279%, +178 to +380%, $p<0.001$), IL-10 (+207%, +63 to +351%, $p=0.0001$), and IL-RA (+63%, +40 to +85%, $p<0.0001$). After 240 min of recovery IL-6 (+70%, +25 to +115%, $p=0.12$), IL-10 (+101%, +4 to +198%, $p=0.38$) began to regress towards resting concentrations, with IL-1RA (+51%, +39 to +64%, $p<0.0001$) remaining elevated relative to rest.

DISCUSSION

The present study examined short-term dietary blackcurrant supplementation for potential benefits on physiological responses, gastrointestinal barrier damage, microbial translocation, and circulating cytokines following a single bout of exertional heat stress. We present three main findings; first we show that the 7-day period of blackcurrant extract supplementation was sufficient to reduce small intestinal permeability and reduce enterocyte damage following an acute bout of exertional heat stress. Second, we show that despite preserved GI barrier function, neither the translocation of microbial products (LBP, sCD14) nor the subsequent systemic inflammatory response of selected cytokines (IL-6, IL-10, IL-1RA) were altered following blackcurrant intake. Third, neither the physiological nor thermoregulatory responses to acute heat stress were altered after blackcurrant supplementation – although there was some evidence for a lower deep body temperature during the blackcurrant trial, the data fall within the accepted measurement error for the technique (~0.1 °C). Collectively, these data suggest that 7 days of blackcurrant supplementation may help prevent heat-induced

impairments in the GI function of non-acclimated individuals. However, these data should be interpreted with appropriate caution as sCD14 and LBP, alternative markers of GI function and microbial translocation, remained unaltered and downstream inflammatory responses were also not impacted after 7 days of blackcurrant supplementation.

The best supported explanations regarding the breakdown of GI barrier function following exertional heat stress relate to hyperthermia-mediated dysregulation of GI tight junctions (Dokladny et al., 2015); splanchnic hypoperfusion-mediated ischemia-reperfusion injury (van Wijck et al., 2012); and alternations in several complex neuroendocrine-immune related interactions (e.g. antimicrobial protein secretion; De Punder & Pruimboom, 2015). Thus, mechanisms behind potential blackcurrant induced preservation of the GI barrier should be considered in relation to these known mediators of GI barrier damage. Changes in deep body temperature are a key predictor of the magnitude of exercise-associated gastrointestinal disturbance, where strong correlations between peak deep body temperature and after-exercise I-FABP concentrations (r = 0.91) have been shown (Pires et al., 2016). Our exertional heat stress protocol led to clinically significant increases in deep body temperature (average peak temperature of 39.4 °C, range 38.6 °C to 40.0 °C). Although we observed slight decreases in exercising deep body temperature (by ~0.1 °C) and a decrease in the delta change in deep body temperature (by ~0.05 °C) during the blackcurrant trial, it is unlikely that these relatively minor physiological differences would have affected small intestinal permeability and/or explained the ~40% reduction in I-FABP AUC.

Elevated perfusion needs of skeletal muscle and cutaneous circulation result in ischemic/hypoxic stress at the gastrointestinal mucosa. The associated gut ischemia and reduced oxygen supply to the splanchnic region alters the normal anti-inflammatory processes at the gut mucosa, resulting in increased NF-KB activation, iNOS and TNFα release - essential components involved in tight junction breakdown and the passage of gram-negative bacteria into systemic circulation (Dokladny et al., 2015). Human research has provided evidence that anthocyanins enhance vascular and endothelial function, which may result in improved muscle

perfusion and enhanced oxygen extraction that could help to preserve blood flow across splanchnic tissue beds (Cook & Willems, 2019). However, it is unlikely that physiologically relevant alterations to splanchnic blood flow occurred in the present study, given that heart rate and mean skin temperature were the same between conditions (this indirectly implies similar rates of blood flow, skin perfusion, and presumably splanchnic perfusion).

We measured plasma concentrations of the acute phase proteins LBP and sCD14 as markers of microbial translocation because they are more stable and are less prone to contamination compared to the direct measurement of plasma LPS (Costa et al., 2017; Ogden et al., 2020). Moreover, these markers do not appear to be as rigorously subjected to consistent neutralizing and clearance because of immune surveillance, phagocytosis, and circulatory and lymphatic elimination (Snipe et al., 2018). Post exercise changes to both sCD14 and LBP do not consistently increase in response to exercise where body temperature remains below 39.0 °C (Russo et al., 2021a, 2021b). In the present study, in which most participants exceeded 39.0 °C, we observed an ~80% increase in LBP immediately after exercise that remained elevated through 60 minutes of exercise recovery. These response kinetics are similar to previous exertional heat stress protocols (e.g. ~150% increase, Extebarria et al., 2021), and these concentrations are still comfortably within the healthy range (5 – 15 ug/mL), with peak values ranging from ~3.7 to 12.8 ug/mL, representing an increase of ~3 ug/mL after exercise (range +0.8 to +6.8 ug/mL). Previous exertional heat protocols also report sub-clinical increases of ~2.0 ug/mL (Selkirk et al., 2008), and ~5.3 ug/mL (Wallett et al., 2021). The increase in LBP was accompanied by an increase in plasma sCD14, a phosphatidylinositol-linked membrane glycoprotein on polymorphonuclear leucocytes that serves as a receptor for endotoxin (Costa et al., 2017). When taken together, these data indicate that the translocation of bacterial products into the systemic circulation occurred at a sub-clinical level and were not impacted by the minor reductions in small intestinal permeability and enterocyte damage seen following blackcurrant intake. This also likely explains the comparable cytokine responses between study conditions. Further insight into polyphenol/anthocyanin effects on GI barrier function

could be gained by incorporating systemic and intracellular markers, which would help to elucidate potential mechanisms of any protective action provided through supplementation (Falgiano et al., 2018).

The present experimental design may not perfectly mimic real-life activity, as our exercise was performed in the fasted state. However, recent recommendations for maximizing performance in endurance athletes do call for some exercise sessions to be performed under fasted conditions, the so-called Sleep-Low model (Riis et al., 2019). Moreover, a fasted exercise state was required to ensure accuracy and validity of the dual sugar absorption test, and to minimise the effects of dietary intake on I-FABP release (Etxebarria et al., 2021). It is plausible that the observed effects to small intestinal permeability and plasma I-FABP may be lost when exercise is performed following adequate dietary carbohydrate/protein intake prior to and during exercise (Snipe et al., 2017b). Other dietary manipulations (e.g. low FODMAP diets, and appropriate macronutrient intake prior to exercise) have been shown to be effective at ameliorating GI damage/GI symptoms during exercise (Gaskell et al., 2019). The extent to which additional supplement interventions improve GI barrier function and reduce intestinal damage remains a current gap within the literature. It is recommended that future work investigate diet-supplement interactions.

The 7-day dosing regimen used herein is consistent with previous studies supplementing with blackcurrant anthocyanins (Cook et al., 2015, 2017), and has been suggested to be a sufficient period of time to allow the build-up of anthocyanin metabolites over time (Costello et al., 2021). Only one previous investigation has explored the effects of acute blackcurrant ingestion (~105 mg anthocyanins) to determine changes to the anthocyanin metabolite profile (Costello et al., 2021), with no studies exploring metabolite changes over the commonly used 7-day dosing period. A more in-depth understanding of anthocyanin-metabolite changes over time (e.g., throughout a 7-day dosing period) is required to refine and optimise dosing protocols. Prolonged periods of supplementation could be considered burdensome for wider implementation in scenarios where short-notice deployments to exertional heat-stress could

be encountered (for example military deployments, firefighting). In these scenarios the use of other effective supplements (e.g. curcumin, glutamine) with a less demanding dosing period could be preferred should evidence regarding their effectiveness warrant their consideration.

A limitation of the present investigation (and a prevailing limitation within the nutraceutical literature) is that we did not compare the active component of the blackcurrant supplement against another compound known to preserve GI barrier function following exertional heat stress (for example curcumin, bovine colostrum, glutamine) (Syzmanski et al., 2018; McKenna et al., 2020; Zuhl et al., 2014). While our comparison of a blackcurrant supplement with an inactive placebo pill is a common approach that can provide initial proof of concept data regarding the effectiveness of a particular nutritional or pharmacological compound, this method does not provide practitioners with the information they truly want – namely which supplement or supplements are the most effective for a given situation. Furthermore, follow-up studies that directly compare two different active supplements are rarely performed, despite such an approach providing more robust information for end-users.

CONCLUSION

Blackcurrant extract supplementation reduces small intestinal permeability and enterocyte damage following an acute bout of exertional heat stress, but the utility of that potential benefit needs to be balanced against the limited alterations in physiological function and inflammatory markers that were observed in the present study. Future work should also consider making comparisons against other effective nutraceutical/pharmacological supplementation regimes, enabling a more efficient down-selection of effective nutritional compounds aimed at preserving GI barrier function during and after exertional heat stress.

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M.R. Kuennen with assistance from P.A. Romano, L.G. Greisler and G.W. Hill; Data interpretation and manuscript preparation were undertaken by B.J. Lee, M.R. Kuennen and M.E.T Willems. All authors approved the final version of the manuscript. The authors have no conflicts of interest. The authors would like to thank all participants for volunteering their time and effort to facilitate completion of this study. We thank Mr Simon Hasted for his hospitality and willingness to share resources throughout data collection.

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Table 1. Summary of dietary intake, ambient conditions, cardiorespiratory and thermoregulatory responses. Respiratory variables are presented as the average recorded over 60 minutes of treadmill running. Data for thermal and CV strain are reported as the peak value recorded during the 60-minute treadmill run. Urine specific gravity and osmolality were recorded at rest and before trial commencement. Data are mean (SD) for n=12.

Variable group	Variable	Placebo	Blackcurrant	P-value
Dietary intake	Total energy intake (mJ/day)	8.4 (1.9)	8.4 (2.0)	0.343
	Carbohydrate (g)	202 (39)	200 (46)	0.822
	Protein (g)	105 (34)	118 (49)	0.235
	Fat (g)	78 (25)	77 (24)	0.662
	Habitual anthocyanin intake (mg/day)	116 (39)	111 (47)	0.778
Ambient conditions	Temperature (°C)	34.2 (0.4)	34.2 (0.3)	0.927
	Humidity (%)	43.7 (3.9)	42.2 (2.6)	0.510
Exercise workload	Running speed (km/hr)	10.4 (1.2)	10.3 (1.2)	0.871
	% peak oxygen consumption	70 (4)	69 (4)	0.128
Indirect calorimetry	Oxygen consumption ($L \cdot min^{-1}$)	3.10 (0.29)	3.06 (0.28)	0.295
	Carbon dioxide production ($L \cdot min^{-1}$)	2.78 (0.28)	2.66 (0.27)	0.076
	RER	0.90 (0.03)	0.87 (0.04)	0.001
Thermal & CV strain	Rectal temperature (°C)	39.47 (0.43)	39.36 (0.46)	0.003
	Δ Rectal temperature (°C)	+2.86 (0.46)	+2.81 (0.42)	0.783
	Mean skin temperature (°C)	34.99 (0.69)	34.89 (0.74)	0.664
	Mean body temperature (°C)	37.18 (0.36)	37.07 (0.34)	0.319
	Heart rate (bpm)	184 (9)	185 (9)	0.868
	Physiological strain index	9.8 (0.7)	9.6 (0.6)	0.799
Hydration	Urine specific gravity	1.010 (0.006)	1.008 (0.004)	0.394
	Urine osmolality	355 (172)	284 (128)	0.231
	Fluid ingestion (L/hour)	0.91 (0.36)	0.86 (0.50)	0.682
	Sweat rate (L/hour)	2.09 (0.59)	2.24 (0.73)	0.574
	Body mass (kg)	79.9 (9.6)	80.1 (9.7)	0.581

Dietary intake and hydration variables were assessed via a paired samples t-test. All other variables were assessed via an ANOVA, with the p-value shown for the main effect of condition.

639 **Figure Legends**

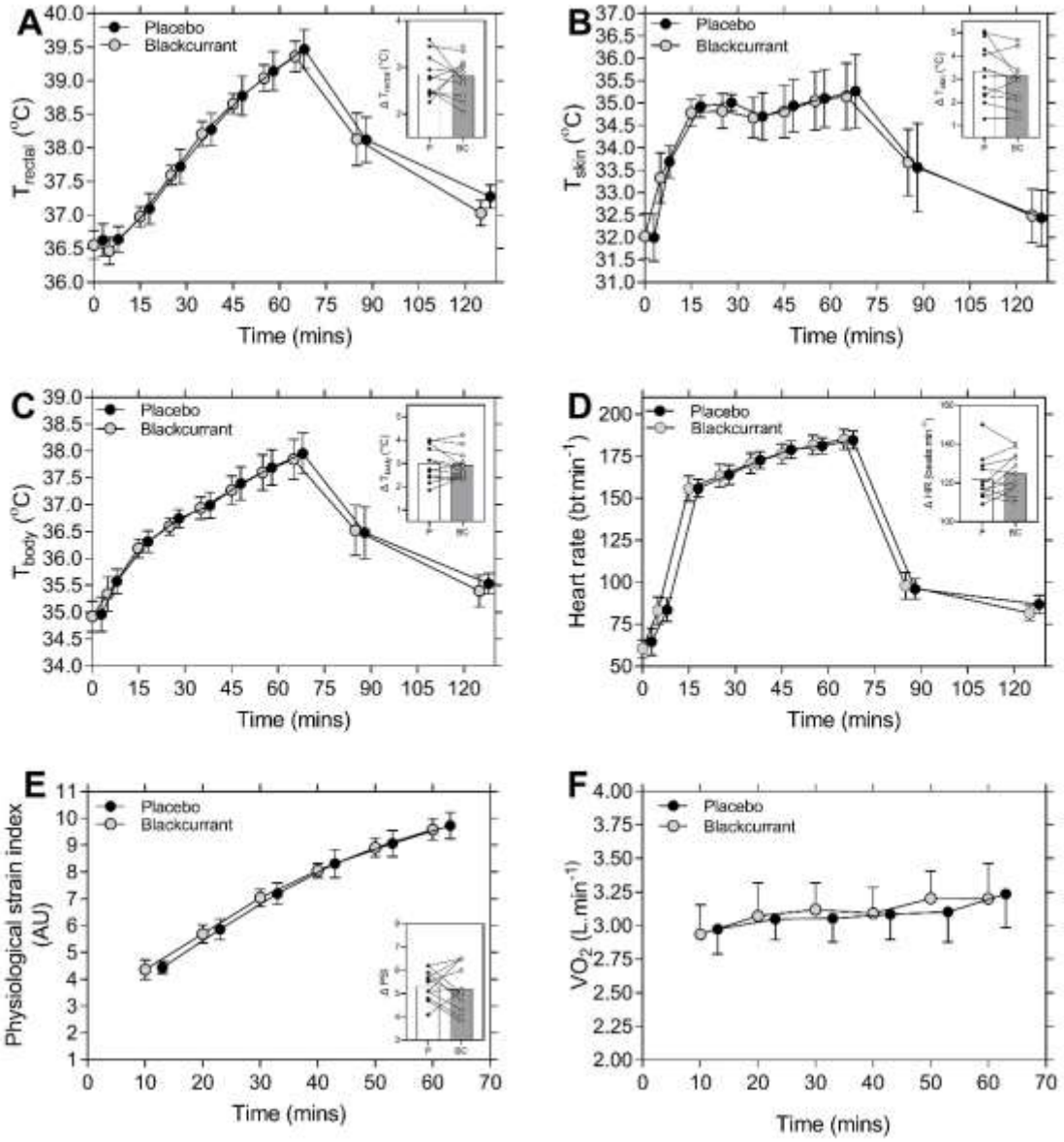
640 **Figure 1.** Short term (7 days) blackcurrant supplementation does not alter thermal or
641 cardiovascular strain during 60 min of submaximal exercise performed in hot conditions. Deep
642 body temperature **(*A*)**, mean skin temperature **(*B*)**, mean body temperature **(*C*)**, heart rate **(*D*)**,
643 physiological strain index **(*E*)**, and oxygen consumption ***(F)*** in response to 60 min of treadmill
644 exercise performed at a workload equivalent to 70% $\dot{V}o_{2max}$. *A–D*: Rest and exercise data at
645 10-min intervals (main figure) and as individual delta values (*insets, P=Placebo, BC=*
646 *Blackcurrant*); *E-F*: exercise data at 10-min intervals. Data are mean and 95% confidence
647 interval for n=12.

648 **Figure 2.** Estimation plot for lactulose/rhamnose ratio. On the left is the individual L/R data for
649 the Familiarization (n=8), Placebo and Blackcurrant conditions (both n=12). The right side
650 displays the mean difference and 95% confidence interval of the difference and includes all
651 individual responses (diamonds). The data show that 10/12 participants had an attenuation in
652 GI barrier permeability following blackcurrant supplementation ($p<0.01$). BC = blackcurrant, P
653 = placebo.

654 **Figure 3.** Time course and individual AUC for plasma I-FABP (***A, B; n=12***) LBP (***C, D; n=12***)
655 and sCD14 (***E, F; n=12***). Time-course data was assessed by a two-way mixed linear model
656 and presented as the mean ± 95% CI. For clarity, only differences between conditions are
657 shown on time-course figures. AUC data show each individual response (lines). Right-hand y-
658 axes represent the difference between conditions for AUC, showing all individual responses
659 and summary statistics (mean ± 95% CI). Paired two-tailed t-tests were used to compare the
660 AUC between conditions. AUC, Area under the curve.

661 **Figure 4.** Time-course and individual AUC for plasma IL-6; **(*A, B; n=10*)**; plasma IL-10 (***C, D***;
662 n=12) and plasma IL-1RA; **(*E, F;* *n=10*)**. Data was assessed by a two-way mixed linear model
663 and presented as the mean ± 95% CI. No condition main effects nor interaction effects were
664 observed for all cytokine responses. AUC data show each individual response (lines). Right-

hand axes represent the difference between conditions for AUC, showing all individual responses and summary statistics (mean ± 95% CI). Paired two-tailed t-tests were used to compare the AUC between conditions. AUC. Area under the curve. Due to 2 participants falling below the detection limit of the assay, data are presented as n=10 for IL-6 and IL-1RA



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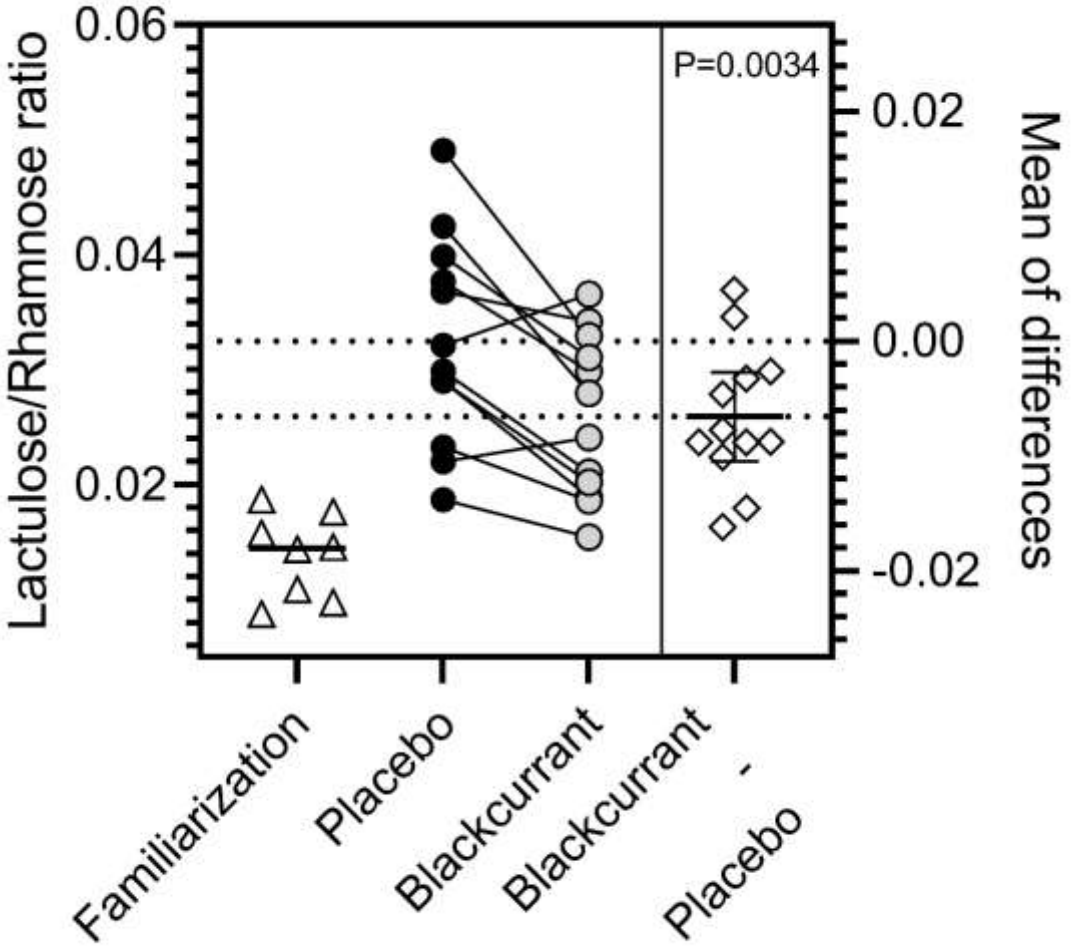

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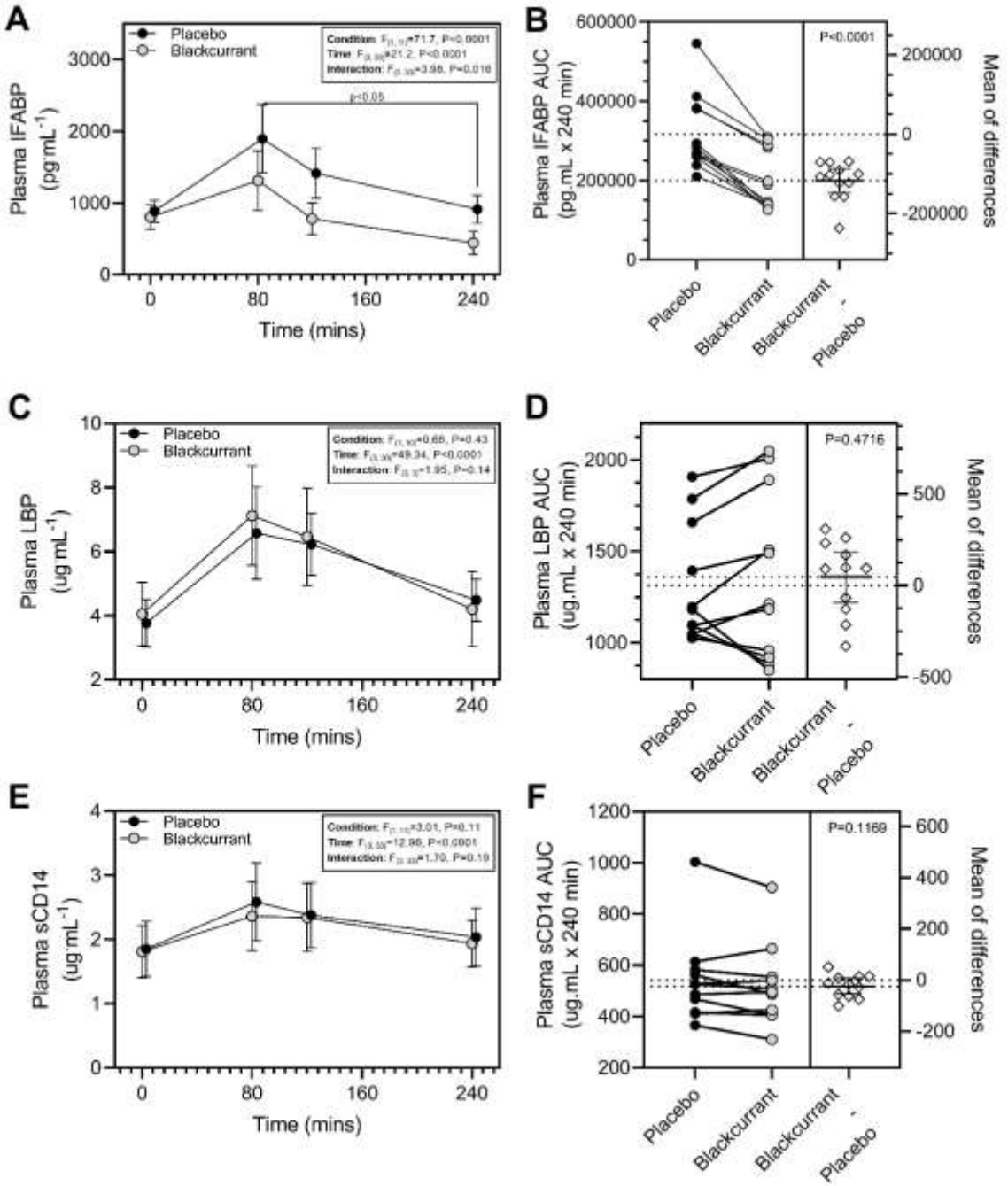

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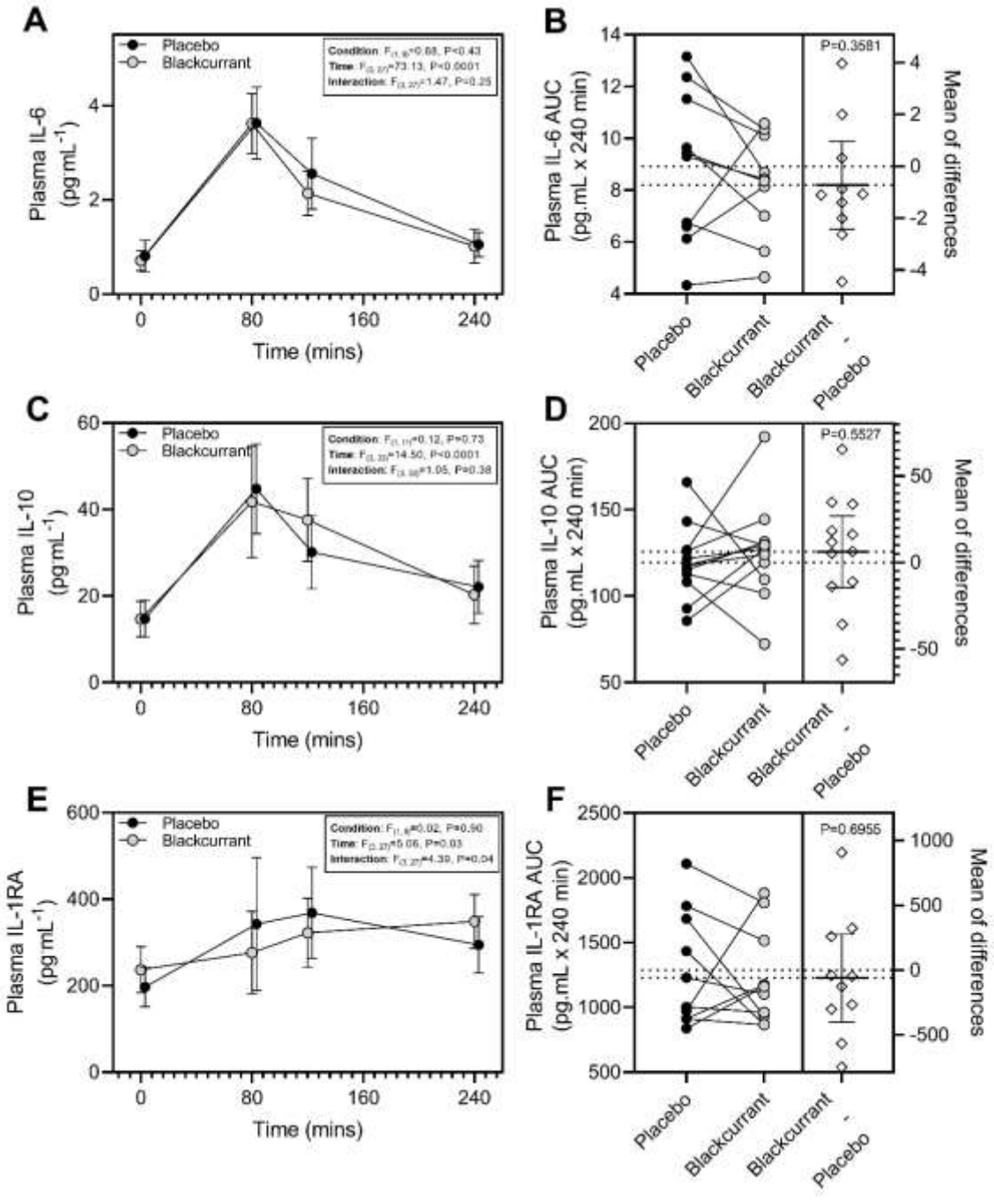

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710 Figure 4.