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Complete List of Authors:	Marshall, Hannah; University of Bedfordshire, Institute of Sport and Physical Activity Research (ISPAR) Chrismas, Bryna; Qatar University, Sport Science Program, College of Arts and Sciences; Suckling, Craig; Anglia Ruskin University Roberts, Justin; Anglia Ruskin University Foster, Josh; University of Bedfordshire, Institute of Sport and Physical Activity Research (ISPAR) Taylor, Lee; Aspetar Qatar Orthopaedic and Sports Medicine Hospital, Athlete Health and Performance Research Centre
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Chronic probiotic supplementation with or without glutamine does not influence the eHsp72 response to a multi-day ultra-endurance exercise event.

Hannah Marshall[1], Bryna Catherine Rose Chrismas[2], Craig Andrew Suckling.[3], Justin D. Roberts[3], Josh Foster [1], Lee Taylor [4*, 5]

[1] Institute of Sport and Physical Activity Research (ISPAR), University of Bedfordshire, Bedford, UK.

[2]Sport Science Program, College of Arts and Sciences, Qatar University, Doha, Qatar.

[3]Cambridge Centre for Sport and Exercise Sciences, Department of Life Sciences, Anglia Ruskin University, Cambridge, UK.

[4]ASPETAR, Athlete Health and Performance Research Centre, Qatar Orthopaedic and Sports Medicine Hospital, Aspire Zone, Doha, Qatar.

[5]School of Sport, Exercise and Health Sciences, Loughborough University, Loughborough, UK.

Corresponding Author: Dr. Lee Taylor, ASPETAR, Athlete Health and Performance Research Centre, Qatar Orthopaedic and Sports Medicine Hospital, Aspire Zone, Doha, Qatar. Email: Lee.Taylor@aspetar.com. Phone: + (974) 4413 2403.

Abstract

Probiotic and glutamine supplementation increases tissue Hsp72, but their influence on extracellular Hsp72 (eHsp72) has not been investigated. The aim of this study was to investigate the effect of chronic probiotic supplementation, with or without glutamine, on eHsp72 concentration before and after an ultramarathon. Thirty-two participants were split into three independent groups, where they ingested probiotic capsules (PRO, $n = 11$), probiotic + glutamine powder (PGLn, $n = 10$) or no supplementation (CON, $n = 13$), over a 12-week period prior to commencement of the Marathon des Sables (MDS). eHsp72 concentration in the plasma was measured at baseline, 7 days pre-race, 6-8 hours post-race, and 7 days post-race. The MDS increased eHsp72 concentrations by 124% ($F_{1,3}$ = 22.716, $p < 0.001$), but there was no difference in the response between groups. Additionally, PRO or PGLn supplementation did not modify pre- or post-MDS eHsp72 concentrations compared to CON ($p > 0.05$). In conclusion, The MDS caused a substantial increase in eHsp72 concentration indicating high levels of systemic stress. However, chronic PRO or PGLn supplementation did not effect eHsp72 compared to control pre- or post-MDS. Given the role of eHsp72 in immune activation, the commercially available supplements used in this study are unlikely to influence this cascade.

Key words: Ultra-endurance; extracellular heat shock protein 72; probiotics; desert; race; event.

Introduction

Exercise in extreme heat elicits physiological stress upon the human body (Nybo et al. 2001) which can decrement physical performance (Nybo et al. 2014) and cognitive function (Taylor et al. 2015). In addition, strenuous exercise coupled with high environmental temperatures (exercise heat-stress; EHS) is a major risk factor for the development of exertional heat illnesses [EHI; (Armstrong et al. 2007)]. A plethora of physiological responses including elevated core temperature (Binkley et al. 2002), increased heart rate (HR) and a redistribution of blood flow (González-Alonso et al. 2008) are seen in response to EHS (Nybo 2008; 2014). These responses initially act to protect the body from tissue damage and protein denaturation during EHS, yet they can prove catastrophic if the total stress exceeds a highly individualised threshold of tolerance [EHI; (Adams et al. 2015; Goforth et al. 2015; Wijerathne et al. 2016)]. Nonetheless, humans willingly subject themselves to prolonged EHS when completing endurance events such as the Marathon des Sables (MDS). The MDS is a 294.4 km 7 day ultra-marathon completed across the Southern Moroccan portion of Sahara Desert. Ultra-endurance events such as the MDS are becoming increasingly popular, with a substantial rise in competitor numbers in recent years (Knechtle et al. 2011; da Fonseca-Engelhardt et al. 2013).

Exposure to EHS can increase extracellular heat shock protein (eHsp72) concentration (Walsh et al. 2001; Whitham et al. 2007; Sandström et al. 2008; Magalhaes et al. 2010; Périard et al. 2012; Gibson et al. 2014), a protein which is thought to contribute to the immune response to EHS (Mizzen et al. 1988; Asea 2005). This increase in eHsp72 is thought to prepare the immune system (immuno-stimulatory) for subsequent EHS mediated homeostatic perturbations (Johnson et al. 2006). Although discussion regarding the precise role of eHsp72 remain (Johnson and Fleshner 2006; Whitham et al. 2008), evidence suggests that eHsp72 enhances the production of pro-inflammatory cytokines (Asea 2005) and chemokines (Lehner et al. 2000). This implies the importance of eHsp72 during EHS, given the role of pro-inflammatory cytokines in the pathophysiology of heat stroke (Lim et al. 2006; Leon et al. 2010). Previous *in vitro* (Wischmeyer 2002; Petrof et al. 2004; Tao et al. 2006) and *in vivo* (Wischmeyer et al. 2001) research has demonstrated that glutamine increases intracellular Hsp72 [iHsp72 (epithelial cells)] concentrations, thus it is plausible to suggest that such interventions may elevate eHsp72 concentrations. Because the release mechanism of Hsp72 into the extracellular

environment is still not fully defined, it is unknown if interventions that increase iHsp72 also influence eHsp72 concentrations.

Previous research has demonstrated exercise duration to be a key modulator of eHsp72 release (Febbraio et al. 2002; Marshall et al. 2006; Amorim et al. 2008), and therefore ultra-endurance events, defined as lasting > 6 h (Zaryski et al. 2005), are likely to induce a significant increase in eHsp72 concentration. The MDS (294.4 km, 7 days, 20-50°C) is physiologically challenging given that competitors must carry food and survival equipment for the duration of the event, with only rationed water provided each day, subsequently placing competitors at an increased risk of EHS and potentially EHI throughout the race. Therefore, a nutritional intervention which increases eHsp72 concentration prior to a multi-day ultra-endurance event, such as the MDS, could alter the associated immune response patterns in competitors. Whether or not a basal increase in eHsp72 is beneficial or detrimental in this regard is currently unknown. The additions of probiotics and glutamine have been shown to induce an increase in Hsp72 concentration in various cultured cell types (Wischmeyer 2002; Petrof et al. 2004; Tao et al. 2006). However, to our knowledge, no research has yet been conducted in relation to probiotic supplementation and the eHsp72 response to EHS relative to a multi-day ultra-endurance event with logistical and nutritional challenges, such as the MDS. Owing to its immuno-stimulatory actions, a probiotic or glutamine mediated increase in pre-race eHsp72 may influence the immune response pattern to EHS (MDS). This knowledge is important because any alteration in pro/anti-inflammatory responses to EHS could influence the severity of EHI (Lim and Mackinnon 2006). Despite previous studies demonstrating a significant increase in iHsp72 through probiotic and glutamine supplementation (Wischmeyer 2002; Petrof et al. 2004), no research has investigated if this alters eHsp72 concentrations prior to and following EHS.

This preliminary study aimed to investigate the effects of chronic (12-weeks) probiotic supplementation with (PGLn) or without (PRO) glutamine (both commercially available), on the eHsp72 response pre- and post-MDS completion. Owing to the effects of probiotics and glutamine on Hsp72 synthesis, it was hypothesised that PRO and PGLn would increase eHsp72 concentrations pre-MDS in comparison to a control group (CON). Additionally, it was hypothesised that the nutritional

interventions would reduce the stress and thus the inflammatory response to exercise, marked by a blunted post-MDS eHsp72 concentration when compared to CON.

Materials and methods

Participants

Male and female entrants (aged 18-60 years) were contacted via email through the UK organiser (RunUltra UK). Thirty-two MDS 2015 competitors (6 female, 24 male, age 41; range 23-53 years, height 1.75 ± 0.08 m, body mass 76.89 ± 2.04 kg) met the inclusion criteria and were recruited for this study. Participants were excluded from taking part if: (1) unable to attend all testing sessions; (2) outside of desired age range (18 – 60 years); (3) unsatisfactory health-screen questionnaire completion (i.e. potential history of heart abnormalities, hypertension, heart disease or diabetes); (4) suffering from any musculo-skeletal injury that may have impaired participation in the study and/or endurance training; (5) any known blood related disorder; (6) were taking over-the-counter and/or prescribed medication which may have influenced normal participation (excluding inhalers for exercise-induced asthma or the contraceptive pill); (7) were consuming any other commercial supplementation which conflicted the study parameters (i.e. an alternative probiotic) – and were unwilling to refrain from use of supplementation over the study period; and (8) any participant who was, or had the potential to become, pregnant during the study. All participants provided written informed consent, were deemed healthy and able to take part, and verbally confirmed that they were not currently undertaking any probiotic or glutamine supplementation regime. Participants were instructed to avoid heat acclimation/acclimatisation training sessions in the 48 h prior to all data collection visits (excluding the immediate post-race sample). Of the 32 recruited participants, 94% were compliant to this requirement, with one participant in both the PRO and CON groups undertaking a bout of heat acclimation training 48 h prior to data collection visit 2. Between data collection visit 2 and departure for the MDS, 31.25% ($n = 10$) of participants undertook at least one heat training session [PRO: 2 sessions ($n = 5$), 3 sessions ($n = 1$); PGLn: 3 sessions ($n = 1$); CON: 2 sessions ($n = 2$), 4 sessions ($n = 1$)]. All procedures were approved by the Anglia Ruskin University Ethics Committee, and conformed to the Declaration of Helsinki.

Study overview

Following a randomised independent measures design; participants were assigned to one of three experimental conditions utilizing commercially available dietary supplements:

Probiotic capsules (PRO; n = 11): Participants were required to consume one capsule (Bio-Acidophilus Forte, Biocare Ltd., Birmingham, UK) per day for the duration of the 12-week intervention period. Each multi-strain capsule contained 150 $mg.d^{-1}$ Lactobacillus acidophilus (10 billion $CFU.d^{-1}$, Lactobacillus acidophilus CUL-60 [NCIMB 30157] and 10 billion $CFU.d^{-1}$ Lactobacillus acidophillus CUL-21 [NCIMB 30156]), 16.8 $mg.d^{-1}$ *Bifidobacterium bifidum and lactis* (9.5 billion $CFU.d^{-1}$, *Bifidobacterium bifidum* CUL-20 [NCIMB 30172] and 0.5 billion $CFU.d^{-1}$ *Bifidobacterium animalis subspecies lactis* CUL-34 [NCIMB 30153]), and 55.8 $mg.d^{-1}$ *fructooligosaccharides*.

Probiotic + glutamine powder *(PGLn; n = 10)*: Participants were required to consume 5 g powder (GI Complex, UK) per day, mixed well in water or food. 2 billion, *Lactobacillus acidophilus* CUL-60 (NCIMB 30157), 2 billion; *Lactobacillus acidophilus* CUL-21 (NCIMB 30156); 50 million, *Bifidobacterium bifidum* CUL-20 (NCIMB 30172); 0.95 billion, Bifidobacterium animalis subsp. Lactis CUL-34 (NCIMB 30153); 5 billion, Lactobacillus salivarius CUL61 (NCIMB 30211), each 5 g dose also contained 0.9 g L-Glutamine.

Control (CON; n = 9): maintenance of regular diet.

Apparent daily adherence to intervention and control was self-reported at 100% for all groups. Due to the nature of the study, blinding of groups was not possible, however, the PGLn group were unaware of the addition of glutamine to their probiotic supplement.

Data was collected at four time points across the duration of the study, which consisted of three laboratory visits (visits 1, 2, and 4, Anglia Ruskin University, Cambridge) and one field-based data collection point (visit 3, Ouarzazate, Morocco), as detailed below (Fig. 1a).

Visit 1

This baseline data collection took place 12-weeks prior to the MDS. Participants arrived at the environmentally controlled laboratory (18°C, 35% RH) in a fasted state (minimum 4 h fasted), with confounding variables of alcohol (Taylor et al. 2010a), caffeine (Lu et al. 2008), generic supplementation (Hillman et al. 2011) and smoking (Anbarasi et al. 2006) all controlled in line with previous work in the field (Taylor et al. 2011; Taylor et al. 2012a; Taylor et al. 2012b), self-reported adherence was confirmed at 100% for all participants. Upon arrival, participants rested in a semi supine position to provide a venous blood sample via venepuncture from the antecubital fossa for analysis of eHsp72, followed by the assessment of maximal oxygen uptake [($\dot{V}O_{2max}$) as shown in Fig. 1b]. Participants were provided with the appropriate supplementation regime and information upon departure, and were required to adhere to instructions for the following 12-weeks.

Visit 2

Following 12-weeks of supplementation, participants returned to the laboratory and followed the same procedures as described for visit 1. Participants then departed the UK to undertake the MDS 2015 the following week.

Visit 3

The MDS 2015 took place from 5th – 11th April, and covered a total distance of 249.4 km across the Sahara Desert, Morocco (maximum temperature 39°C), over 7 days; each stage commenced at 0900h (Fig. 1a indicates distances for each stage of the MDS phase of the experimental design). The MDS required competitors to be self sufficient, meaning they were to carry their own food (minimum 2000

kcal per day), equipment, and sleeping materials for the duration of the race. Water was rationed to ~ 9.0 – 10.5 L/day per competitor, dependent on the distance of the stage.

Upon completion of the race, participants boarded coaches and were taken from the Desert back to the city of Ouarzazate, for post-race data collection (Fig. 1c). A team of trained experimenters collected post-race venous blood samples; this data collection took place 6 - 8 h post-race completion.

Visit 4

Finally, participants attended the sport science laboratories 7 days post-race, whereby venous blood samples and body composition were measured in line with visits 1 and 2. Participants were not required to undertake a $\dot{V}O_{2max}$ test during the final visit.

****Please insert Fig. 1a-c here****

$\dot{V}O_{2max}$ Test

A graded exercise test to maximal exhaustion on a motorized treadmill (Pulsar, HP Cosmos, UK) began with a 5 min self-paced warm up. Thereafter, speed was increased by 1 km/h^{-1} every 2 min, after 4 stages (8 min) speed remained constant and treadmill incline increased by 1% every 2 min until volitional exhaustion (Winter et al. 2006). Online breath-by-breath analysis (Metalyser 3B, Cortex, UK) was used to determine $\dot{V}O_{2max}$. Measures of HR (Polar, FS1, UK) and ratings of perceived exertion (RPE) were recorded every 2 min to be used as secondary criteria. The $\dot{V}O_{2max}$ was considered as the highest $\dot{V}O_2$ obtained in any 10 s period, and in line with end point criteria guidelines of the ACSM; which required participants to meet a plateau in $\dot{V}O_2$, plus 2 of the 3 following criteria: a failure of HR to increase with increasing exercise intensity, respiratory exchange ratio (RER) of > 1.15, and RPE > 17 (ACSM 2013).

Blood collection and analysis

Venous blood samples were collected at all four visits from the antecubital fossa via venepuncture (Safety blood collection set and holder, Vacuette®, Greiner Bio-One, UK), directly into three separate Vacuette® tubes (4 ml; Vacuette® Grenier Bio-One, UK) treated with K3 Ethylenediaminetetraacetic acid (EDTA) coagulant. Whole blood samples were centrifuged (EBA 200, Hettich, Germany) at 3000 rpm for 10 min for plasma separation, after which the plasma was aliquoted and stored at - 80°C until analysed in duplicate for eHsp72 using a commercially available high-sensitivity enzyme-linked immunosorbent assay (ELISA) kit. Whilst the measurement of plasma volume change would have been beneficial, the applied nature of the study and the logistical challenges present precluded the analysis of this variable. However, previous research has demonstrated that long-endurance exercise causes no significant increase in haemoglobin and haematocrit values (Gomez-Merino et al. 2006; Ruell et al. 2006; Suzuki et al. 2006), thus the changes may have been negligible (Suzuki et al. 2000; Suzuki et al. 2003). Incubation of the 96-well kit was performed on an incubital shaker (Heidolph Titramax 1000, Fischer Scientific, UK) at 500 rpm, and read by a plate reader (VICTOR™ X, Perkin Elmer, UK) using absorption at 450 nm. Intra-assay variability was 9.93% which was in line with previous work in the field: Campisi et al. (2003): < 10%; Gibson et al. (2014): 10.5%; Périard et al. (2012): 5%; Walsh et al. (2001): < 10%; Whitham et al. (2006): 6.3%.

Statistical analysis

All statistical analyses were performed using the *'psych'*, '*nlme*' and '*stats*' packages in R version 3.3.2 (R Core Development Team, 2014). Normality assumptions were checked using quantile-quantile plots. Normally distributed data is presented as mean standard deviation (SD), and non-normally distributed data is presented as median and range. The Akaike information criteria (AIC) was used to determine fit of the full model relative to the null model (Akaike 1976). A linear mixed model with fixed ('condition', 'time') and random ('subject id') effects was fitted with a compound symmetric correlation structure to determine the effect of PRO and PGLn on eHsp72 compared with CON [Time (4 levels): baseline, pre-race, 6-8 h post-race, 7-d post-race × Condition (3 levels): CON, PRO, PGLn]. In accordance with previous literature, eHsp72 concentrations were also presented as a percentage change from baseline, to account for high individual variance in baseline values and responses (Suzuki et al. 2006; Morton et al. 2007; Sandström et al. 2008; Taylor et al. 2010a; Taylor et al. 2010b; Peart et

al. 2011; Gibson et al. 2014). The sex of the participants (male or female) was not included as an independent variable due to the low number of females in the experiment (4 females, 30 males). A one-way analysis of variance (ANOVA) was used to compare time to completion (minutes) between the CON, PRO, and PGLn groups. The two-tailed alpha level of significance was set as $p \leq 0.05$, and 95% confidence intervals are presented to denote the imprecision in the point estimate.

Results

Anthropometric data

Participant characteristics are displayed in Table 1. For height, those in the PRO group were 5.3 ± 1.5 cm and 6.5 ± 1.6 cm taller than those in the CON and PGLn group, respectively ($F_{2, 125} = 9.372$, $p < 0.001$). For body mass at baseline, the mass of those in the PGLn group was 8.17 ± 2.46 kg and 8.50 ± 2.57 kg less than those in the PRO and CON group, respectively ($F_{2, 125} = 7.196$, $p = 0.001$). For body mass at pre-race, the mass of those in the PGLn group was 7.20 ± 2.35 kg and 7.40 ± 2.45 kg less than those in the PRO and CON group, respectively ($F_{2, 125} = 5.992$, $p = 0.003$). For $\dot{V}O_{2max}$ at baseline, those in the PGLn group had a score 5.33 ± 2.00 ml.kg.min^{-1} greater than those in the CON group ($F_{2, 125} = 3.909$, $p = 0.023$). There were no differences between groups for age and $\dot{V}O_{2max}$ at pre-race ($p > 0.05$).

eHsp72

The eHsp72 response was not different between nutritional groups (CON, PRO, PGLn). There was no main effect for group ($F_{1,2} = 3.252$, $p = 0.053$) or interaction (condition × time) effect ($F_{1,6} = 1.051$, $p = 0.399$) for eHsp72 responses. There was a main effect for time ($F_{1,3} = 22.716$, $p < 0.001$), showing that eHsp72 was elevated by 0.81 ± 0.36 ng·ml^{-1} (95% CI = 0.20 to 1.43 ng·ml^{-1}) at 6-8 h post-race compared with baseline ($p = 0.02$). The boxplots in Fig. 2 show the variability in the responses for each group over time. Table 2 displays the mean ± SD (range) of eHsp72 for each condition at each time point. Table 3 displays the model's fixed effects coefficients and random effect variances. Individual

responses over time are displayed in Fig. 3 a-c, where the change is expressed as a % change from baseline.

Time to completion

The time to completion was not different ($F_{1, 2}$ = 0.615, p = 0.548) between the CON (2819 ± 846 min, 95% CI = 2213 to 3425 min), PRO (2481 ± 525 min, 95% CI = 2105 to 3424 min), and PGLn (2570 ± 707 min, 95% CI = 2064 to 3075 min) groups. The boxplots in Fig. 4 show the variability in time to completion for each group.

**** Insert Table 2 near here please ****

****Insert Table 3 near here please****

**** Insert Fig 2, 3 a-c and 4 near here please ****

Discussion

The primary aim of this study was to investigate the effects of chronic PRO and PGLn commercially available supplementation on the eHsp72 response to the MDS. The experimental hypothesis was not accepted in this paper, as PRO and PGLn supplementation had no effect on eHsp72 concentration over the 12-week supplementation period. In addition, post-MDS eHsp72 concentration was significantly elevated in all groups, however there was no difference in the magnitude of change in the CON group compared to PRO or PGLn. These findings indicate that 12-week supplementation with PRO or PGLn had no influence on eHsp72 concentrations.

A mean 124% increase in eHsp72 concentration was seen across all conditions post-MDS, compared to pre-MDS values (Fig. 2a), supporting previous data related to the EHS response (Walsh et al. 2001; Gomez-Merino et al. 2006; Suzuki et al. 2006; Gibson et al. 2014). The magnitude of this response post-MDS was not different between groups, indicating that the utilised nutritional intervention had no influence on pre-MDS eHsp72 concentrations, despite a likely increase in iHsp72 pre-race (Tao et al.

2006). The novelty of the present study (i.e. MDS exercise model and 12-week nutritional interventions) renders it challenging to make direct comparisons to previous research. Indeed, to the author's knowledge only two previous studies have reported the eHsp72 response to ultra-endurance exercise performance (Gomez-Merino et al. 2006; Suzuki et al. 2006). These studies demonstrated an increased eHsp72 concentration of ~ 2200% (Suzuki et al. 2006) and ~ 1674% (Gomez-Merino et al. 2006) following an ironman triathlon and 100 km run, respectively.

The difference in magnitude of response between the present study (~124% increase) and others (Gomez-Merino et al. 2006; Suzuki et al. 2006) could be attributed to a number of factors. Firstly, the logistical issues associated with field-based research, particularly the MDS location, determined that post-exercise sampling was only feasible 6-8 h post-MDS. It is therefore possible that the greatest phase of the response was not recorded. Fehrenbach et al. (2005) demonstrated that eHsp72 concentration returned to baseline levels within 24 h of exercise completion, following a marathon run, with values already significantly different at 3 h post-exercise in comparison to immediately post-exercise. It is therefore likely that the 6-8 h time course between MDS completion and sample collection in the present study elicited lower eHsp72 concentration changes from pre-race in comparison to the values that would have been displayed immediately post-race. Future research should aim to collect data as close to race completion as possible to obtain the most representative results.

Secondly, the difference may be due to a habituation effect, given the consecutive and multi-day nature of the MDS compared to the discrete within one-day ironman triathlon and 100 km run. Following 7 consecutive days of EHS, participants would likely begin to undergo heat acclimatisation, given heat acclimation/acclimatisation has been shown to commence in as few as 4 exercise-heat exposures (Petersen et al. 2010), with 'full' heat acclimation taking typically 7-14 days (Tyler et al. 2016). Thus, by the 7th consecutive day of EHS, it is likely that participants would have developed a level of acclimation to these conditions. Consequently, resting eHsp72 concentration prior to the commencement of the final MDS stage would potentially have been greater than recorded pre-race values. For example, Sandström et al. (2008) investigated the effects of 15 consecutive days heat acclimation on eHsp70, and demonstrated the impact of acclimation upon eHsp70 concentration. In

that paper, resting eHsp70 following 7 days heat acclimation was 45% greater than baseline concentration. In addition, the pre- to post-acclimation change on day 1 (54%) showed a greater effect in comparison to that of day 7 (-4.5%). This speculation could aid understanding regarding the less substantial increase in eHsp72 post-exercise in comparison to previous ultra-endurance research (Gomez-Merino et al. 2006; Suzuki et al. 2006). It is possible that the MDS induced a level of acclimatisation, which subsequently impacted the eHsp72 concentrations recorded following completion of the race, masking any influence of the probiotic supplement. However, as all participants completed the race, it is likely that the possible heat acclimatisation stimulus of MDS completion, and any subsequent confounding effects of such acclimatisation, would have been similar across participants. Whether within-race heat acclimatisation did mask any potential benefits of the probiotics cannot be excluded within the present design. Future research is recommended to analyse additional markers (such as IL-6, TNF- α), in order to obtain a greater understanding regarding the response to ultra-endurance exercise and probiotic supplementation.

Previous *in vitro* research has suggested that a key mechanism behind the protective role of probiotic (Petrof et al. 2004; Tao et al. 2006) and glutamine (Wischmeyer et al. 2001) is elevations in basal iHsp72 concentrations. Tao et al. (2006) stated that the induction of iHsp72 following probiotic treatment *in vitro* may be due to changes in gene transcription through binding of heat shock factor-1, a key activator responsible for transcription of heat shock genes (Zuhl et al. 2014). Because the systematic concentrations of Hsp72 may be proportional to the iHsp72 concentration, it was important to investigate if probiotic supplementation also influenced eHsp72 concentrations. In contrast to previous *in vitro* research (Petrof et al. 2004; Tao et al. 2006), the present study utilised commercially available supplements, which may have contained additional ingredients and subsequently impacted the response (Maughan 2005). Future research utilising similar experimental designs should develop a standardised and pharmacologically optimised probiotic supplement strain (which may likely be hybridised), to avoid the previously documented lack of quality control of probiotics (Tuomola et al. 2001). It may also be suggested that the prescribed glutamine dosage was insufficient to induce a significant eHsp72 response *in vivo*. Previous research has utilised a dosage relative to the individuals' body mass (Zuhl et al. 2014; Zuhl et al. 2015), however the present study implemented the suppliers' recommended dosage, and subsequently may have been insufficient to induce a significant response.

Should the present study have implemented the individualised dosages as utilised by Zuhl et al. (2014), dosages would range from 19.75 – 67.05 g PRO/PGLn supplementation per day (based on fat free mass range 21.95 – 74.5 kg of participants in the present study). However, only 0.9 g/day was administered in this study, demonstrating a deviation of 18.85 – 66.15 g/day from the aforementioned dosage (Zuhl et al. 2014). Evidently, these differences are substantial, and thus the effect of PRO and PGLn from the dosage utilised in the present study may have been insufficient to induce a significant response. It is important to note, however, that competitors would have utilised the commercially available dietary supplements in the same manner (i.e. in line with the supplier recommendations) as the design employed by the present study. Another consideration is the makeup of the probiotic supplement. Whilst there are limited studies in this area (particularly using the specific strains supplied in the present study), the predominance of Lactobacillus and Bifidobacterium bifidum species in this supplement indicate that these would have the strongest influence upon the eHsp72 response (if any). Although the lactobacillus and Bifidobacterium strains activate the innate immune system (Bellavia et al. 2013; Lescheid 2014), it is unclear what specific strains interact with eHsp72. Additionally, whilst there was a level of prebiotic included within the supplement, this very small dosage (55.8 mg.d-1 fructooligosaccharides; FOS) is unlikely to have had an effect on eHsp72 concentration.

It is evident that participation in a multi-day ultra-endurance event imposes a significant level of stress upon the human body, leading to an increase in eHsp72 concentration. Such increases are likely for stimulation of pro-inflammatory cytokine and chemokines, which is typical during EHS. However, the implementation of commercially available PRO and PGLn supplementation, in line with supplier recommendations, did not increase basal eHsp72 concentration prior to the race, or alter the eHsp72 response seen in all groups post-race.

Experimental limitations, practical applications and future research directions

Collection of data within an applied setting involves a number of challenges, particularly when in a harsh environment, such as the Sahara Desert. Due to methodological limitations in the present work, it is recommended that future research collect samples immediately / within 1 h of race completion, and should implement a dosage relative to the body mass of the individual taking part, in addition to

utilising robustly standardised and optimised nutritional supplements. This will confirm if PRO or PGLn increases basal eHsp72 values prior to ultra-endurance events, particularly those completed within thermally challenging environments. Additionally, future research should focus on controlling the outlined confounding variables alongside analysis of additional biological markers, such as inflammatory markers (IL-6, TNF- α), intracellular markers (iHsp72), and markers of endotoxemia/gut damage (LPS); to greater understand the implicated biological cascades such as gut damage. To increase internal validity within similar research designs, all participants should complete the exact same robust heat acclimation regime (or completely avoid any acclimation) prior to leaving for the MDS, to control the potential confounding influence of heat acclimation/acclimatisation upon eHsp72 concentrations pre and during race. Therefore, whilst the presented results should be interpreted carefully relative to these limitations, the findings remain relevant to athletes and coaches, given we have shown that the specific commercially available probiotic supplements and chronic administration strategies utilised are unlikely to interfere with the pathways regulating immune responses to multi-day ultra-endurance running exercise in the heat.

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Table 1. Anthropometrical characteristics of age, height, mass; body fat percentage, and $\dot{V}O_{2max}$ data (week 1 and week 12) are reported as means ± SD (age is reported as range due to non-normal distribution).

		Baseline		Pre-race	
Group	**Age (years)**	**Mass (Kg)**	**$\dot{V}O_{2max}$ (ml.kg.min^{-1})**	**Mass (Kg)**	**$\dot{V}O_{2max}$ (ml.kg.min^{-1})**
PRO	25 – 50	78.87 ± 6.93	56.42 ± 8.26	77.49 ± 6.52	59.40 ± 6.33
PGLn	31 – 53	71.34 ± 12.92	56.50 ± 5.95	70.09 ± 12.31	59.25 ± 6.92
CON	23 – 60	79.50 ± 14.07	51.17 ± 12.68	77.29 ± 12.92	55.25 ± 11.96

Table 2. Mean ± SD (range) eHsp72 (ng.ml^{-1}) concentration between groups at each data collection time point.

Group	**Baseline**	**Pre-race**	**6 hours post race**	**7 d post-race**
CON	1.68 ± 1.65 (0.59 – 3.81)	1.82 ± 1.98 (0.63 – 1.93)	3.45 ± 3.35 (0.78 – 3.30)	2.05 ± 2.49 (0.67 – 2.17)
PRO	1.20 ± 0.58 (0.68 – 2.70)	1.58 ± 0.87 (0.75 – 2.96)	2.77 ± 1.21 (1.82 – 7.30)	1.80 ± 1.27 (0.82 – 4.13)
PGLn	1.77 ± 2.51 (0.59 – 1.90)	1.03 ± 0.45 (0.73 – 2.19)	2.20 ± 0.76 (1.57 – 4.16)	1.04 ± 0.41 (0.71 – 2.16)

Kg: kilograms; **$\dot{V}O_{2max}$:** maximal volume of oxygen consumption; **ml.kg.min^{-1}:** millilitres per kilogram per minute. **PRO:** Probiotic; **PGLn:** Probiotic + Glutamine; **CON:** Control

Table 3. Beta coefficients (*B*), 95 % confidence intervals (CI), and alpha values (*p*) are reported for the fixed components (Supplement & time) before and after the MDS race. The standard deviation of the intercept and residual are reported for the random effect (subject ID).

	eHsp72 ($ng.ml^{-1}$)		
	B	*95% CI*	*p*
Fixed Parts			
(Intercept)	1.31	0.79 – 1.83	**<.001**
Condition Effect			
PRO	0.02	-0.72 – 0.75	0.968
PGLn	-0.32	-1.09 – 0.45	0.421
Time Effect			
Pre-Race	-0.1	-0.70 – 0.50	0.742
6-8 h Post-Race	0.81	0.20 – 1.43	**0.011**
1-Week Post-Race	-0.04	-0.67 – 0.60	0.911
Condition: Time Interaction			
PRO: Pre-Race	0.27	-0.59 – 1.13	0.541
PGLn: Pre-Race	0.13	-0.75 – 1.01	0.770
PRO: 6-8 h Post-Race	1.01	0.15 – 1.87	**0.023**
PGLn: 6-8 h Post-Race	0.38	-0.51 – 1.28	0.401
PRO: 1-Week Post-Race	0.6	-0.31 – 1.50	0.198
PGLn: 1-Week Post-Race	0.09	-0.82 – 1.00	0.851
Random Parts			
σ^2		0.511	
$\tau_{00,\ ID}$		0.258	

673 **Figure legends**
674
675 **Fig. 1 a-c** Experimental schematic outlining: a: the full experimental study design, b: procedures of
676 Visits 1, 2 and 4, c: procedure of Visit 3
677
678 **Fig. 2** Boxplots showing the variability in responses for each group over time. #Denotes a significant
679 effect of time on eHsp72 concentration
680
681 **Fig. 3 a-c** Individual eHsp72 responses within groups, as a % change from baseline (a: PRO, b: PGLn,
682 c: CON)
683
684 **Fig. 4** Boxplots showing the variability in time to completion for each group

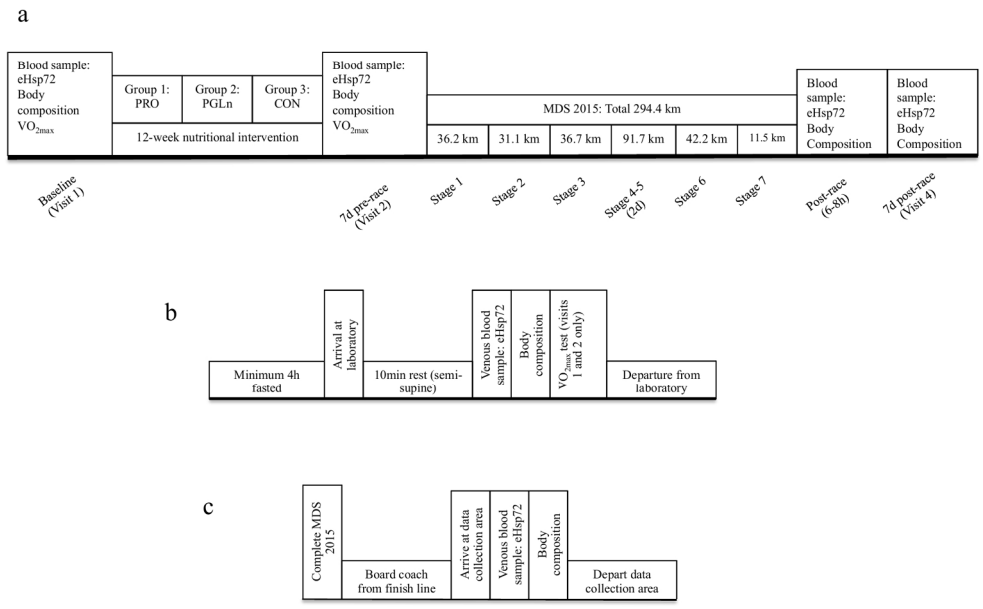

Fig. 1 a-c Experimental schematic outlining: a: the full experimental study design, b: procedures of Visits 1, 2 and 4, c: procedure of Visit 3

254x190mm (200 x 200 DPI)

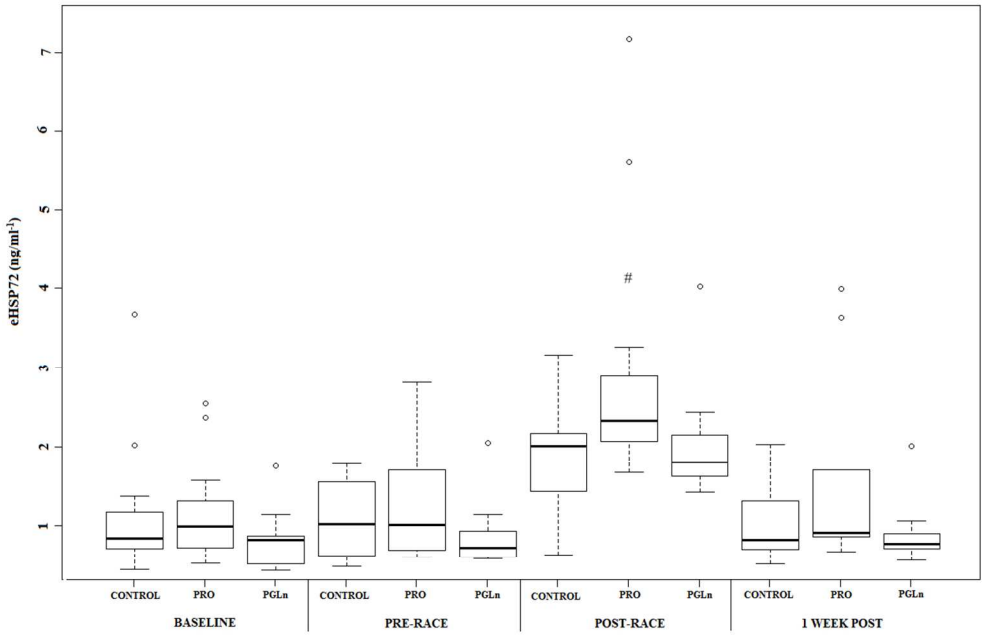

Fig. 2 Boxplots showing the variability in responses for each group over time. #Denotes a significant effect of time on eHsp72 concentration

336x222mm (96 x 96 DPI)

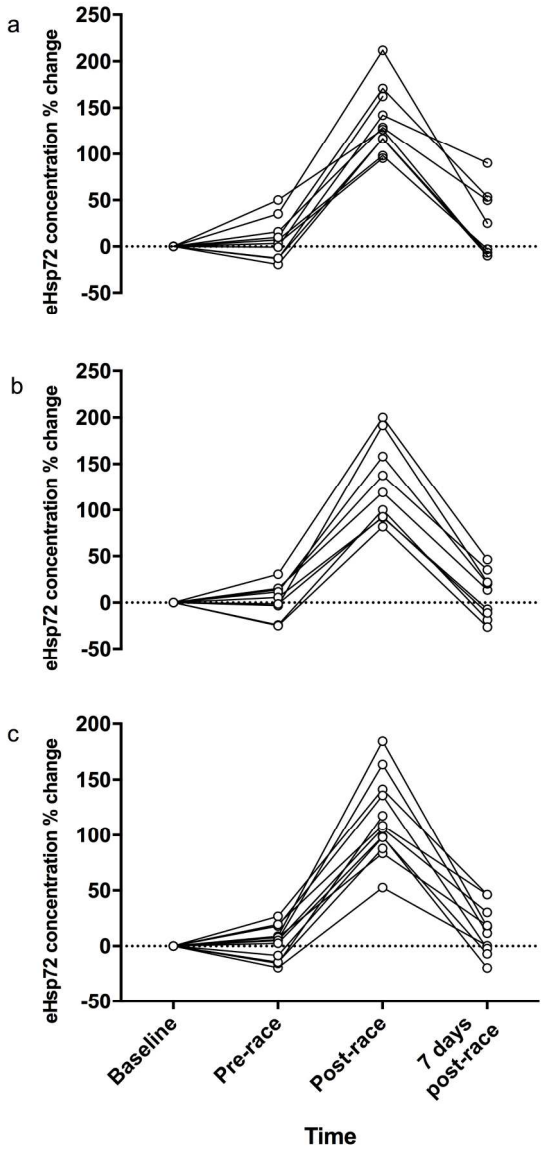

Fig. 3 a-c Individual eHsp72 responses within groups, as a % change from baseline (a: PRO, b: PGLn, c: CON)

110x233mm (300 x 300 DPI)

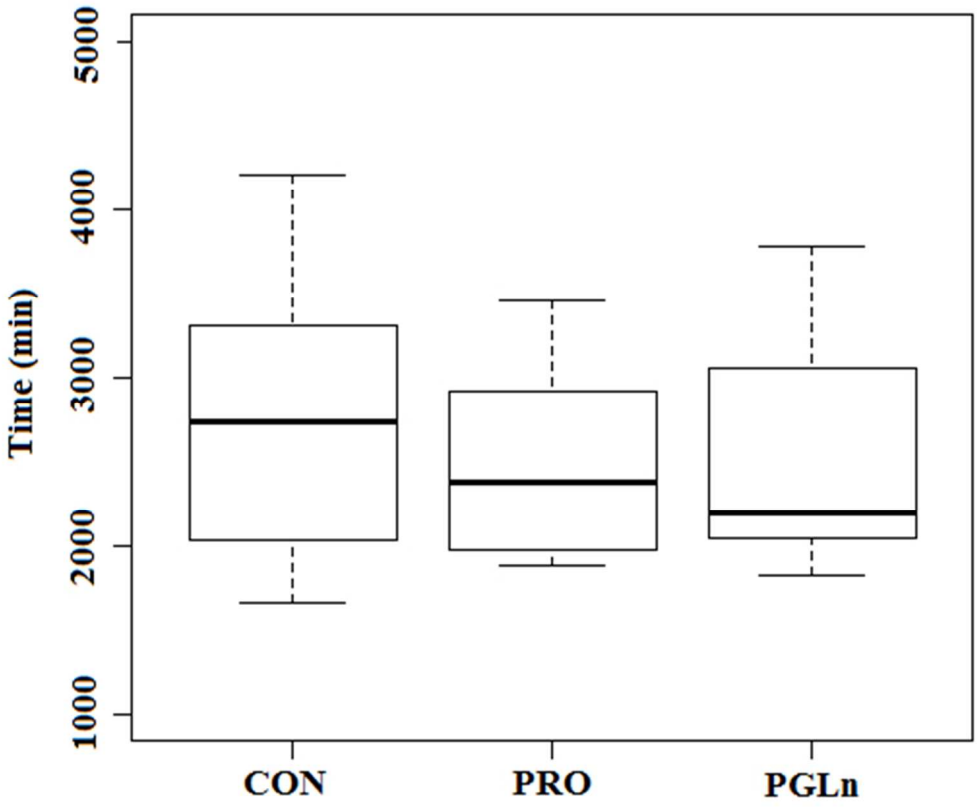

Fig. 4 Boxplots showing the variability in time to completion for each group

136x116mm (96 x 96 DPI)