

TITLE

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1 **Title:** Beetroot supplementation improves the physiological responses to incline walking

2

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26

27 **Abstract**

28 **Purpose:** We investigated the effects of an acute 24-h nitrate-rich beetroot juice supplement
29 (BR) on the energy cost, exercise efficiency and blood pressure responses to intermittent
30 walking at different gradients. **Methods:** In a double-blind, cross-over design, eight
31 participants were provided with a total of 350 ml of nitrate-rich (~20.5 mmol nitrate) BR or
32 placebo (PLA) across 24-h before completing intermittent walking at 3 km/h on treadmill at
33 gradients of 1%, 5%, 10%, 15% and 20%. **Results:** Resting mean arterial pressure (MAP)
34 was ~4.1% lower after BR (93 vs. 89 mmHg; P = 0.001), as well as during exercise (102 vs.
35 99 mmHg; P = 0.011) and recovery (97 vs. 94 mmHg; P = 0.001). Exercising (1227 vs. 1129
36 ml/min P < 0.001) and end-stage (1404 vs. 1249 ml/min; P = 0.002) oxygen uptake ($\dot{V}O_2$) was
37 lower in BR compared to PLA, which was accompanied by an average reduction in phase II
38 $\dot{V}O_2$ amplitude (1067 vs. 940 ml/min; P = 0.025). Similarly, recovery $\dot{V}O_2$ (509 vs. 458
39 ml/min; P = 0.001) was lower in BR. Whole-blood potassium concentration increased from
40 pre-post exercise in PLA (4.1 ± 0.3 vs. 4.5 ± 0.3 mmol/L; P = 0.013) but not BR (4.1 ± 0.31
41 vs. 4.3 ± 0.2 mmol/L; P = 0.188). **Conclusions:** Energy cost of exercise, recovery of $\dot{V}O_2$,
42 MAP and blood markers were ameliorated after BR. Previously reported mechanisms explain
43 these findings, which are more noticeable during less efficient walking at steep gradients (15-
44 20%). These findings have practical implications for hill-walkers.

45

46 **Key words:** Nitrate; efficiency; energy cost; blood pressure.

47

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49

50

51 **Abbreviations**

- 52 Adenosine triphosphate (ATP)
- 53 Beetroot juice (BR)
- 54 Blood lactate concentration (B[La])
- 55 Blood potassium concentration ($[K^+]$)
- 56 Blood pressure (BP)
- 57 Coefficient of variation (CV%)
- 58 Diastolic (DP)
- 59 Energy cost of walking (Cw)
- 60 Glucose concentration ([Glu])
- 61 Gross efficiency (GE%)
- 62 Heart rate (HR)
- 63 Mean arterial pressure (MAP)
- 64 Nitrate (NO_3^-)
- 65 Nitrite (NO_2^-)
- 66 Nitric oxide (NO)
- 67 Oxygen uptake ($\dot{V}O_2$)
- 68 Placebo (PLA)
- 69 Rating of perceived exertion (RPE)
- 70 Sarcoplasmic reticulum calcium (SR- Ca^{2+})
- 71 Systolic pressure (SP)
- 72 Ventilatory threshold (V_T)
- 73
- 74
- 75
- 76
- 77

78 **Introduction**

79 Supplementation of inorganic nitrate (NO_3^-), in the form of a highly-concentrated beetroot
80 juice (BR) supplement (5.2 - 8.2 mmol of NO_3^- per day), improves endurance performance
81 (Bailey et al. 2009, 2010, 2015; Bryan et al. 2008; Lansley et al. 2011a; Wylie et al. 2013).
82 These effects are thought to be facilitated by the oxygen independent reductions of NO_3^- to
83 nitrite (NO_2^-) and nitric oxide (NO) (Bailey et al. 2012). The NO_3^- - NO_2^- -NO cascade
84 increases circulating NO_2^- , which can be further reduced to NO under certain physiological
85 conditions (Bryan et al. 2008). Thus, the availability of NO can be preserved when enzymatic
86 synthesis of NO via nitric oxide synthase (NOS) has been compromised (Bryan et al. 2008).
87 The role of the NO_3^- - NO_2^- -NO pathway is potentiated during intense exercise, in particular,
88 owing to the mild hypoxic and/or acidic conditions induced in the working skeletal muscle
89 (Jones et al. 2016).

90

91 Nitric oxide is a key regulator of blood pressure (BP) and a signalling molecule for smooth
92 muscle surrounding the endothelium (Gilchrist et al. 2011). These mechanisms permit
93 vasodilation of blood vessels and enhance blood flow to the working musculature (Ferguson
94 et al. 2013). Accordingly, reduced BP is common after NO_3^- supplementation during rest
95 (Bailey et al. 2009; Bond et al. 2013, 2014; Kapil et al. 2010; Vanhatalo et al. 2010; Webb et
96 al. 2008) and exercise (Bond et al. 2013, 2014; Choi et al. 2016; Kenjale et al. 2011; Oggioni
97 et al. 2017). The combination of increased muscle blood flow and reduced oxygen extraction
98 at the site of the working musculature leads to enhanced exercise tolerance (Bailey et al.
99 2010). The latter finding has been attributed to an enhanced efficiency of oxidative adenosine
100 triphosphate (ATP) turnover in the mitochondria (Larsen et al. 2011). Mammalian studies
101 have demonstrated that other factors, such as improved sarcoplasmic reticulum calcium (SR-
102 Ca^{2+}) handling, might also contribute to improved exercise tolerance following NO_3^-

103 supplementation (Hernández et al. 2011). However, recent studies have demonstrated that
104 longer (> 7 days) supplementation periods would be necessary to induce such changes in
105 calcium handling proteins and point to alternative mechanisms, such as increased emission of
106 mitochondrial reactive oxygen species and their possible effects on muscle contractile
107 apparatus (Whifield et al. 2016). Nevertheless, these mechanisms contribute to a decreased
108 ATP turnover for a given power output (Bailey et al. 2010), thus improving exercise
109 economy across a variety of work-rates, with a notable 12 % reduction in the O₂ cost ($\dot{V}O_2$
110 amplitude) of walking at fixed gradients of 1% (Lansley et al. 2011b) and preferable
111 alterations in the pulmonary oxygen uptake ($\dot{V}O_2$) kinetics of older adults (Kelly et al. 2013).

112

113 BR supplementation has not improved endurance performance or lowered exercising $\dot{V}O_2$ in
114 all cases, particularly when administered to elite endurance athletes (Christensen et al. 2012;
115 Peacock et al. 2012; Boorsma et al. 2014). The inconsistent findings have been ascribed to
116 the aerobic fitness level of the athletes (Porcelli et al. 2015) and might relate to the lower
117 reliance on the NO₃⁻-NO₂⁻-NO pathway or the presumed higher proportions of type I muscle
118 fibre among well-trained endurance athletes. This is consistent with the evidence that the
119 effects of NO₃⁻ are potentiated in type II muscle fibre (Hernández et al. 2011; Ferguson et al.
120 2013). As such, certain forms of locomotion that mandate the recruitment of type II muscle
121 fibre could be affected differently by BR supplementation compared to those without,
122 particularly when performed by non-elite athletes.

123

124 Incline walking is a popular and effective mode of exercise for physical fitness (Ainslie et al.
125 2002, 2005; Gottschall and Kram 2006) but will present a challenge to certain populations or
126 vocations. The energy cost of walking (C_w) increases as a function of surface incline (Minetti
127 et al. 2006). The external work done to walk on gradients between 1% and 20% comprises a

128 combination of negative and positive contributions (Minetti et al. 2006). On extreme uphill
129 slopes (20%), this work becomes entirely positive and reaches a mechanical efficiency (i.e.
130 ratio of mechanical work for vertical displacement to the energy expended) equivalent to
131 concentric muscle contraction (Margaria et al. 1963). Negative work is more efficient (~
132 80%), yet diminishes as a function of walking gradient, meaning there is a greater reliance on
133 less efficient (~ 25%) positive work (Minetti et al. 2006). When walking uphill at
134 progressively steeper inclines, muscle recruitment strategies are proportionally altered to
135 coincide with the leading leg performing longer and more forceful concentric muscle
136 contractions (i.e. more positive work) (Gottschall and Kram 2006). Consequently, greater
137 lower-limb muscle activation (Vernillo et al. 2017), anaerobic metabolism (Sloniger et al.
138 1997; Staab et al. 1992) and perception of effort (Kolkhorst et al. 1996) is evident during
139 uphill locomotion, compared to level exercise at the same speed. These observations reflect a
140 change in the muscle recruitment patterns of the lower limbs and a logical reliance on type II
141 muscle fibres to support the more forceful and concentrically-biased muscle contraction.

142

143 Whilst it is known that dietary NO_3^- can enhance muscle efficiency in humans, rodent studies
144 have shown that NO_3^- supplementation can improve muscle O_2 delivery in musculature with a
145 high type II fibre distribution (Ferguson et al. 2013), where it also improves SR- Ca^{2+} storage
146 and release (Hernández et al. 2011). It has recently been proposed that the ergogenic
147 properties of NO_3^- are fibre type dependent (Jones et al. 2016). In support of this, Breese et al.
148 (2013) reported faster $\dot{V}\text{O}_2$ kinetics and improved time to exhaustion during step-exercise,
149 designed to elicit the recruitment of higher order motor units. It is therefore feasible that
150 consumption of NO_3^- can improve the efficiency of uphill walking, owing to the constrained
151 changes in gait pattern toward a less efficient form of locomotion and an increased reliance
152 on type II muscle fibres as the gradient increases. Indeed, a recent study demonstrated

153 lowered $\dot{V}O_2$ after BR supplementation during loaded uphill walking at 10% gradients in
154 hypoxic (fraction of inspired $O_2 = 11.7\%$) conditions (Shannon et al. 2017). However, the
155 effects of BR supplementation on the physiological responses to incline walking at different
156 gradients have not been systematically investigated among unloaded subjects in normoxic
157 conditions. Accordingly, this study aimed to assess the effects of an acute 24-h NO_3^- -rich BR
158 supplement on the energy cost, exercise efficiency and BP responses to intermittent walking
159 at different gradients. We hypothesized that the BR supplement would reduce the cost of
160 exercise as a function of the exercise intensity and ameliorate recovery between exercise
161 bouts, including a reduction in BP during rest and exercise.

162

163 **Methods**

164

165 *Participants and design*

166 Eight participants (5 male (age 27 ± 2 years; stature 1.74 ± 0.09 m; body mass 81.4 ± 7.3 kg;
167 $\dot{V}O_{2max}$ 56.8 ± 7.8 ml/kg/min) and 3 female (age 29 ± 5 years; stature 166 ± 4 cm; body mass
168 60.0 ± 6.1 kg; $\dot{V}O_{2max}$ 38.0 ± 10.4 ml/kg/min) provided written informed consent to take part
169 in this study. The participants were all recreationally active and regularly hill-walked as part
170 of their occupation or leisure activities. The participants were all naive to the effects of
171 beetroot juice on exercise. After baseline measures and familiarisation, in a double-blind,
172 randomized cross-over design, the participants were either supplemented with a total of 350
173 ml of BR, in the form of beetroot shots, which were consumed at three evenly-spaced periods
174 in the 24-h before the exercise trial, or a placebo (PLA), one week apart. After 24-h of
175 supplementation, the participants completed a randomized, intermittent, inclined walking
176 protocol on a treadmill, while their physiological ($\dot{V}O_2$, blood lactate concentration (B[La]),
177 blood potassium concentration ($[K^+]$), heart rate (HR) and continuous BP), perceptual (rating

178 of perceived exertion; RPE) and gross mechanical work was measured. The participants were
179 instructed to arrive at least 3-h post prandial, abstain from heavy exercise, caffeine intake,
180 anti-bacterial mouthwash and NO₃⁻-rich foods in the 48-h before and during the study. Food
181 diaries were completed to ensure compliance with these instructions. This study was granted
182 institutional ethical approval and was conducted in accordance with the ethical standards laid
183 down in the 1964 Declaration of Helsinki.

184

185 **Procedure**

186 *Baseline measurements*

187 The participants visited the laboratory on three occasions. On their first visit, the participants
188 were screened to ensure low-risk stratification, including assessment of resting BP and the
189 completion of extended health questionnaires (Exercise Sport Science Australia). Resting BP
190 was measured from the left brachial artery, using an automated sphygmomanometer (Omron
191 M7 BP Monitor, OMRON Healthcare Europe, Hoofddrop, Netherlands) after sitting for 15-
192 min in a quiet room. The average systolic (SP), diastolic (DP) and mean arterial pressures
193 (MAP) of three measurements was recorded, calculated as: (MAP) (MAP = DBP + 0.33
194 (SBP-DBP)). This method of BP measurement was adopted at baseline, for screening
195 purposes, whereas continuous measurements were performed for all subsequent visits. A HR
196 monitor was strapped across the participants' chest and synchronized with a wrist watch
197 (V800, Polar, Kempele, Finland) to record resting HR. Whilst seated in the same position,
198 baseline venous blood was drawn from the participants' right arm at the antecubital fossa,
199 using a hypodermic needle and a lithium-heparinized vacutainer (4 ml). The whole blood was
200 immediately tested for blood glucose ([Glu]) and [K⁺] concentrations using a mobile analyser
201 (iSTAT analyser, Abbott Point of Care Inc, Princeton, NJ) with a manufacturer-reported
202 coefficient of variation (CV%) of 1.1%. The remaining and whole blood was then centrifuged

203 at 4,000 r/min and 4 °C for 10-min, after which the plasma was immediately stored at -80 °C.
204 Blood plasma was later thawed and analysed for NO₃⁻ using a colorimetric assay, with an
205 intra-assay CV% of 2.7, and an ELISA with an intra-assay CV% of 6.3, respectively (see
206 http://www.abnova.com/products/products_detail.asp?catalog_id=KA1342). Plasma insulin
207 was measured using a solid phase two-site enzyme immunoassay, with an intra-assay CV%
208 of 6.4 and an inter-assay CV of 8.5 (see
209 http://www.abnova.com/products/products_detail.asp?catalog_id=KA0921).

210

211 The participants also completed an incremental, maximal treadmill test on their first visit, to
212 establish $\dot{V}O_{2max}$ and ventilatory threshold (V_T). An adapted Bruce protocol was performed on
213 a calibrated motorized treadmill (Mercury® Med, hpcosmos sports & medical gmbh;
214 Nussdorf-Traunstein, Germany), beginning at 2.7 km/h and 0% gradient and increasing by
215 speed and gradient every 3-min until volitional exhaustion. This test was selected to
216 familiarize the participants to gradient walking, as well as incorporating a warm-up stage into
217 the test and providing a measurement of $\dot{V}O_{2max}$. Pulmonary gas was measured continuously
218 using a breath-by-breath gas analyser (Jaegar, Oxycon Pro, Viasys Healthcare, Hoechberg,
219 Germany). The gas analyser and flow turbine were calibrated before each test using a known
220 gas mixture (15% O₂ and 5% CO₂) and a 3-L syringe, respectively (Hans Rudolph, Kansas
221 City, KS). $\dot{V}O_{2max}$ was determined as the mean value recorded over the final 30-s of the test.
222 The same gas analyser was used throughout the study and calibrated identically, with a CV%
223 of 4.3. The V_T was determined using the V-slope method, which has previously been used
224 during 3-min incremental stages (Amman et al. 2004). The vertical mechanical power output
225 (\dot{W}) was calculated at the V_T according to Minetti et al. (2006) as:

226

227 $\dot{W}_{vert} = gv \sin (\text{arc tan } [i])$ [Eq. 1]

228

229 where g = gravity (m/s/s), v = treadmill velocity (m/s) and i = the treadmill gradient.

230

231 The power output was subsequently used to delineate moderate and heavy exercise domains.

232 The aim of the final experimental protocol was to exercise participants below the heavy

233 exercise domain, at fixed gradients and speeds. As such, the power output at V_T was

234 measured to ensure that it was higher than the highest power output used in the final test

235 protocol. The mean of the highest power output to be performed during the experimental trial

236 was calculated *a-priori* as 108 ± 18 W, while the mean power output at V_T was 165 ± 29 W,

237 with no participants achieving a V_T power output lower than the highest power output in the

238 experimental protocol. Therefore, all participants were exercising below the heavy intensity

239 domain during the study. The mean $\% \dot{V}O_{2\max}$ during the highest intensity (20 % gradient)

240 trials was 52 ± 14 % and 48 ± 13 % in the PLA and BR conditions, respectively.

241

242 ***Experimental protocol***

243 At baseline testing, the participants were randomly assigned to one of the two conditions and

244 returned to the laboratory 24-h later (am) to receive their first of three supplements, in the

245 form of two NO_3^- -rich BR shots (2 x 70 ml ~ 4.1 mmol NO_3^- per shot; Beet it, James White

246 Drinks Ltd., Ipswich, UK) or PLA (isocaloric fruit juice and maltodextrin mixture). The

247 drinks were opened 10-min before their arrival at the laboratory and provided in identical

248 opaque containers. The drinks were consumed within 30-s by all participants. The

249 participants visited the laboratory to take their supplements on two further occasions across

250 the subsequent 24-h period, with the second beverage (2 x 70 ml ~ 4.1 mmol NO_3^- per shot or

251 PLA) consumed mid-way between the first and final serving. The final drink (1 x 70 ml ~ 4.1

252 mmol NO_3^- per shot or PLA) was consumed 1.5-h prior to testing (~2-2.5-h prior to exercise).

253

254 On the morning of each visit to the laboratory, resting BP, HR was measured and venous
255 blood was drawn after 15-min of seated rest. The participants then mounted the treadmill and
256 were fitted with a continuous BP monitor (Finometer MIDI, Finapres Medical Systems,
257 Amsterdam, The Netherlands), designed to measure brachial BP using photoelectric
258 plethysmography of the finger pressure waveform (Wesseling 1995). The Finapres® device
259 was fitted to the left arm of each participant. Specifically, the device was fitted by securing an
260 inflatable plastic bladder (50 μ m and 55 mm length) between the distal and proximal inter-
261 phalangeal joint of the middle finger, which housed an infrared light-emitting diode (950 nm
262 emission) and detector. This was connected to a front-end unit via an air hose. The front-end
263 unit was attached to the left wrist using a Velcro strap. The front end was connected to the
264 main unit and pump inside the Finometer MIDI. A height correction unit was also fitted to
265 the front-end unit to automatically correct for hydrostatic pressure changes due to movement
266 in hand position relative to the heart. During the experimental protocol, the participants
267 walked with a natural arm carriage, with their fingers relaxed and without holding on to the
268 treadmill. A laptop was connected to the Finometer MIDI and the raw 200 Hz data were
269 analysed using the BeatScope® software (Version 1.1, Finapres Medical Systems BV,
270 Arnhem, Netherlands). BeatScope® software was used to reconstruct mean brachial pressure
271 from the finger-pulse pressure waveform. The Finapres® was activated 1-min prior to
272 recording to allow for calibration. The calibration is performed automatically through the
273 PhysioCal software in the device, which was activated during the trials. Any interruption of
274 the recording owing to calibration was removed from the data set. No further filtering was
275 applied to the data. MAP was reported during exercising and resting conditions. The inter-
276 day walking MAP CV of this device ranged from 1.6-3.2 %, increasing as a function of
277 exercise intensity (i.e. gradient).

278

279 Prior to exercise, a finger-prick capillary sample of blood was then drawn from the index
280 finger of the participant whilst standing on the treadmill, which was measured for B[La]
281 using a hand-held analyser (Lactate Pro; Arkray KDK Corp., Kyoto, Japan), which has a
282 CV% of 3.1. The participants were then fitted with the face mask of the breath-by-breath gas
283 analyser, where 3-min measurements of $\dot{V}O_2$, respiratory exchange ratio (RER), HR, and
284 continuous BP were recorded during quiet standing. These measurements were continued for
285 the remainder of the protocol. After quiet standing measurements, the participants began the
286 intermittent walking trial, which comprised 5 × 5-min bouts of walking at randomized 1%,
287 5%, 10%, 15% or 20% inclines, at a fixed speed of 3 km/h, with each exercise stage
288 interspersed by 5-min of quiet standing. No talking was permitted by participants during the
289 trial. B[La] was measured 1-min post exercise bout, while RPE was provided by the
290 participants in the final 15-s of each bout. Final venous blood draws were performed no more
291 than 5-min after the final exercise stage.

292

293 The rest-recovery configuration of the protocol permitted the measurement of on-pulmonary
294 $\dot{V}O_2$ kinetics during intermittent exercise and prevented interference of $\dot{V}O_2$ responses
295 between repeated exercising bouts. Raw $\dot{V}O_2$ data were filtered to remove errant breaths more
296 than 4 SD from the local mean. After interpolating breath-by-breath data to 1-s values, the
297 $\dot{V}O_2$ on-kinetics were modelled using a non-linear least squares fitting procedure in a custom
298 programme (Microsoft Excel). After excluding the first 20-s of data on-transients, a mono-
299 exponential model was used to characterize all trials, as they were performed below the
300 heavy intensity domain. On-transients were modelled based eq. 2:

301

302
$$\Delta\dot{V}O_2(t) = \text{base}\dot{V}O_2 + A [1 - e^{-(t - TD)/\tau}] \quad [\text{Eq. 2}]$$

303

304 Where $\Delta\dot{V}O_2(t)$ = absolute $\dot{V}O_2$ at a given time t ; $\text{base}\dot{V}O_2$ = baseline resting $\dot{V}O_2$; τ =
305 fundamental time constant; TD = time delay and $A = \dot{V}O_2$ amplitude (i.e. $\Delta\dot{V}O_2 > \text{base}\dot{V}O_2$).

306

307 The overall mean response time (MRT) was resolved by fitting a mono-exponential model to
308 the $\dot{V}O_2$ data from 0-s to end exercise, without adding the time delay. The external
309 mechanical work (W) was calculated based on published equations (Minetti et al. 2006; see
310 Eq.1) and was used to determine gross efficiency (GE%) as the ratio of mechanical work
311 done and metabolic energy, as measured via gas analysis. The energy cost of walking (Cw)
312 was expressed as in joules (J) / kg body mass / min (J/kg/min), using the thermal equivalent
313 of oxygen (20.9 J/L O₂) (Minetti et al. 2006).

314

315 **Statistical analyses**

316 All data are presented as means \pm SD, unless otherwise stated. A two-way repeated measures
317 analysis of variance was used to evaluate the effects of condition (BR or PLA), gradient (1%,
318 5%, 10%, 15% and 20%) and their interaction. If tests of Sphericity were violated, the
319 Greenhouse-Geisser correction was used. In the event a statistical difference was identified, a
320 *post-hoc* Bonferroni test was used to identify differences. An alpha level of $P < 0.05$ was set
321 for all analyses. Effect sizes (d), calculated as the mean difference divided by the pooled
322 standard deviation of scores, were included for pairwise comparisons to provide information
323 on the magnitude of any effect. Effect sizes were defined as: trivial = 0.2; small = 0.21–0.6;
324 moderate = 0.61–1.2; large = 1.21–1.99; very large > 2.0 (Hopkins et al. 2009). Statistical
325 analysis was conducted through IBM SPSS (Software V22.0, IBM, New York, USA).

326

327

328 **Results**

329 *Plasma [NO₃]*

330 BR significantly elevated plasma [NO₃] ($340 \pm 58 \mu\text{M}$) prior to exercise compared to
331 baseline ($90 \pm 11 \mu\text{M}$, $P < 0.001$; $d = 5.3$) and PLA ($89 \pm 12 \mu\text{M}$, $P = 0.001$; $d = 5.3$). After
332 the exercise protocol, plasma [NO₃] was significantly reduced in BR ($309 \pm 45 \mu\text{M}$) but
333 remained higher than PLA ($86 \pm 11 \mu\text{M}$, $P < 0.001$; $d = 6.0$).

334

335 ******Insert Table 1 here******

336 *MAP during rest and exercise*

337 Resting MAP was ~4.1% lower after BR consumption compared to PLA ($P = 0.001$, $d = 1.5$).
338 There were also main effects (independent of the exercise stage) for recovery ($P = 0.001$) and
339 exercise MAP ($P = 0.011$), which in both cases was lower (average ~3.9% and ~2.4%,
340 respectively) in BR than PLA (Figure 1). The interaction between exercise stage and
341 supplement for exercise MAP approached significance ($P = 0.056$), with the highest mean
342 difference in MAP present at a gradient of 1% (~4.1%; $d = 0.98$) and lowest at 20% (~1.7%;
343 $d = 0.3$).

344

345 ******Insert Figure 1 here******

346

347

348

349 *Cardiorespiratory measurements*

350 There were no differences between BR and PLA for resting ($P = 0.699$), exercise ($P = 0.257$)
351 or recovery HR ($P = 0.512$) (Table 1). Pulmonary $\dot{V}\text{O}_2$ kinetics are presented in Table 1 and
352 Figure 2, whilst average $\dot{V}\text{O}_2$ during each exercise stage and subsequent recovery is shown in
353 Figure 3. Average (~18%, $P < 0.001$) and end-stage (~11%, $P = 0.002$) $\dot{V}\text{O}_2$ during each

354 exercise stage was lower in BR compared to PLA. This was accompanied by an average
355 reduction in phase II $\dot{V}O_2$ amplitude (~12%, $P = 0.025$), and a trend for a lower phase II time
356 constant (~12%, $P = 0.07$) with BR in response to exercise. There was an effect of the incline
357 on the MRT ($P = 0.04$) and a trend for BR ($P = 0.08$), but no pairwise differences or
358 interactions ($P > 0.05$) were found. There were also main effects for recovery $\dot{V}O_2$ ($P =$
359 0.001), which on average was lower in BR (~10%).

360

361

362 *******Figure 2 here*******

363

364

365

366 *******Figure 3 here*******

367

368

369

369 The energy cost of exercise was significantly lower across each exercise stage with BR (~8%,
370 $P < 0.001$). There was also a significant interaction between exercise gradient and condition
371 for gross efficiency ($P < 0.001$), which was lower with BR when walking at 10% ($P < 0.001$;
372 $d = 1.79$), 15% ($P = 0.001$; $d = 1.79$) and 20% ($P = 0.002$; $d = 1.05$) gradients, but not
373 different to PLA at any other stage (Figures 4 and 5).

374

375

376 *******Figure 4 here*******

377

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380

381 *******Figure 5 here*******

382

383

384

385

386 ***Blood measurements***

387 BR had no effect on blood lactate ($P = 0.122$), which increased across each exercise stage (P
388 $= 0.002$). Plasma insulin did not change significantly from the start to the end of the exercise
389 protocol ($P = 0.104$) and was not different between BR and PLA ($P = 0.332$). Similarly,
390 blood glucose did not change across gradients ($P = 0.799$) or between conditions ($P = 0.379$).
391 However, there was a significant interaction ($P = 0.005$) for K^+ , which was higher after
392 exercise (4.5 ± 0.3 mmol/L) than before (4.1 ± 0.3 mmol/L) in PLA ($P = 0.013$; $d = 1.33$), but
393 did not change in BR from pre-to-post exercise (4.1 ± 0.3 *cf.* 4.3 ± 0.2 mmol/L respectively,
394 $P = 0.188$).

395

396

*****Table 1 here*****

397

398 **Discussion**

399 The main findings of this study further the current understanding of the physiological
400 responses to exercise mediated by NO_3^- supplementation. In support of our hypothesis, we
401 have demonstrated that the O_2 cost of exercise, the exercise-induced increase in blood $[K^+]$
402 and both resting and exercising MAP was reduced, alongside favourable alterations in the
403 pulmonary $\dot{V}O_2$ kinetics during rest-to-exercise transitions, after consuming NO_3^- -rich BR
404 across a 24-h period. Collectively, these findings support, and extend upon, the reported
405 physiological effects conferred by BR supplementation, which have many potential
406 implications for people walking on inclined surfaces as part of their daily tasks. Furthermore,
407 our findings suggest that the form of locomotion (uphill walking) permits the physiological
408 changes induced by dietary NO_3^- to manifest during exercise, despite the moderate intensity
409 of the task. The $MAP/\dot{V}O_2$ at higher external intensities (i.e. 15-20% inclines) also reveals a
410 phenomenon previously unreported after dietary NO_3^- supplementation.

411

412 We have demonstrated that acute supplementation of a NO₃⁻-rich BR supplement lowered
413 MAP at baseline (rest) and during inclined walking, with seemingly larger effects at gradients
414 of 1% - 10%, as well as during post-exercise recovery (Figure 1). However, there were no
415 differences in HR between conditions (Table 1). These findings imply that the net systemic
416 vasodilatory effect of NO on blood vessels was more active during rest and lower-intensity
417 exercise ($Cw < \sim 7$ J/kg/min) than when exercise intensity was increased ($Cw = > \sim 8$
418 J/kg/min) via inclined walking. Indeed, since MAP was also lower during rest, the net
419 increase in MAP during 15% and 20% inclines was actually larger in the BR compared to the
420 PLA condition. We did not anticipate these responses and, consistent with most studies
421 (Bond et al. 2013; Choi et al. 2016; Kenjale et al. 2011), it was hypothesized that MAP would
422 be systematically lower throughout the rest and exercise protocol.

423

424 Oggioni et al. (2017) reported very similar findings to that of the current study, albeit among
425 older adults (64.7 ± 3.0 years), where reductions in diastolic BP were observed at lower
426 external cycling power output of 20 W – 80 W after BR consumption. During their
427 subsequent exhaustive trial, both systolic and diastolic BP was equal between PLA and BR
428 conditions. However, other studies have reported a consistent reduction in BP with increasing
429 exercise intensity (Bond et al. 2013; Choi et al. 2016; Kenjale et al. 2011). The reasons for
430 conflicting findings between studies is unclear, but it is noteworthy that each of the studies
431 with contrasting data to ours used manual sphygmomanometry to measure BP responses,
432 which rely on human operation and only provide a single caption of end-exercise values,
433 rather than continuous recordings. Indeed, ambulatory BP recordings have previously been
434 reduced among hypertensive individuals (Kapil et al. 2015), which avoids the technical
435 expertise needed to measure manual exercising BP. One explanation for our MAP

436 observations could be a greater increase in cardiac output (via stroke volume) in the BR
437 condition at higher gradients; however, changes in cardiac output have not been observed
438 after BR supplementation (Oggioni et al. 2017). Redistribution of blood flow towards the
439 working skeletal musculature has also been demonstrated following BR supplementation
440 (Horiuchi et al. 2017) and the seemingly necessary exercise-induced net increase in central
441 BP might also be explained by a similar mechanism. Further investigations are needed to
442 explore such mechanisms associated with nitrate-induced changes in MAP during different
443 exercise tasks.

444

445 Consistent with other studies (Bailey et al. 2009; Larsen et al. 2011; Lansley et al. 2011b), we
446 found a reduction in the O₂ cost of exercise, which translated into improvements in GE at
447 10%, 15% and 20% gradients, among the BR condition compared to PLA (Figure 5). These
448 findings are most comparable with the reported 12%-14% reductions in O₂ cost of exercise
449 (Lansley et al. 2011b), despite the lower treadmill velocity (3 km/h vs. 4 km/h). The only
450 other study to use an inclined treadmill protocol (Kenjale et al. 2011) did not increase the
451 incline beyond 6% and reported minimal effects of BR on $\dot{V}O_2$ responses, despite exercising
452 to exhaustion. We attribute our findings to the type of exercise being performed at higher
453 gradients.

454

455 When walking uphill at progressively steeper inclines, muscle recruitment strategies are
456 proportionally altered, such that the leading leg performs longer and more forceful concentric
457 muscle contractions (i.e. more positive work) (Gotschall and Kram 2006). Based on
458 Henneman's size principle, the leading legs would require recruitment from a greater number
459 of larger motor units (type II fibre). These muscle fibres provide more force but are less
460 fatigue-resistant and are recruited during a period of increased metabolic demand (Minetti et

461 al. 2006). Given the lower-limb forces required to walk on inclined terrain, coupled with the
462 above-mentioned changes in gait, it is feasible that intermittent occlusion of blood flow will
463 also result in metabolic perturbation at the working musculature. The increased metabolic
464 cost, intensity and duration of contractions, coupled with an intermittent occlusion of local
465 blood flow that occurs during uphill walking, would lead to a mildly hypoxic and acidic
466 environment, which has been suggested to potentiate the effects of NO at the muscle (Jones et
467 al. 2016).

468

469 Plasma insulin and glucose concentrations were unaffected by the BR condition compared to
470 PLA, which has been reported before (Gilchrist et al. 2013), despite the previously described
471 improvements in glucose homeostasis induced by dietary NO_3^- after intense exercise (Wylie
472 et al. 2013). Our findings are most likely related to the lower intensity of the exercise or the
473 health status of the participants, who did not present with symptoms of metabolic disease.
474 There were also no differences in B[La] between conditions after each trial. However, owing
475 to the organization of the protocol, B[La] was recorded in the 1-min after exercise cessation,
476 which is unlikely to permit a larger accumulation of lactate in the blood. There was an
477 interaction between time and condition for $[\text{K}^+]$, indicating that BR supplementation
478 attenuated its exercise-induced increase. This confirms the findings of other analyses (Wylie
479 et al. 2013), which were explained by the accelerated NO_3^- - NO_2^- -NO pathway in acidic
480 environments, potentially blunting the rise in extracellular $[\text{K}^+]$ in this state. We also
481 speculate that the increased ATP availability (Larsen et al. 2011) for Na^+/K^+ pumping or
482 increases in muscle blood flow (Ferguson et al. 2013) found with NO_3^- consumption would
483 facilitate clearance of K^+ . We are the first to show this during moderate intensity exercise and
484 also attribute this finding to the type of exercise performed. Plasma $[\text{K}^+]$ is increased after
485 exercise, reflecting a transient disturbance in muscle ion homeostasis and reduced muscle

486 excitability. Intermittently walking at 1%-20% inclines for a total of 55-min at an intensity
487 below the heavy domain was, therefore, sufficient to induce K^+ flux into the blood. The
488 recruitment of muscle fibres when fixed into an inefficient gait cycle is likely to be type II
489 preferential, thus relying upon musculature that is more fatigable owing to the lower O_2
490 muscle tension (Jones et al. 2016). The observed increase in $[K^+]$ in this study can be
491 considered as a marker of peripheral fatigue that is increased during inclined walking and
492 attenuated by BR supplementation. Future studies should explore the mechanisms through
493 which BR exerts this effect.

494

495 $\dot{V}O_2$ on-kinetics were improved with BR supplementation. Reductions in the amplitude and
496 non-significant ($P = 0.07$), yet small effect sizes ($d = 0.2 - 0.5$), for phase II time constant
497 from rest to exercise support the findings of others during different exercise types (Bailey et
498 al. 2009, 2010; Lansley et al. 2011b). To the best of the authors' knowledge, this is the first
499 study to demonstrate enhanced recovery of pulmonary O_2 after BR supplementation.
500 Recovery was improved by reducing the resting $\dot{V}O_2$ during the intermittent 5-min recovery
501 periods. These results were accompanied by a lower resting MAP, reflecting reduced
502 cardiorespiratory stress in response to exercise and, thus, improved exercise tolerance after
503 BR supplementation.

504

505 There are a variety of occupations in which employees are required to regularly perform
506 physically demanding tasks. For example, the daily duties of outdoor occupations, such as
507 military, farming and emergency services, comprise walking activities performed over-
508 ground of varying topography (Ruby et al. 2002). Similarly, physically challenging outdoor
509 activities, such as mountaineering and hiking, are increasingly popular recreational pursuits,
510 requiring prolonged sub-maximal hill-walking (Ainslie et al. 2002, 2005; Gottschall and

511 Kram 2006). For people engaged in such habitual activities, uphill locomotion is perhaps one
512 of their most challenging tasks and can lead to an earlier onset of fatigue (Vernillo et al.
513 2017). Indeed, there is a significant metabolic cost associated with hill-walking, which
514 increases proportionally with the gradient of the slope and limits the capacity for mechanical
515 energy transfer (Gottschall and Kram 2006; Minetti et al. 2006). Interventions that increase
516 hill-walking performance and presumably delay the onset of fatigue via reduced metabolic
517 costs delay the onset of fatigue, without the need for long-term training adaptation, are
518 therefore applicable to habitual hill-walkers. Beyond those employed in hazardous
519 occupations, the capacity to improve the physiological responses to daily exercise, such as
520 commuting, might also confer a functional and health benefit. Given its apparent capacity to
521 reduce the appearance of fatigue-related metabolites and lower the O₂ cost of exercise, acute
522 NO₃⁻ supplementation (BR) could provide a practical intervention for such populations.

523

524 **Conclusion**

525 The Cw of walking is reduced and muscle efficiency is increased, particularly at steeper
526 gradients (10%-20%), after acute BR supplementation. Intermittent recovery of pulmonary
527 O₂ uptake and MAP is ameliorated and selected blood markers of peripheral fatigue are
528 attenuated by BR supplementation. BP was particularly lowered whilst walking at smaller
529 gradients (1-10%) after BR supplementation but approaches PLA conditions at higher (15-
530 20%) gradients, despite a lower O₂ cost. We collectively attribute these findings to the
531 inefficient form of locomotion that is required to walk at these gradients, necessitating the
532 recruitment of more fatigable muscle fibre types during a period of increased metabolic
533 demand and intermittently hypoxic or mildly acidic environment, known to augment the
534 reduction of NO₂⁻ to NO. However, further research is required to confirm these suggestions.

535 These findings have implications for those exercising on terrain of varying topography in
536 their daily tasks.

537

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539

540 **Compliance with Ethical Standards**

541

542 **Conflict of Interest:** The authors declare that they have no conflict of interest.

543 **Ethical approval:** All procedures were performed in accordance with the ethical standards of
544 the institutional and/or national research committee and with the 1964 Helsinki declaration
545 and its later amendments or comparable ethical standards.

546 **Informed consent:** Informed consent was obtained from all individual participants included
547 in the study

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737 **Figure legends**

738

739 **Figure 1.** Mean arterial pressure responses to walking at 1%, 5%, 10%, 15% and 20%
740 inclines and intermittent recovery in beetroot and placebo conditions ($n = 8$). *Significant ($P <$
741 0.05) main effect of BR supplementation

742

743 **Figure 2.** Pulmonary $\dot{V}O_2$ (L/min) responses of a representative subject walking at 1%, 5%,
744 10%, 20% and 15% inclines and intermittent recovery in beetroot and placebo conditions ($n =$
745 8). Data are shown in 5-s averages.

746

747 **Figure 3.** Mean pulmonary $\dot{V}O_2$ (ml/min) responses to walking at 1%, 5%, 10%, 15% and
748 20% inclines and intermittent recovery in beetroot and placebo conditions ($n = 8$).
749 *Significant ($P < 0.05$) main effect of BR supplementation

750

751 **Figure 4.** Energy cost (J/kg/min) of walking at 1%, 5%, 10%, 15% and 20% inclines in
752 beetroot and placebo conditions ($n = 8$). *Significant ($P < 0.05$) main effect of BR
753 supplementation

754

755 **Figure 5.** Gross efficiency (%) of walking at 1%, 5%, 10%, 15% and 20% inclines in
756 beetroot and placebo conditions ($n = 8$). *Significant ($P < 0.01$) interaction between BR and
757 PLA.

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762 **Table title**

763

764 **Table 1.** Cardiorespiratory responses to walking at 1%, 5%, 10%, 15% and 20% inclines in
765 beetroot (BR) and placebo (PLA) conditions ($n = 8$). *Significant ($P < 0.05$) main effect of
766 BR supplementation

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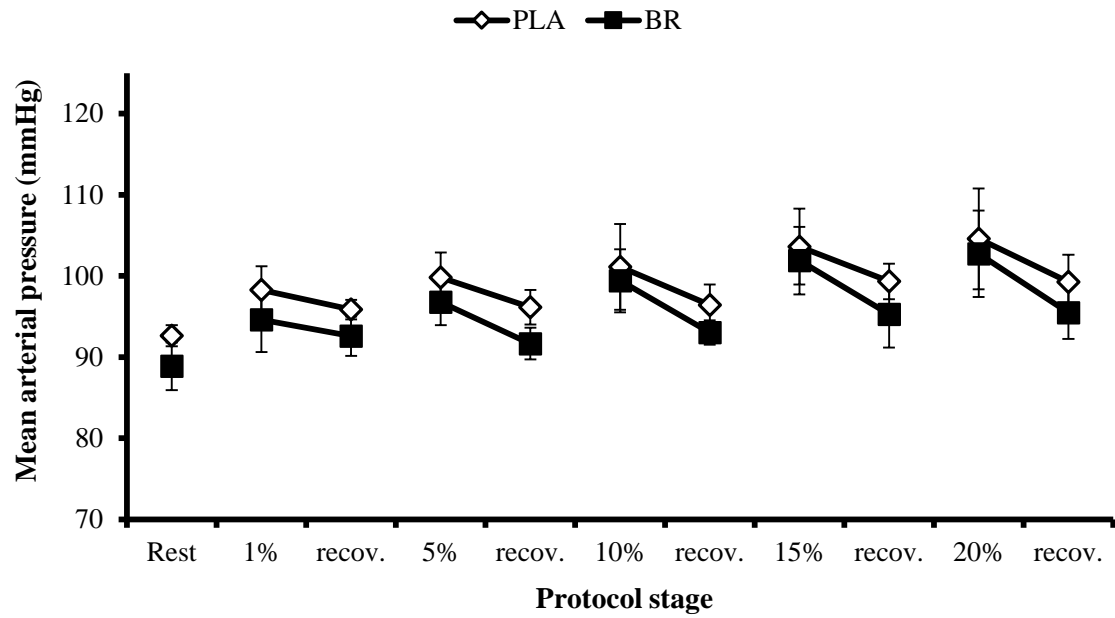
	PLA	BR	PLA	BR	PLA	BR	PLA	BR	PLA	BR
	1%		5%		10%		15%		20%	
771										
772 End exercise										
773 $\dot{V}O_2$ (ml/min)*	794 ± 204	643 ± 137	1076 ± 277	931 ± 221	1363 ± 285	1249 ± 264	1707 ± 287	1550 ± 310	2083 ± 389	1877 ± 352
774 On phase II amplitude (ml/min)*	472 ± 125	349 ± 101	742 ± 265	591 ± 237	1078 ± 303	1086 ± 383	1319 ± 291	1158 ± 278	1722 ± 306	1514 ± 368
775 On phase II time constant (s)	30 ± 16	26 ± 13	35 ± 12	29 ± 7	43 ± 11	38 ± 16	24 ± 10	26 ± 11	37 ± 18	30 ± 11
776 Mean response time (s)	41 ± 18	39 ± 14	47 ± 12	42 ± 9	56 ± 12	52 ± 16	32 ± 11	37 ± 13	48 ± 18	42 ± 9
777 Exercising Heart rate (b/min)	80 ± 11	79 ± 12	88 ± 12	86 ± 13	98 ± 13	97 ± 12	113 ± 19	109 ± 20	120 ± 17	116 ± 17
778 Resting heart rate (b/min)	71 ± 10	68 ± 12	78 ± 12	76 ± 10	81 ± 13	77 ± 11	87 ± 20	79 ± 14	89 ± 18	84 ± 18

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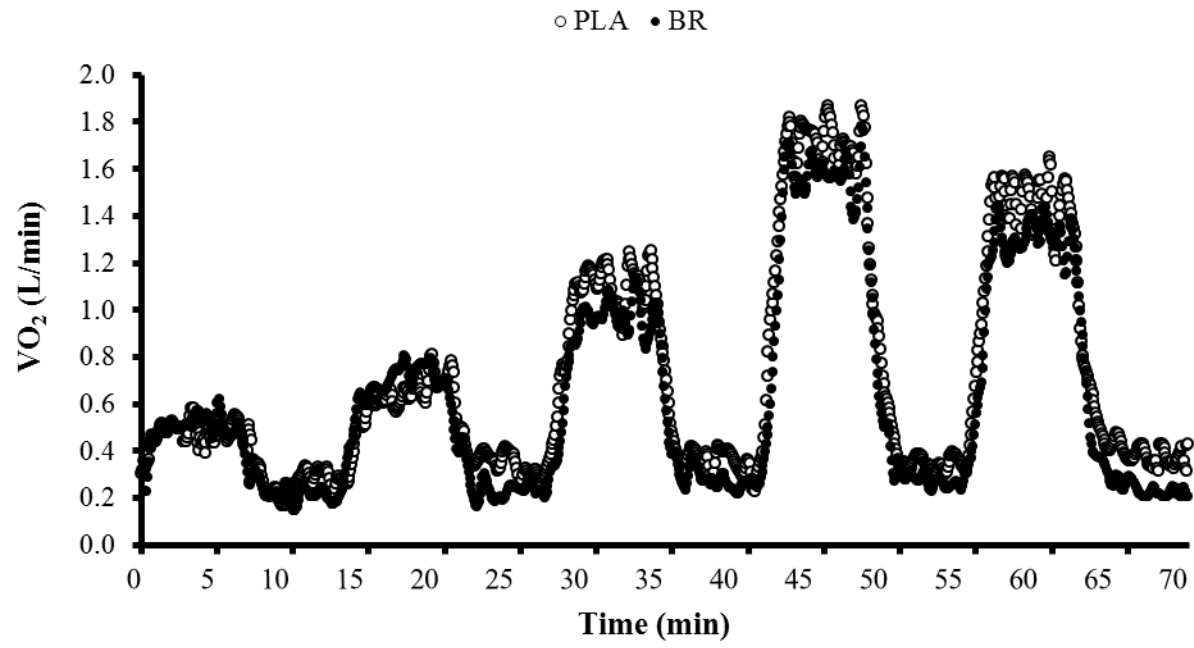
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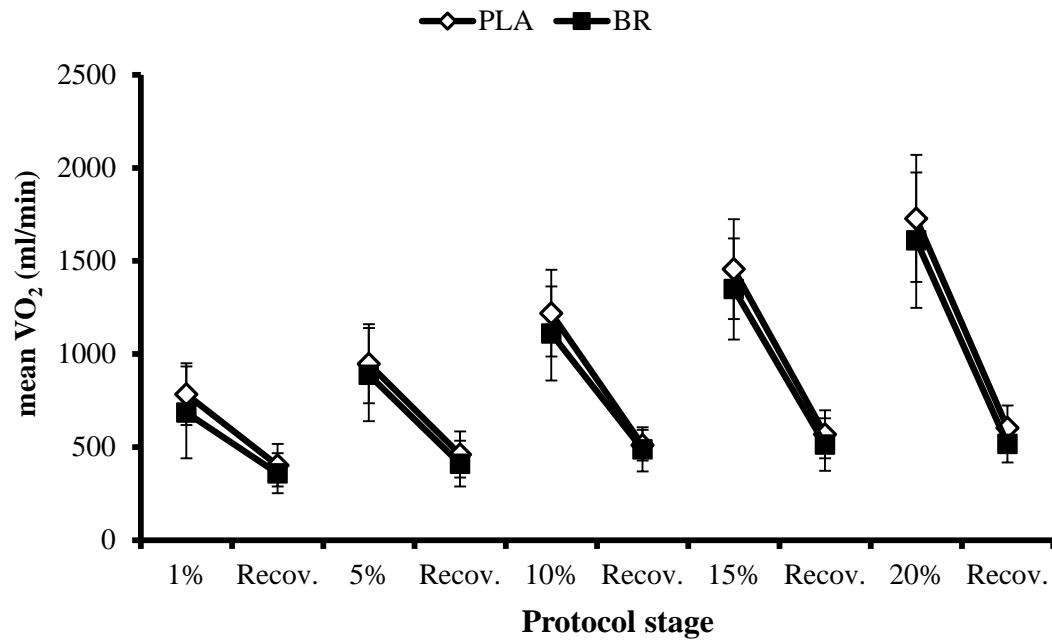
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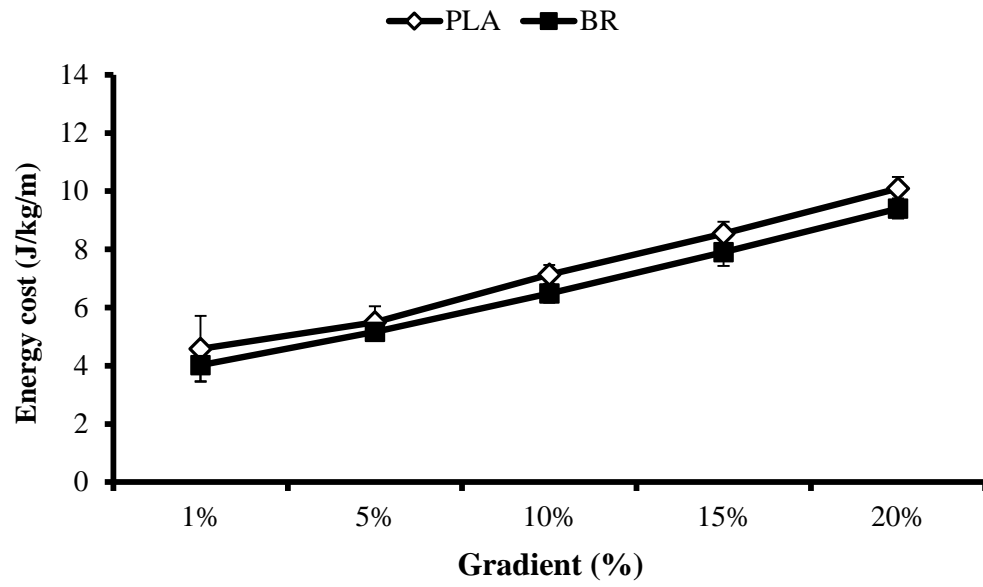
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