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1 Probiotic supplementation increases carbohydrate metabolism in
2 trained male cyclists: a randomized, double-blind, placebo-controlled
3 cross-over trial

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30
31 Abbreviations: CHO - carbohydrate
32 ELISA - Enzyme-linked immunosorbent assay
33 GI - Gastrointestinal
34 HR - Heart rate
35 I-FABP - Intestinal-fatty acid binding protein
36 LR - Lactulose:rhamnose ratio
37 NEFA – non-esterified fatty acid
38 NSAID - non-steroidal anti-inflammatory drug
39 RPE - Ratings of perceived exertion
40 SGLT1 - Sodium-glucose co-transporter 1

41

42 Conflict of interest: Aliment Nutrition © supplied the probiotic supplements and have provided partial
43 financial support for the studies of JP. The funding sponsors had no role in the design of the study; in
44 the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision
45 to publish the results.

46 **Abstract**

47 We hypothesised that probiotic supplementation (PRO) increases the absorption and oxidation of
48 orally ingested maltodextrin during 2h endurance cycling, thereby sparing muscle glycogen for a
49 subsequent time trial (simulating a road race). Measurements were made of lipid and carbohydrate
50 oxidation, plasma metabolites and insulin, gastrointestinal permeability, and subjective symptoms of
51 discomfort. Seven male cyclists were randomized to PRO (bacterial composition given in methods) or
52 placebo (PLC) for four weeks, separated by a 14-day washout period. After each period, cyclists
53 consumed a 10% maltodextrin solution (initial 8 mL·kg⁻¹ bolus and 2 mL·kg⁻¹ every 15 min) while
54 exercising for 2h at 55% W_{max} followed by a 100 kJ time trial. PRO resulted in small increases in peak
55 oxidation rates of the ingested maltodextrin (0.84 ± 0.10 vs 0.77 ± 0.09 g·min⁻¹, $P = 0.016$), and mean
56 total carbohydrate oxidation (2.20 ± 0.25 vs 1.87 ± 0.39 g·min⁻¹, $P = 0.038$), while fat oxidation was
57 reduced (0.40 ± 0.11 vs 0.55 ± 0.10 g·min⁻¹, $P = 0.021$). During PRO small but significant increases were
58 seen in glucose absorption, plasma glucose and insulin concentration and decreases in NEFA and
59 glycerol. Differences between markers of GI damage and permeability and time trial performance
60 were not significant ($P > 0.05$). In contrast to the hypothesis, PRO led to minimal increases in
61 absorption and oxidation of the ingested maltodextrin and small reductions in fat oxidation, while
62 having no effect on subsequent time trial performance.

63

64 Introduction

65 Adequate CHO availability, as the main fuel for skeletal muscle and the central nervous system during
66 endurance exercise lasting 1-2 h, and the maintenance of high CHO oxidation rates is a critical
67 component for optimal performance. Liver and muscle glycogen stores are limited and oral ingestion
68 of CHO before and during exercise has been reported to improve performance (11) and delay fatigue
69 during cycling and running (9, 51). This performance benefit has since been reported in numerous
70 publications, with exogenous carbohydrate ingestion showing ergogenic effects for endurance
71 performance in most of these studies (39, 49). However, oxidation rates of orally ingested glucose and
72 maltodextrin (glucose polymer) solutions appear to plateau around $1 \text{ g}\cdot\text{min}^{-1}$ (or $60 \text{ g}\cdot\text{h}^{-1}$) (53), even
73 with ingestion rates as high as $2.6 \text{ g}\cdot\text{min}^{-1}$ (26). A finding that has been shown many times (12). The
74 capacity of the sodium-glucose transporter (SGLT1) in the small intestine is generally regarded as the
75 limiting factor for glucose absorption and the oxidation rate of glucose and maltodextrin ingested
76 during endurance exercise (21). While there appears to be a mean maximal rate of exogenous glucose
77 oxidation of $1 \text{ g}\cdot\text{min}^{-1}$ there appears to be variation between individuals in one study (53) and also
78 between studies (23). Environmental factors can also reduce the maximal oxidation of consumed
79 carbohydrates. Reductions in exogenous carbohydrate oxidation have been seen at increased
80 environmental temperatures (20). This has been related to reductions in splanchnic blood flow and
81 compromised intestinal absorption (47). Strategies that may increase the maximal oxidation rate of
82 orally ingested carbohydrates either above the previously established $1 \text{ g}\cdot\text{min}^{-1}$, or above an
83 individual's own maximal oxidation rate under normal or compromised environmental conditions
84 could be of benefit to endurance athletes.

85 One such method proposed to increase the oxidation of ingested CHO during exercise is
86 supplementation with probiotic bacteria. *In vitro* research has shown that co-incubation of Caco-2
87 cells (enterocyte model) with as yet unidentified, heat labile metabolites from bacterial strains from
88 the *Lactobacilli* species increases glucose uptake (46). Probiotics can also modulate luminal short chain
89 fatty acid production (44) which are shown to increase both the abundance and activity of SGLT1 (50)
90 as well as increasing insulin secretion following CHO ingestion (28). The latter of these could have
91 wider implications on total CHO oxidation given that insulin suppresses lipolysis and lipid oxidation
92 during exercise (19). There are then potential mechanisms by which probiotics could increase
93 absorption and so the subsequent oxidation of consumed glucose, as well as altering total
94 carbohydrate oxidation, and these findings could have practical and relevant implications for athletes
95 if replicable during endurance exercise.

96 As well as the potential to increase CHO absorption and oxidation, probiotics have also been proposed
97 to be beneficial to performance via positive effects on GI permeability and damage. It has been shown

98 that probiotic supplementation, or inoculation with the metabolites of probiotic bacteria, can prevent
99 epithelial apoptosis (57), increase mucin secretion (6), inhibit attachment of pathogenic bacteria (3),
100 as well as increase expression of tight junction proteins and decrease secretion of pro-inflammatory
101 cytokines (33). Given that endurance exercise has been shown to increase markers of GI permeability
102 (e.g. serum lactulose:rhamnose ratio; LR) and damage (e.g. intestinal-fatty acid binding protein; I-
103 FABP) (52), probiotic supplementation could attenuate such changes in GI physiology. While there is
104 some evidence that probiotic supplementation can attenuate exercise induced increases in GI
105 permeability and circulatory endotoxin concentrations (45), we have previously shown there to be no
106 effect (43). However, it is difficult to draw definitive conclusions from such field based studies.
107 Laboratory controlled investigations should hopefully provide more insight into the effects of
108 probiotics on exercise-induced GI damage.

109 The aim of the current study was to investigate whether probiotic supplementation increases the
110 oxidation of an ingested maltodextrin drink and total CHO oxidation during 2 h of cycling exercise at
111 55% W_{\max} . It is hypothesised that 4 weeks of probiotic supplementation would increase the intestinal
112 digestion and absorption rate of the maltodextrin drink, the percent contribution of the drink to
113 carbohydrate oxidation rates and total carbohydrate oxidation rates. It is also hypothesised that the
114 ingestion of the probiotic supplement would significantly reduce the LR ratio and intestinal damage
115 (I-FABP) and improve performance during the 2 h of cycling exercise. These hypotheses have been
116 tested using a double blind placebo-controlled cross-over design.

117

118 **Methods**

119 **Participants**

120 Seven trained cyclists participated in this study (mean \pm SD; age 23 ± 4 yrs, body mass 73.4 ± 7.1 kg,
121 $\dot{V}O_{2\text{peak}}$ 64.0 ± 2.2 mL \cdot kg $^{-1}\cdot$ min $^{-1}$). None of the participants used medication (e.g. NSAIDs,
122 antidepressants, or diuretics) or nutritional supplements or reported a history of GI-related medical
123 issues (e.g. irritable bowel disease or abdominal surgery). After explaining the nature and risks of the
124 experimental procedures to the subjects, their informed written consent was obtained. The study was
125 approved by the institutional ethics committee of Liverpool John Moores University and conformed
126 to the standards set by the Declaration of Helsinki.

127 **Pre-testing**

128 At least 7 days prior to the first experimental trial, subjects completed preliminary testing. $\dot{V}O_{2\text{peak}}$ and
129 maximal aerobic power output (W_{\max}) were determined on an electronically braked cycle ergometer
130 (Lode Excalibur Sport, Groningen, The Netherlands) during an incremental exhaustive exercise test.

131 Work rate commenced at 95 W for 3 min, followed by incremental steps of 35 W every 3 min until
132 volitional exhaustion. Oxygen uptake was measured continuously during exercise using an on-line gas
133 analysis system (Moxus modular metabolic system, AEI technologies Inc, Pennsylvania, USA). $\dot{V}O_{2peak}$
134 was determined from highest recorded $\dot{V}O_2$ value from 10 s averages. W_{max} was calculated from the
135 last completed work rate, plus the fraction of time spent in the final non-completed work rate
136 multiplied by the work rate increment (22). After a rest period of 30-60 minutes, participants then
137 completed 1 h of cycling exercise at 55% W_{max} following the prescribed drinking protocol and followed
138 by a time trial to familiarise themselves to the real testing procedures described in the following
139 paragraphs.

140 Treatment allocation

141 In a randomized, double-blind, placebo-controlled crossover design, each subject completed two 28
142 day periods of supplementation as well as consuming an additional supplement capsule on the
143 morning of the trials at the end of each intervention period, one hour before commencing exercise.
144 This 28 day supplement period is in line with our previous work showing lower GI symptoms during
145 exercise (43). Each supplement period was separated by a 14 day washout period. This wash out
146 period was based upon unpublished data showing the probiotic strains used are undetectable from
147 stool samples after this time. It also has been shown that this period allows for a number of complete
148 epithelial cell turnovers within the small intestine (2). Participants were randomized to consume either
149 a capsule of a commercially available probiotic (PRO) or a visually identical placebo daily for 28 days.
150 The PRO supplement contained the active strains *Lactobacillus acidophilus* (CUL60), *Lactobacillus*
151 *acidophilus* (CUL21), *Bifidobacterium bifidum* (CUL20) and *Bifidobacterium animalis* subsp. *lactis*
152 (CUL34) (Proven Probiotics, Port Talbot, UK). The minimum concentration was 25 billion colony-
153 forming units (CFU). This probiotic supplement has previously been shown to survive the GI tract
154 during consumption (31) and have beneficial effects for endurance athletes (43, 45). During the
155 supplementation period, participants were informed to avoid consumption of probiotic foods such as
156 fermented foods and yogurts. The PLC capsules were visually identical and consisted of starch only
157 (Proven Probiotics, Port Talbot, UK). Subjects were instructed to swallow the capsule daily after their
158 first meal. The randomization code was held by a third party (Cultech Ltd) and unlocked for statistical
159 analyses by the authors upon sample analysis completion.

160 Experimental trials

161 Each subject underwent four experimental trials; one prior to and at the end of each supplementation
162 period. Trials consisted of 120 min of cycling at 55% W_{max} , in line with previous work examining
163 exogenous oxidation during cycling exercise (55, 58). This was followed by a time trial amounting to
164 100 kJ of work, simulating the final sprint in a competitive long distance road race. Subjects were

165 instructed not to perform any strenuous exercise 24 hr prior to testing and to avoid caffeine, alcohol,
166 and any spicy food. Subjects also recorded their food intake in the 24 hr before the first trial and
167 repeated this for each subsequent visit.

168 Subjects reported to the laboratory at the same time (~7:30am) for each trial after an overnight fast
169 of at least 12 hours. A cannula (Safety Lock 22G, BD Biosciences, West Sussex UK) was inserted into
170 the antecubital vein and baseline blood sample was taken. Resting breath samples were collected over
171 a 5 min period (Moxus modular metabolic system, AEI technologies Inc, Pennsylvania, USA) and
172 exetainer tubes were filled directly from the mixing chamber to determine the $^{13}\text{C}/^{12}\text{C}$ ratio in expired
173 CO_2 . Subjects then began cycling at 55% W_{max} for 120 minutes. Heart rate (Polar FT1 HRM, Polar
174 Electro, Kempele, Finland) and ratings of perceived exertion (RPE) (4) were recorded every 15 minutes.
175 Immediately following the 120 minutes of steady state cycling, simulated cycling time trials were
176 undertaken with the ergometer set in a cadence-dependent power output (linear) mode for subjects
177 to complete 100kJ of work. Power output was therefore a function of cadence and a fixed factor (alpha
178 value) was used, as described in the following equation: Power (W) = $L \times \text{rpm}^2$, in which the rpm is the
179 pedalling rate, and L is a linear factor. This factor was chosen in a way that would evoke 100% peak
180 aerobic power output at a pedalling rate of 90 rpm.

181 Maltodextrin drink

182 During exercise subjects consumed a 10% CHO drink enriched with the stable isotope [$\text{U-}^{13}\text{C}$]glucose
183 (CK Isotopes, Istock, UK). 176.4 g maltodextrin (Myprotein®Inc, Northwich, UK) and 3.6 g [$\text{U-}^{13}\text{C}$]
184 ^{13}C]glucose were dissolved in water and made up to a total volume of 1800ml. For the two participants
185 in which the oxidation rate of the ingested maltodextrin was not measured, the drink consisted of 10%
186 maltodextrin only. Total drink volume was prescribed according to participant weight with an $8 \text{ mL} \cdot \text{kg}^{-1}$
187 ^1bw bolus in the first 3 minutes of exercise followed by $2 \text{ mL} \cdot \text{kg}^{-1}\text{bw}$ each subsequent 15 min during
188 120 min cycling exercise (24). Total fluid volume and carbohydrate intakes prescribed were $1790 \pm$
189 152 mL and $179 \pm 15.2 \text{ g}$ respectively. An elemental analyser isotope ratio mass spectrometer (EA-
190 IRMS; Europa Scientific 20–20, Iso-Analytical Ltd, Crewe, UK) was used to accurately measure the ^{13}C -
191 enrichment of freeze-dried samples of the maltodextrin/[$\text{U-}^{13}\text{C}$]glucose drinks and the natural ^{13}C -
192 background enrichment of the maltodextrin powder expressed as $\delta^{13}\text{C} \text{ ‰}$ vs PDB. The ^{13}C -enrichment
193 of the consumed drinks was very high at 1681 ‰ vs PDB. The drinks also contained $35 \text{ mmol} \cdot \text{L}^{-1}$ of
194 sodium chloride as sodium in the $30\text{-}50 \text{ mmol} \cdot \text{L}^{-1}$ range leads to better fluid delivery and retention in
195 endurance trained individuals (32).

196 $^{13}\text{C}/^{12}\text{C}$ analysis of breath CO_2

197 Breath samples were analysed using an isotope ratio mass spectrometer (Delta XP, coupled to a Gas
 198 Bench II and GC Pal autosampler (ThermoElectron, Bremen, Germany). The breath tubes were held
 199 in a heated sample tray at 26°C . The breath sample was continuously transferred through a Valco
 200 sampling port in a flow of helium. Carbon dioxide was separated from the presence of other gases by
 201 using a capillary column (PoraPLOTQ; Agilent JW columns) with dimensions of $27.5\text{ m} \times 0.32\text{ mm} \times 10$
 202 μm . The oven temperature was kept constant at 68°C . Nafion water traps removed H_2O from the
 203 sample. Multiple analysis of each sample was achieved by switching the contents of the sample loop
 204 to the GC column every 50 seconds. Each switch corresponded to starting the GC separation of the
 205 sample coming from the loop. Ions m/z 44 and 45 were monitored for CO_2 and $^{13}\text{CO}_2$ respectively. The
 206 ^{13}C enrichment results from breath samples were expressed as $\delta^{13}\text{C} \text{‰}$ vs PDB. The $\delta^{13}\text{C} \text{‰}$ vs PDB
 207 results of the maltodextrin powder, the maltodextrin/[U- ^{13}C]glucose drinks and breath samples were
 208 converted to the tracer-to-tracee ratio (TTR) by using the following equation:

209
$$\text{TTR } (^{13}\text{C}:^{12}\text{C}) = [(\delta^{13}\text{C}\text{‰}/1000) + 1] \times 0.0112372 \text{ (14)}$$

210 **Analysis of plasma [U- ^{13}C]glucose enrichment**

211 Plasma glucose isotope enrichment was measured as the tracer/tracee ratio (TTR) by gas-
 212 chromatography mass spectrometry using a trimethyl silyl-O-methyloxime derivative according to
 213 methods previously described (48). The peak areas of the ions m/z 319.2 and m/z 323.2, for natural
 214 glucose and [U- ^{13}C]glucose respectively, were measured by GC-MS on a Agilent 5975C Inert XL EC/CI
 215 MSD (Agilent Technologies, Wokingham, Berks, UK).

216 The concentration of [U- ^{13}C]glucose in the plasma ([U- ^{13}C]Glu_p) was calculated as follows:

217
$$[\text{U-}^{13}\text{C}]\text{Glu}_p = \text{Glu}_p \times \text{TTR}$$

218 where Glu_p is the plasma glucose concentration in mmol.L^{-1} and TTR of [U- ^{13}C]glucose in the plasma.

219 At any given time point, the concentration of unlabelled glucose ([^{12}C]Glu_p mmol.L^{-1}) in the plasma
 220 that had originated from the maltodextrin drink was derived from the known enrichment of the
 221 glucose in the drink as follows:

222
$$[^{12}\text{C}]\text{Glu}_p = ([\text{U-}^{13}\text{C}]\text{Glu}_p / 3.1) \times (100 - 3.1)$$

223 where 3.1 is the % isotopic enrichment of the test drink.

224 The total concentration of glucose in plasma that originated from the maltodextrin drink (Glu_m) was
 225 calculated as:

226
$$\text{Glu}_m = [^{12}\text{C}]\text{Glu}_p + [\text{U-}^{13}\text{C}]\text{Glu}_p$$

227 and the difference between this value and total plasma glucose concentration was assumed to have
 228 been derived endogenously. This calculation distinguishes between $[^{12}\text{C}]\text{Glu}$ produced endogenously
 229 and $[^{12}\text{C}]\text{Glu}$ in the test drink.

230 **Indirect calorimetry and calculations**

231 Respiratory gas exchange variables were measured using a mixing chamber (Moxus modular
 232 metabolic system, AEI technologies Inc, Pennsylvania, USA) with oxygen uptake ($\dot{V}\text{O}_2$), carbon dioxide
 233 output ($\dot{V}\text{CO}_2$), and respiratory exchange ratio (RER) measured during a 4 min interval and sample
 234 frequency of 15 seconds after every 15 min during the 2 h of cycling exercise. Breath samples were
 235 collected in duplicate directly from the mixing chamber of the MOXUS system into sealed vacutainer
 236 collection tubes again every 15 min. Total CHO and fat oxidation rates were calculated from indirect
 237 calorimetry data assuming negligible protein oxidation (27):

238
$$\text{Glucose oxidation} = 4.55 \times \dot{V}\text{CO}_2 - 3.21 \times \dot{V}\text{O}_2$$

239
$$\text{Fat oxidation} = 1.67 \times \dot{V}\text{O}_2 - 1.67 \times \dot{V}\text{CO}_2$$

240 Exogenous glucose oxidation was calculated using the formula (35):

241
$$= \dot{V}\text{CO}_2 \times (\delta\text{Exp} - \delta\text{Expbkg}) / (\delta\text{Ing} - \delta\text{Expbkg}) / k$$

242 in which δExp is the ^{13}C enrichment of expired air during exercise at different time points, δIng is the
 243 $[\text{U-}^{13}\text{C}]$ enrichment of the ingested maltodextrin drink, δExpbkg is the ^{13}C enrichment of expired air
 244 before exercise (background), and k is the amount of CO_2 (in litres (L)) produced by the oxidation of 1
 245 g of glucose ($k = 0.7467 \text{ L CO}_2/\text{g glucose}$).

246 Plasma glucose oxidation was calculated using the formula:

247
$$= \dot{V}\text{CO}_2 \times (\delta\text{Exp} - \delta\text{Expbkg}) / (\delta\text{PG} - \delta\text{PGbkg}) / k$$

248 in which, δPG is the plasma $[\text{U-}^{13}\text{C}]$ glucose enrichment during exercise and δPGbkg is the plasma $[\text{U-}$
 249 $^{13}\text{C}]$ glucose enrichment before ingestion of the maltodextrin/ $[\text{U-}^{13}\text{C}]$ glucose drink and the start of
 250 exercise (background sample). In the above calculations, the ^{13}C -enrichments were all expressed as
 251 tracer to tracee ratio (TTR) for all analysed samples (breath, enriched maltodextrin drinks, unenriched
 252 maltodextrin powder, plasma glucose).

253 Because plasma glucose oxidation represents the oxidation of both glucose coming from the gut
 254 (exogenous glucose) and the contribution of the liver (glycogenolysis and gluconeogenesis), liver-

255 derived glucose oxidation and muscle glycogen oxidation could be calculated by the following
256 formulas:

257 Liver-derived glucose oxidation = plasma glucose oxidation – exogenous glucose oxidation

258 Muscle glycogen oxidation = total CHO oxidation – plasma glucose oxidation

259 Blood parameter analysis

260 Plasma glucose, lactate, non-esterified fatty acids, and glycerol were analysed using a Randox Daytona
261 spectrophotometer and commercially available kits (Randox Laboratories, Ireland). Analysis for
262 lactulose and rhamnose ratio (LR) as a marker of GI permeability and intestinal fatty acid binding
263 protein (I-FABP) were performed as previously described (42). Cytokine concentrations were
264 measured using cytometric bead array (CBA, BD Biosciences, San Diego, USA) for the cytokines IL-1 α
265 IL-6, IL-8, and IL-10 using the manufacturer's instructions with four bead populations with distinct
266 fluorescence intensities coated with capture antibodies specific for IL-1 α IL-6, IL-8, and IL-10 proteins.
267 Following acquisition of sample data using the flow cytometer, the sample results were generated in
268 graphical and tabular format using the BD CBA Analysis Software. Post exercise and 1hr post exercise
269 sample concentrations were corrected for plasma volume changes as described by Dill and Costill (13).

270 Assessment of gastrointestinal damage and symptoms

271 Intestinal permeability was assessed by analysing serum samples using a previously published protocol
272 (15), with the modification of using rhamnose instead of mannitol as the monosaccharide probe.
273 Briefly, immediately after the 100kJ time trial, a 50 mL sugar probe solution (5 g lactulose, 2 g
274 rhamnose) was consumed and the ratio of the sugars was measured from serum samples 60 minutes
275 after ingestion. Concentrations of intestinal-fatty acid binding protein (I-FABP) were measured pre,
276 post and 1 hour post exercise from EDTA plasma using an ELISA (Hycult Biotechnology, Uden, the
277 Netherlands; detection window 47 - 5000 pg·mL⁻¹) according to the manufacturer's instructions.
278 Specific GI symptoms were recorded every 30 min during exercise whereby a visual analogue scale
279 was used to assess specific symptoms such as bloating, nausea, urge to vomit, urge to defecate were
280 assessed. GI symptoms were scored on a 10-point scale (0 = no symptoms and 9 = very severe
281 symptoms) with a score > 4 being regarded as moderate. To ensure understanding, specific symptoms
282 were explained and described to participants.

283 Statistical analysis

284 ANOVA for repeated measures was used to compare differences in substrate utilization and in blood
285 related parameters over time between the trials. A Tukey's post hoc test was applied in the event of
286 a significant F-ratio. Where appropriate, the comparison of variables between the two conditions was

287 conducted by using a Student's t-test for paired samples. For plasma metabolites, area under the curve
288 (AUC) calculation was completed using and differences between conditions were compared using a
289 Student's t-test for paired samples. To detect a meaningful increase in exogenous CHO oxidation of
290 $0.1 \text{ g}\cdot\text{min}^{-1}$ with a standard deviation (SD) of $0.05 \text{ g}\cdot\text{min}^{-1}$ (58) at 80% power, a minimum of 5
291 participants would be required. All values are expressed as means \pm SD. Statistical significance was set
292 at $P < 0.05$.

293 Results

294 Physiological response to exercise

295 Participants cycled for 2 hours at $180 \pm 20 \text{ W}$ across trials corresponding to 55% of their W_{max} . There
296 were no significant differences between mean heart rate (149 ± 18 vs $146 \pm 16 \text{ b}\cdot\text{min}^{-1}$), $\dot{V}O_2$ ($34.5 \pm$
297 3.9 vs $34.2 \pm 3.4 \text{ mL}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$), or RPE (12 ± 1 vs 12 ± 1) for PLC and PRO, respectively. CHO and fat
298 oxidation during each hour are presented in Table 1. CHO oxidation was lower during the second hour
299 in both trials. CHO oxidation was higher ($P = 0.019$) in the second hour in PRO compared to PLC. Fat
300 oxidation was lower in PRO during both the first ($P = 0.026$) and the second ($P = 0.004$) hour compared
301 to PLC. Energy expenditure did not differ between PLC ($6292 \pm 644 \text{ kJ}$) and PRO ($6232 \pm 493 \text{ kJ}$) ($P =$
302 0.662).

303 Substrate utilisation during 60 – 120 min exercise period

304 Plasma $[\text{U-}^{13}\text{C}]\text{glucose} / ^{12}\text{C}$ glucose ratios increased as a result of maltodextrin/ $[\text{U-}^{13}\text{C}]\text{glucose}$ drink
305 and was stable during the 60-120 min period (Figure 1A). Baseline ^{13}C -enrichments from resting breath
306 samples were comparable between PLC ($-25.2 \pm 3.6 \text{ ‰}$ vs PDB) and PRO ($-25.0 \pm 1.8 \text{ ‰}$ vs PDB) ($P >$
307 0.05). Changes in enrichment after ingestion of the drink at the start of 2 h of endurance exercise at
308 55% W_{max} are shown in Figure 1B. $^{13}\text{CO}_2$ enrichments levelled off from 45 min during both trials and
309 there were no significant differences at any time point between PLC and PRO.

310

311 Mean CHO substrate oxidation during 60-120 min is summarised in Table 2. Mean oxidation of the
312 ingested maltodextrin/ $[\text{U-}^{13}\text{C}]\text{glucose}$ drink was higher in PRO compared to PLC (Table 2), as was the
313 maximal oxidation observed (0.84 ± 0.10 vs $0.77 \pm 0.09 \text{ g}\cdot\text{min}^{-1}$, $P = 0.016$) which was achieved at 120
314 min during both trials (Fig S1). There was no difference in mean liver-derived glucose oxidation and
315 muscle glycogen oxidation tended to be higher in PRO but did not reach statistical significance.

316

317 Blood metabolites

318 At the start of exercise, plasma glucose, lactate, NEFA and glycerol concentrations were all similar in
319 both trials (Figure 2). Plasma glucose increased during the first 30 min of exercise before decreasing
320 at 45 min and remained stable for the rest of the exercise bout. When expressed as AUC, there was a
321 significant difference between PRO and PLC ($P = 0.013$) (Figure 2A). After the ingestion of the
322 maltodextrin drink, there were significant and concomitant increases and decreases in exogenously
323 derived and endogenous plasma glucose concentrations, respectively ($P < 0.05$) (Figure 3). Plasma
324 glucose concentrations derived from exogenous glucose was significantly greater at 15 min in PRO
325 compared to PLC ($P = 0.01$) (Figure 3A). Plasma lactate increased in response to exercise to $\sim 2 \text{ mmol}\cdot\text{L}^{-1}$
326 ¹ and then gradually declined during the course of the exercise bout (Figure 2B). Insulin concentrations
327 were higher at 30, 45 and 75 min in PRO compared to PLC ($P < 0.05$) with a significant difference
328 between AUC data ($P = 0.04$). NEFA concentrations reduced at the onset of exercise and subsequently
329 increased from 60 min in both trials, with a significant difference between trials by 120 min ($P = 0.043$)
330 (Figure 2D). Plasma glycerol increased during exercise in both trials, with significantly lower
331 concentrations at 1200 min during PRO compared to PLC ($P > 0.001$) (Figure 2E).

332

333 Markers of GI permeability, damage and cytokines

334 Individual data points for LR during PRO and PLC are presented in Figure 4A. There was no significant
335 difference in LR between PRO (0.045 ± 0.02) and PLC (0.052 ± 0.03) ($P = 0.436$). For I-FABP, there was
336 no significant difference between PRO and PLC pre ($P = 0.364$), post ($P = 0.374$) or 1hr post exercise (P
337 $= 0.393$) for PRO and PLC, while there was also no effect of exercise (Figure 3B). Plasma cytokine
338 concentrations for pre and post-exercise are presented in Table 3. For pre-exercise measures, IL-1 α
339 and IL-6 concentrations were lower in PRO, while IL-6 was also lower post-exercise in PRO.

340

341 GI symptoms and time trial performance

342 During exercise trials, individual GI symptoms assessed were low (< 4 on scale of 0-10), even when
343 using maximum values from each trial. During the 100kJ time trial there was no significant difference
344 in the time to complete between placebo ($308 \pm 69 \text{ s}$) and probiotic ($301 \pm 74 \text{ s}$) ($P = 0.714$).

345 Data supplements can be found here: <https://doi.org/10.24377/LJMU.d.00000050>

346 Discussion

347 The main aims of this study were to investigate the potential of 4 weeks of probiotics supplementation
348 (PRO) compared to placebo (PLC) to alter exercise metabolism and improve performance in trained
349 athletes during 2 h of cycling exercise at moderate intensities. One of our hypotheses was that PRO
350 would exert this effect via positive effects on GI permeability and prevention of GI damage. This was
351 difficult to ascertain given that there were no increases in exercise-induced damage/permeability,
352 most likely relating to the exercise intensity. The other hypothesis was that PRO would increase the
353 maximal rate of the oxidation of maltodextrins to a value higher than the maximal value of $1 \text{ g}\cdot\text{min}^{-1}$
354 which we observed in a previous study (53). To the authors' knowledge, this is the first study to
355 investigate whether PRO, compared to PLC, has the potential to increase the oxidation rate of both
356 total carbohydrates and orally ingested maltodextrins during endurance exercise. The use of stable
357 isotope tracer methodologies, in combination with indirect calorimetry, has enabled us to quantitate
358 total CHO and total fat oxidation and the gradual change in the fraction of plasma glucose originating
359 from the ingestion of the maltodextrin drink and estimate muscle glycogen and liver glucose utilisation
360 over the 120 min exercise period (Fig. 3).

361 We have shown for the first time that PRO leads to a small but significant increase in total
362 carbohydrate oxidation in the 60-120 min exercise period (Table 1) by increasing both the oxidation
363 of the ingested maltodextrins ($P=0.024$) and muscle glycogen (NS) compared to placebo (Table 2). The
364 increase in total CHO oxidation coincided with a decrease in total fat oxidation (Table 1). It is well-
365 established that the ingestion of multiple transporter carbohydrates (i.e. glucose and fructose) can
366 improve the oxidation of exogenous CHO well above the values reported here ($\sim 1.5 \text{ g}\cdot\text{min}^{-1}$ vs $0.8\text{-}0.9$
367 $\text{g}\cdot\text{min}^{-1}$) (11, 25). However, the proof of principle shown here that PRO may alter substrate utilisation
368 and increase the oxidation of glucose derived from ingested maltodextrins warrants further
369 investigation to both replicate this finding and observe if this apparent difference is observed when
370 both glucose and fructose are consumed during exercise. If the results here are replicated, this would
371 at the very least be of interest to those exercising for 1-2.5 hrs, for which current CHO intake
372 recommendations are 30-60 g per hour during exercise (5).

373 We, in this and previous studies (53), have chosen to orally administer a 10% maltodextrin solution as
374 maltodextrins are rapidly hydrolysed into free glucose and absorbed into the vena porta. The rapid
375 hydrolysis of maltodextrins in the human GI tract is an important reason for their frequent use in
376 commercial sports drinks (18). In the previous study (53) we have used maltodextrins naturally
377 enriched with ^{13}C . In the current study we have mixed the maltodextrin solution with a $[\text{U-}^{13}\text{C}]$ glucose
378 tracer to thus achieve a high plasma glucose TTR. This was required in this study to facilitate accurate
379 estimates of the rates of the oxidation of the ingested maltodextrins and the estimation of the

380 production and oxidation of glucose by the liver (sum of liver glycogen breakdown and
381 gluconeogenesis). Pilot data in the first two participants revealed that the plasma [U-¹³C]glucose
382 enrichment reached a plateau in the 30-120 min period, therefore excluding a difference in
383 appearance kinetics between the maltodextrin solution and the [U-¹³C]glucose tracer. The profile of
384 the ¹³C-enrichment curves of the breath gas samples was similar to the profiles seen in a previous
385 study in which we used naturally enriched maltodextrins (53). For breath gas samples, a plateau was
386 reached towards the end of the second hour in both studies. However, the variation between
387 individuals in plateau enrichment was lower here than with naturally enriched maltodextrin
388 consumption (53). Such variations in plateau enrichments have been shown to be the consequence of
389 variations in the natural ¹³C-enrichment of individuals' endogenous carbohydrate stores (54). In the
390 current study the [U-¹³C]glucose enrichment was >100-fold higher than seen in Wagenmakers, Brouns,
391 Saris and Halliday (53), and the variation between the 7 participants for the plasma [U-¹³C]glucose TTR
392 and breath ¹³CO₂ enrichment (δ per mil vs PDB) were minimal (Figure 1A and 1B). We believe that the
393 current study is therefore the first to validate the use of a mixture of naturally enriched maltodextrins
394 with a [U-¹³C]glucose tracer to estimate the oxidation of the maltodextrin solution during prolonged
395 exercise.

396 It is clear in the present study that there are small but significant differences in fuel selection between
397 PRO and PLC. The AUC data for plasma glucose and plasma insulin concentrations were higher for PRO
398 than for PLC (Fig 2), suggesting that the intestinal absorption of glucose, particularly in the first 30 min
399 of exercise, was higher for PRO than for PLC. This is supported by the higher plasma glucose
400 concentration originating from the ingested maltodextrins after 15 minutes of exercise during PRO
401 compared to PLC (Figure 3A). Higher duodenal glucose absorption rates are known to stimulate insulin
402 production by the pancreatic β-cells (40). Higher systemic insulin concentrations, as are seen during
403 PRO compared to PLC (Fig 1C), have previously been shown to suppress lipolysis in subcutaneous
404 adipose tissue (7). It has been shown that oral ingestion of glucose before and during exercise reduced
405 lipolysis both in subcutaneous adipose tissue stores and lipolysis of the intramuscular triglyceride
406 (IMTG) stores in skeletal muscle (10). This led to a substantial reduction in the oxidation rate of plasma
407 FA, IMTG and total fat during exercise (10). These mechanisms contribute to the lower plasma NEFA
408 and glycerol levels (Figure 2D and 2E) and the reduction in total fat oxidation in PRO compared to PLC
409 (Table 1). However, the small difference in intestinal absorption suggested here does not likely fully
410 explain the differences in plasma insulin and glycerol. Other GI related factors, such as the intestinal
411 incretin response have been shown to effect insulin response to an oral glucose load (16). It may then
412 be plausible that differences in other GI related factors that following PRO supplementation, explain
413 some of the differences in plasma insulin and glycerol observed here.

414 During the 2 h of exercise there is a gradual increase in the fraction of plasma glucose originating from
415 the oral ingestion of the maltodextrin drink (Fig 3A), while at the same time there is a reduction in the
416 fraction of glucose produced by the liver (sum of liver glycogen breakdown and gluconeogenesis; Fig.
417 3B). The plasma glucose concentration (sum of the 2 fractions) remains constant. These data
418 collectively support the concept that the liver acts as a 'glucostat' (8, 38, 56). The data in Fig 3A-B and
419 in previous work (10, 26) clearly show that oral ingestion of carbohydrates reduces endogenous
420 glucose production in proportion to the intestinal absorption rate of glucose and oxidation rate of
421 glucose in the exercising muscles. This role of the liver as a 'glucostat' keeping the blood glucose
422 concentration during exercise constant explains the data we obtained in the current study.

423 While we have presented data that demonstrates differences in exercise metabolism following PRO,
424 we did not observe any difference in 100 kJ time trial performance between supplement groups. It
425 was hypothesised that, had PRO resulted in greater consumed CHO absorption and oxidation had been
426 substantially increased, this would have resulted in larger muscle and/or liver glycogen stores. This
427 would then be of benefit during a higher intensity exercise performance test, following an initial
428 endurance exercise, as has been seen previously with exogenous CHO feeding studies (17, 34). A time
429 trial lasting ~240-360s was chosen as this has been shown to be a reliable performance indicator in
430 trained cyclists (30) and suggested to be at the higher end of duration of sustained power output
431 increases during successful breakaways during professional road cycling (1). The failure to observe an
432 effect of PRO on performance is most likely related to the small increase in the oxidation of exogenous
433 CHO during the preceding exercise. Greater increases in exogenous CHO oxidation following PRO
434 would have likely reduced muscle and/or liver glycogen oxidation and thus spared for use during the
435 higher intensity exercise during the time trial. However, it should also be highlighted that a limitation
436 of the time trial performance measure here was the potential lack of statistical power. While the
437 primary outcome, and thus power calculation performed to ensure sufficient sample size, was to
438 detect differences in exercise metabolism, this may have been under powered for a number of
439 secondary measures, including the time trial performance and GI measures.

440 The presented GI permeability and damage data did not show an increase in measures for I-FABP or
441 GI symptoms during 120 min of cycling, while GI permeability was not different between PRO and PLC
442 and many values were similar to resting values previously reported in our laboratory (41, 42). GI
443 permeability has previously been reported to only significantly increase compared to resting values at
444 exercise intensity of $\geq 80\% \dot{V}O_{2max}$ at ambient temperatures (37), while increases in plasma I-FABP have
445 also not always been shown during moderate intensity exercise, particularly when subjects maintain
446 euhydration and consume CHO (29). In the present trial, exercise intensity was $55\% W_{max}$ ($\sim 55\dot{V}O_{2peak}$).
447). This exercise intensity, in line with previous investigations (55, 58), was chosen as it mimics exercise

448 intensities experienced by professional road cyclists during competition (36) and the intensity at which
449 they are most likely to consume CHO during exercise.. In regards to GI symptoms, these were generally
450 low, again most likely due the chosen exercise modality (cycling instead of running), duration, and
451 intensity, which do not appear to have led to a sustained functional challenge to the GI system. To
452 better investigate the effects of probiotics on exercise induced GI permeability, damage and
453 symptoms, particularly in the presence of carbohydrate ingestion, exercise of a greater intensity and
454 duration should be considered.

455 We conclude that the presented stable isotope tracer data demonstrate for the first time in humans
456 *in vivo* that after 4 weeks of PRO during 2 h of cycling exercise at 55% W_{max} a small, but significant
457 increase in the oxidation rate of glucose originating from an orally ingested maltodextrin solution was
458 observed. We also show significant increases in the plasma glucose and insulin concentration with
459 significant increases occurring in the 2 h area under the curve (AUC) both for plasma glucose and
460 plasma insulin (Fig 2) following PRO. The higher plasma insulin concentrations during exercise reduce
461 total fat oxidation (Table 2) via inhibition of lipolysis of subcutaneous adipose tissue and IMTG lipolysis
462 as we have shown previously (10). Although these data show that PRO does lead to small, but
463 measurable changes in fuel selection and oxidation during exercise, they do not confirm our prior
464 hypothesis that PRO could lead to larger increases in the absorption and oxidation rate of the ingested
465 maltodextrins or increases the maximal maltodextrin oxidation rates reached a plateau to values
466 higher than $1 \text{ g}\cdot\text{min}^{-1}$.

467

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475 J.P., A.W., and G.C., conceived and planned the experiments. J.P. and D.D. collected performed the
476 exercise trials and data collection. J.P., A.W., and J.M. processed and analysed GI the data. S.F.
477 performed Lactulose:Rhamnose analysis. B.F. completed mass spectrometry analysis and
478 interpretation. J.P. took the lead in writing the manuscript. All authors provided critical feedback and
479 helped shape the research, analysis and manuscript.

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641 Table 1. CHO and fat metabolism during 0-60 and 60-120 min. Data are mean \pm SD. *Significantly different from PLC ($P <$

	0-60 min			60-120 min		
	PLC	PRO	<i>P</i> value	PLC	PRO	<i>P</i> value
RER	0.89 \pm 0.03	0.92 \pm 0.03*	0.037	0.86 \pm 0.03	0.90 \pm 0.02*	0.005
CHO oxidation (g·min ⁻¹)	2.11 \pm 0.45	2.38 \pm 0.32	0.087	1.87 \pm 0.39	2.20 \pm 0.25*	0.038
Fat oxidation (g·min ⁻¹)	0.46 \pm 0.11	0.34 \pm 0.08*	0.041	0.55 \pm 0.10	0.40 \pm 0.11*	0.021

642 0.05)

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644

645 *Table 2. Mean CHO utilisation calculated during 60-120 min. Data are presented as g.min⁻¹ and are mean ± SD. *Significantly*
 646 *different from placebo (P < 0.05)*

	PLC	PRO	P-value
Ingested maltodextrin	0.75 ± 0.09	0.79 ± 0.10*	0.024
Liver-derived glucose	0.21 ± 0.08	0.19 ± 0.04	0.323
Muscle glycogen	0.99 ± 0.41	1.24 ± 0.28	0.087

650

651 *Table 3. Pre and post-exercise cytokine concentrations for PLC and PRO. Data are mean \pm SD.*

	Pre-exercise			120 min		
	PLC	PRO	<i>P</i> value	PLC	PRO	<i>P</i> value
IL-1 α (pg·mL ⁻¹)	1.39 \pm 0.82	0.63 \pm 0.64	0.031*	1.84 \pm 1.23	1.78 \pm 1.40	0.450
IL-6 (pg·mL ⁻¹)	2.32 \pm 1.22	1.22 \pm 0.90	0.037*	3.69 \pm 2.02	2.27 \pm 1.19	0.049*
IL-8 (pg·mL ⁻¹)	2.49 \pm 1.18	2.53 \pm 1.38	0.481	4.70 \pm 1.57	4.49 \pm 2.83	0.409
IL-10 (pg·mL ⁻¹)	1.95 \pm 1.37	1.14 \pm 0.90	0.093	3.13 \pm 2.05	3.25 \pm 3.09	0.433

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654 Figure 1. (A) Plasma glucose tracer/tracee ratio (TTR) and (B) Breath $^{13}\text{CO}_2$ enrichment during exercise. Values are means \pm
 655 SD. PDB, Pee Dee Belemnite
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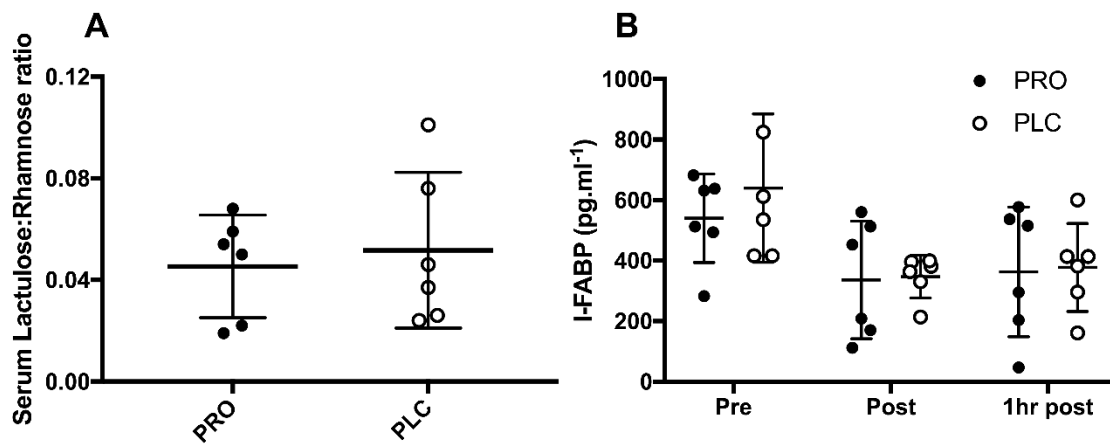
657
 658 Figure 2. Plasma (A) glucose, (B) lactate, (C) insulin, (D) NEFA, and (E) glycerol. *significant difference between PRO and PLC
 659 at the corresponding time point ($P < 0.05$). # significant difference between PRO and PLC
 660

661 Figure 3. Plasma glucose concentration derived from (A) glucose derived from ingested maltodextrin, (B) endogenously-
 662 derived glucose sources *significant difference between PRO and PLC at the corresponding time point ($P < 0.05$).
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 665 Figure 4. A) Serum lactulose:rhamnose ratio for PRO and PLC. B) Intestinal-fatty acid binding protein (I-FABP) pre, post and
 666 1hr post exercise for PRO and PLC
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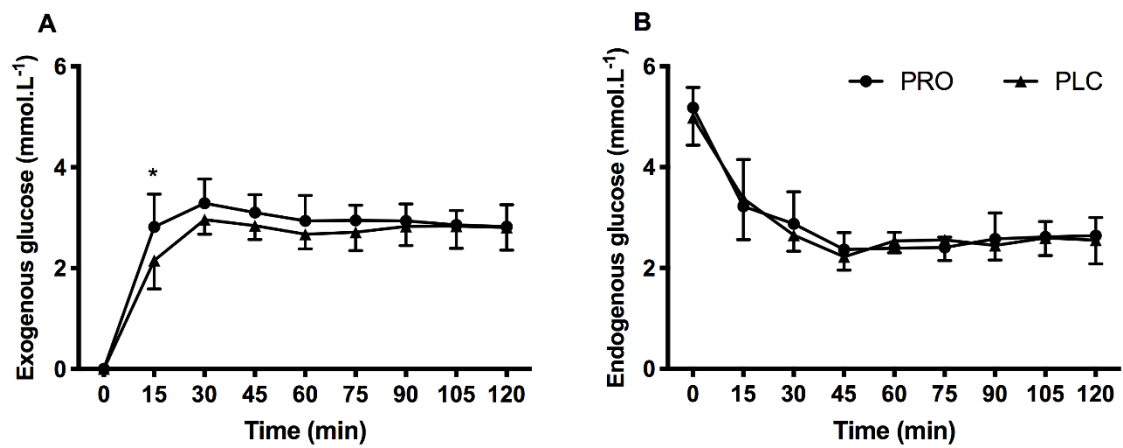
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