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

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

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Abstract

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

Introduction

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

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

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

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

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

Methods

Participants

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

Pre-testing

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

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

Treatment allocation

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

Experimental trials

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

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

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

Maltodextrin drink

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

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

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

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

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

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

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

$$[U\text{-}^{13}C]Glu_p = Glu_p \times TTR$$

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

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

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

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

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

$$Glu_m = [^{12}C]Glu_p + [U\text{-}^{13}C]Glu_p$$

and the difference between this value and total plasma glucose concentration was assumed to have been derived endogenously. This calculation distinguishes between [12C]Glu produced endogenously and [12C]Glu in the test drink.

Indirect calorimetry and calculations

Respiratory gas exchange variables were measured using a mixing chamber (Moxus modular metabolic system, AEI technologies Inc, Pennsylvania, USA) with oxygen uptake ($\dot{V}O_2$), carbon dioxide output ($\dot{V}CO_2$), and respiratory exchange ratio (RER) measured during a 4 min interval and sample frequency of 15 seconds after every 15 min during the 2 h of cycling exercise. Breath samples were collected in duplicate directly from the mixing chamber of the MOXUS system into sealed vacutainer collection tubes again every 15 min. Total CHO and fat oxidation rates were calculated from indirect calorimetry data assuming negligible protein oxidation (27):

$$\text{Glucose oxidation} = 4.55 \times \dot{V}CO_2 - 3.21 \times \dot{V}O_2$$

$$\text{Fat oxidation} = 1.67 \times \dot{V}O_2 - 1.67 \times \dot{V}CO_2$$

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

$$= \dot{V}CO_2 \times (\delta Exp - \delta Expbkg)/(\delta Ing - \delta Expbkg)/k$$

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

Plasma glucose oxidation was calculated using the formula:

$$= \dot{V}CO_2 \times (\delta Exp - \delta Expbkg\delta/\delta PG - \delta PGbkg)/k$$

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

Because plasma glucose oxidation represents the oxidation of both glucose coming from the gut (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-1α
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-1α 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 $pg \cdot mL^{-1}$) 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

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

Results

Physiological response to exercise

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

Substrate utilisation during 60 – 120 min exercise period

Plasma [U-^{13}C]glucose /^{12}C glucose ratios increased as a result of maltodextrin/[U-^{13}C]glucose drink and was stable during the 60-120 min period (Figure 1A). Baseline ^{13}C-enrichments from resting breath samples were comparable between PLC (-25.2 ± 3.6 ‰ vs PDB) and PRO (-25.0 ± 1.8 ‰ vs PDB) ($P > 0.05$). Changes in enrichment after ingestion of the drink at the start of 2 h of endurance exercise at 55% W_{max} are shown in Figure 1B. $^{13}CO_2$ enrichments levelled off from 45 min during both trials and there were no significant differences at any time point between PLC and PRO.

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

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 ~2 mmol·L^{-1}
326 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-1α
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 ± 69 s) and probiotic (301 ± 74 s) (P = 0.714).

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

Discussion

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

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

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

production and oxidation of glucose by the liver (sum of liver glycogen breakdown and gluconeogenesis). Pilot data in the first two participants revealed that the plasma [U-^{13}C]glucose enrichment reached a plateau in the 30-120 min period, therefore excluding a difference in appearance kinetics between the maltodextrin solution and the [U-^{13}C]glucose tracer. The profile of the ^{13}C-enrichment curves of the breath gas samples was similar to the profiles seen in a previous study in which we used naturally enriched maltodextrins (53). For breath gas samples, a plateau was reached towards the end of the second hour in both studies. However, the variation between individuals in plateau enrichment was lower here than with naturally enriched maltodextrin consumption (53). Such variations in plateau enrichments have been shown to be the consequence of variations in the natural ^{13}C-enrichment of individuals' endogenous carbohydrate stores (54). In the current study the [U-^{13}C]glucose enrichment was >100-fold higher than seen in Wagenmakers, Brouns, Saris and Halliday (53), and the variation between the 7 participants for the plasma [U-^{13}C]glucose TTR and breath $^{13}CO_2$ enrichment (δ per mil vs PDB) were minimal (Figure 1A and 1B). We believe that the current study is therefore the first to validate the use of a mixture of naturally enriched maltodextrins with a [U-^{13}C]glucose tracer to estimate the oxidation of the maltodextrin solution during prolonged exercise.

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

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

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

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

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

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

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J.P., A.W., and G.C., conceived and planned the experiments. J.P. and D.D. collected performed the exercise trials and data collection. J.P., A.W., and J.M. processed and analysed GI the data. S.F. performed Lactulose:Rhamnose analysis. B.F. completed mass spectrometry analysis and interpretation. J.P. took the lead in writing the manuscript. All authors provided critical feedback and 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 ± SD. *Significantly different from PLC (P <*

	0-60 min			60-120 min		
	PLC	PRO	*P* value	PLC	PRO	*P* value
RER	0.89 ± 0.03	0.92 ± 0.03*	0.037	0.86 ± 0.03	0.90 ± 0.02*	0.005
CHO oxidation ($g \cdot min^{-1}$)	2.11 ± 0.45	2.38 ± 0.32	0.087	1.87 ± 0.39	2.20 ± 0.25*	0.038
Fat oxidation ($g \cdot min^{-1}$)	0.46 ± 0.11	0.34 ± 0.08*	0.041	0.55 ± 0.10	0.40 ± 0.11*	0.021

642 *0.05)*

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*Table 2. Mean CHO utilisation calculated during 60-120 min. Data are presented as g.min^{-1} and are mean ± SD. *Significantly 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

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

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

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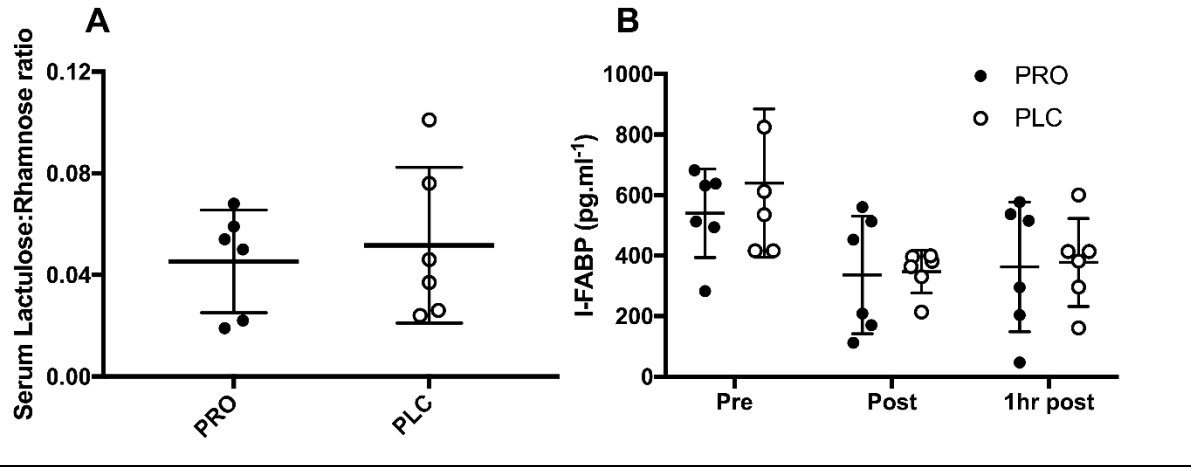
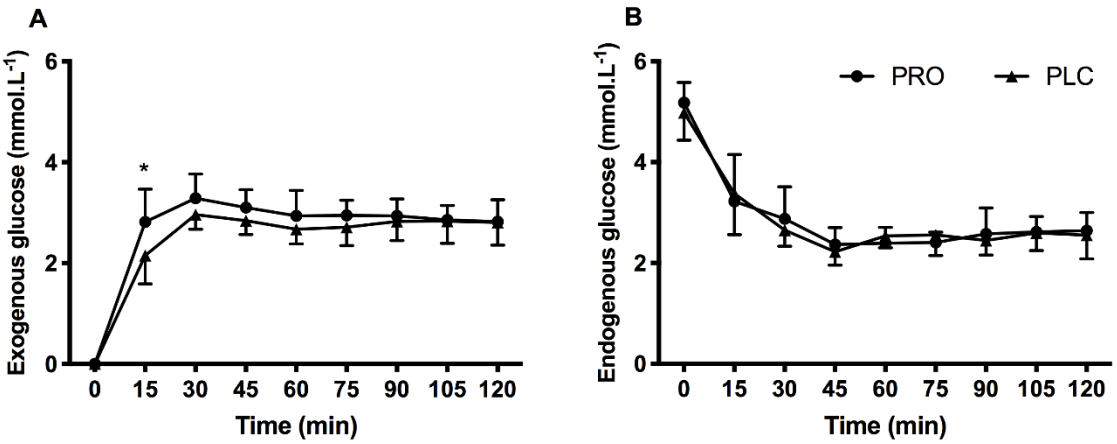
Figure 1. (A) Plasma glucose tracer/tracee ratio (TTR) and (B) Breath $^{13}CO_2$ enrichment during exercise. Values are means ± SD. PDB, Pee Dee Belemnite

*Figure 2. Plasma (A) glucose, (B) lactate, (C) insulin, (D) NEFA, and (E) glycerol. *significant difference between PRO and PLC at the corresponding time point (P<0.05). # significant difference between PRO and PLC*

*Figure 3. Plasma glucose concentration derived from (A) glucose derived from ingested maltodextrin, (B) endogenously-derived glucose sources *significant difference between PRO and PLC at the corresponding time point (P<0.05).*

Figure 4. A) Serum lactulose:rhamnose ratio for PRO and PLC. B) Intestinal-fatty acid binding protein (I-FABP) pre, post and 1hr post exercise for PRO and PLC




A
Exogenous glucose (mmol.L-1)
6
4
2
0
*
0 15 30 45 60 75 90 105 120
Time (min)
B
Endogenous glucose (mmol.L-1)
PRO
PLC
0 15 30 45 60 75 90 105 120
Time (min)

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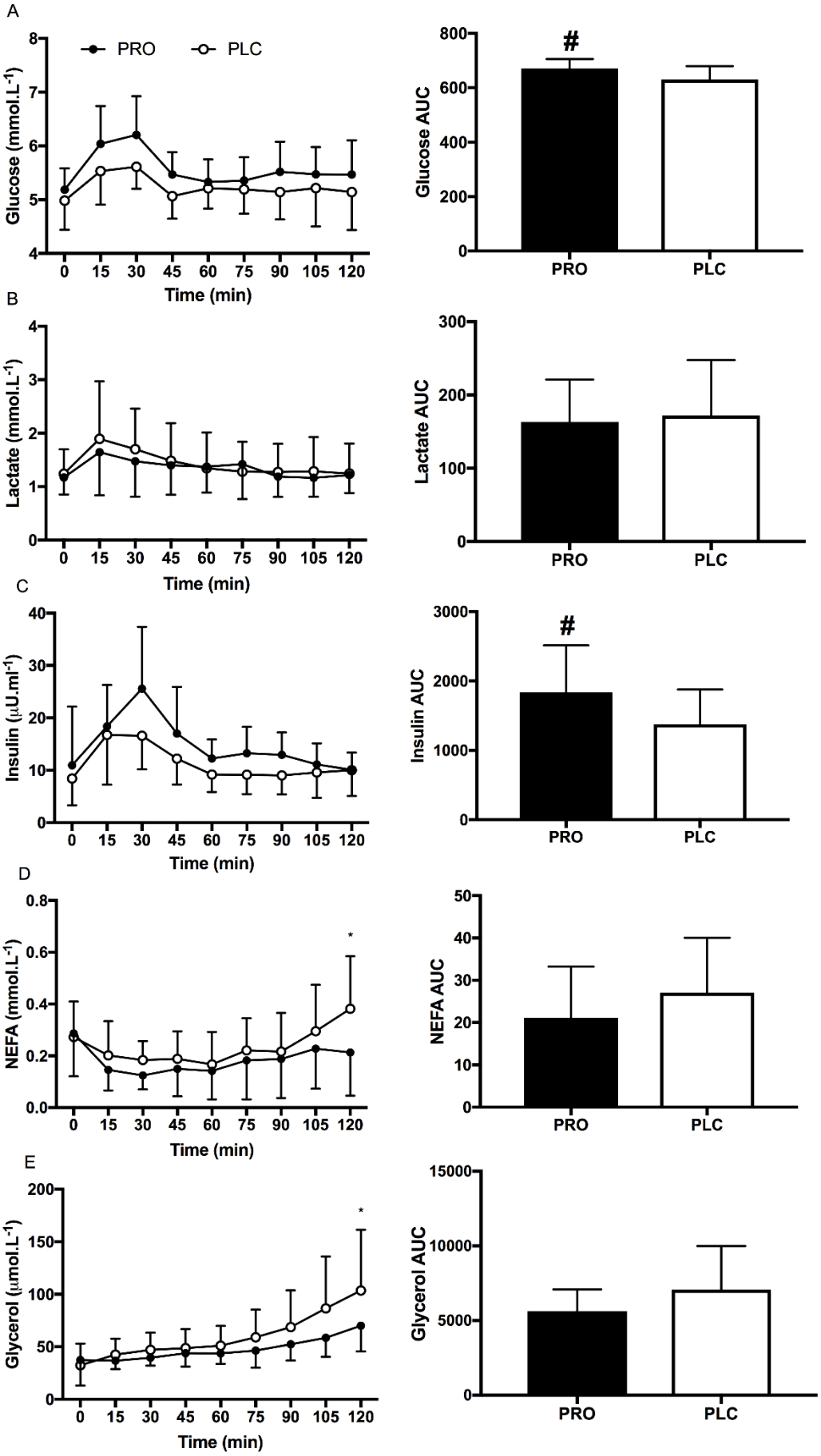
A
PRO
PLC
Glucose (mmol.L-1)
Time (min)
Glucose AUC
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PRO
PLC
B
Lactate (mmol.L-1)
Time (min)
Lactate AUC
PRO
PLC
C
Insulin (µU.ml-1)
Time (min)
Insulin AUC
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Time (min)
Glycerol AUC
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PRO
PLC

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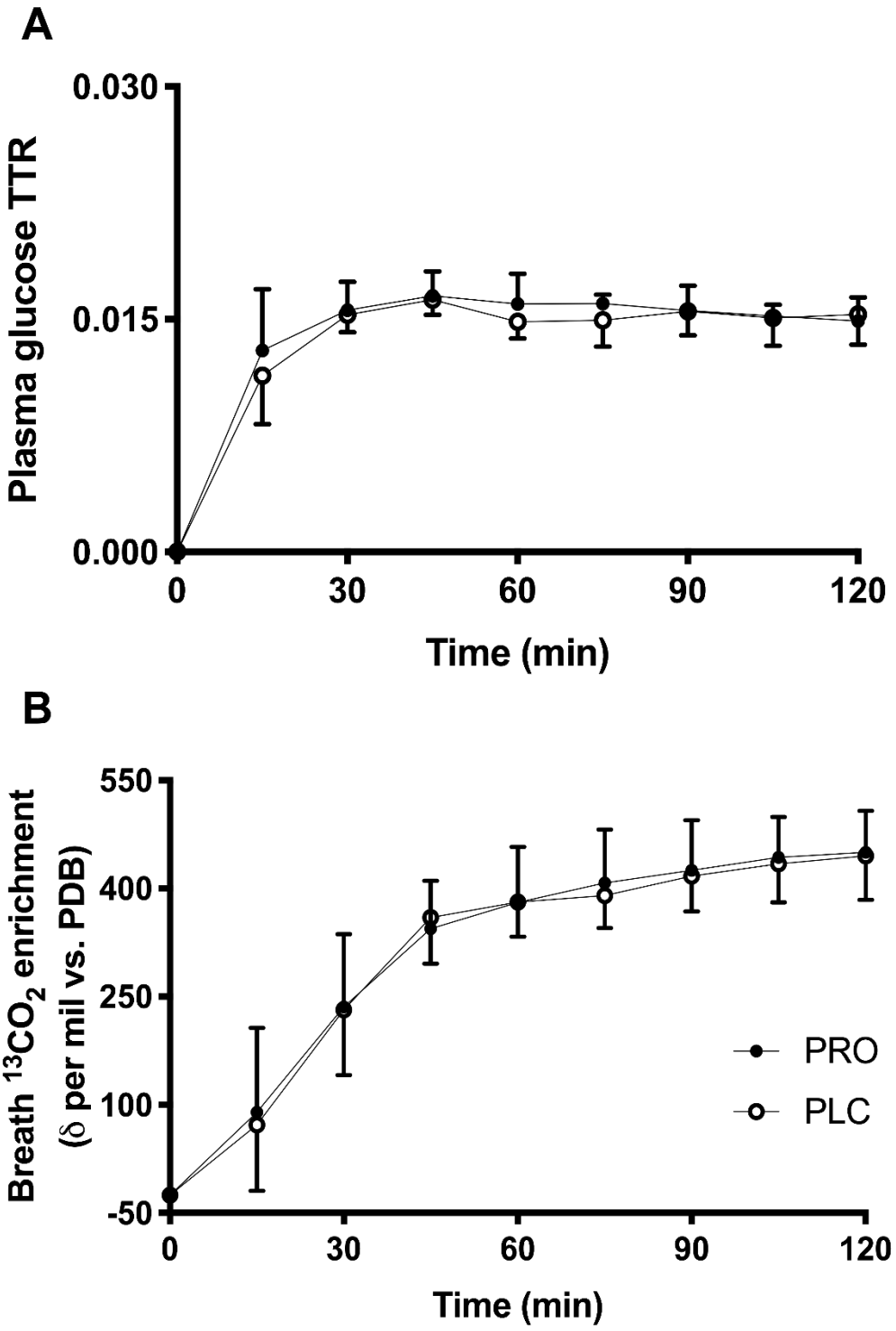
A
Plasma glucose TTR
0.030
0.015
0.000
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30
60
90
120
Time (min)
B
Breath $^{13}CO_2$ enrichment
(δ per mil vs. PDB)
550
400
250
100
-50
0
30
60
90
120
Time (min)
PRO
PLC

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