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Research Article

Safety and efficacy of a probiotic cocktail containing *P. acidilactici* and *L. plantarum* for gastrointestinal discomfort in endurance runners: randomized double-blinded crossover clinical trial

Sarah Lennon[a], **Thomas Lackie**[a], **Adriana Miltko**[a], **Zoey C. Kearns**[a], **Maxime R. Paquette**[a], **Richard J. Bloomer**[a], **Anyou Wang**[b], **and Marie van der Merwe**[a]

[a]Center for Nutraceuticals and Dietary Supplement Research, College of Health Sciences, University of Memphis, Memphis, TN, USA; [b]Harry Feinstone Center for Genomic Research, University of Memphis, Memphis, TN 38152, USA

Corresponding author: **Marie van der Merwe** (email: mvndrmrw@memphis.edu)

Abstract

Probiotics are increasingly used to treat conditions associated with gastrointestinal injury and permeability, including exercise-induced gastrointestinal discomfort. This study assessed safety and efficacy of a probiotic in altering the intestinal milieu and mitigating gastrointestinal symptoms (GIS) in endurance runners. In a double blind, crossover study, 16 runners were randomized to 4 weeks of daily supplementation with a probiotic cocktail containing *Pediococcus acidilactici* bacteria and *Lactobacillus plantarum* or placebo. Fasting blood and stool samples were collected for measurement of gut permeability markers, immune parameters, and microbiome analyses. Treadmill run tests were performed before and after treatment; participants ran at 65%–70% of VO_{2max} at 27 °C for a maximum of 90 min or until fatigue/GIS developed. A blood sample was collected after the treadmill run test. In healthy individuals, 4 weeks of probiotic supplementation did not alter health parameters, although a marginal reduction in aspartate aminotransferase levels was observed with probiotic treatment only ($p = 0.05$). GIS, gut permeability-associated parameters (intestinal fatty acid binding protein, lipopolysaccharide binding protein, zonulin, and cytokines), and intestinal microbial content were not altered by the probiotic supplementation. Post-run measurements of GIS and gut-associated parameters did not differ between groups; however, the observed lack of differences is confounded by an absence of measurable functional outcome as GIS was not sufficiently induced during the run. Under the current study conditions, the probiotic was safe to use, and did not affect gut- or immune-associated parameters, or intestinal symptoms in a healthy population. The probiotic might reduce tissue damage, but more studies are warranted.

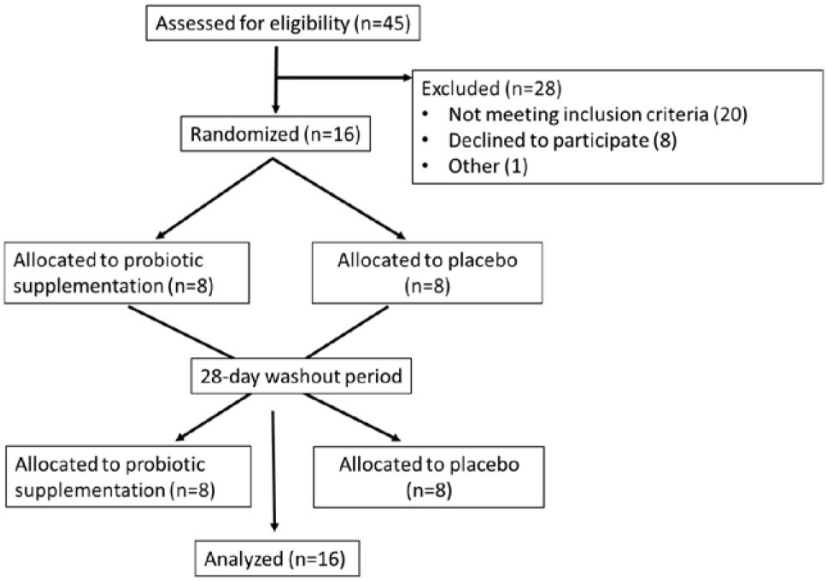
Key words: probiotics, gastrointestinal injury and permeability, endurance runners

1. Introduction

Probiotics have been studied due to their favorable health benefits that include modulating the number and diversity of beneficial gut microbiota (Santos-Marcos et al. 2019) and reducing symptoms associated with gastrointestinal (GI) disorders (Ferrario et al. 2014). The gut microbiome has been shown to regulate many intestinal functions, including barrier maintenance, immune education, and inflammatory status (Jandhyala et al. 2015). In addition, the metabolites from bacterial fermentation, including short chain fatty acids (SCFA), act as an energy source for colonocytes and function as immune modulatory signaling molecules (Ghouri et al. 2014). Due to our improved understanding of the role of various microbes in human health, probiotics are increasingly utilized as a complementary therapy for various pathological conditions. The advantage of using probiotics is an improved safety profile and fewer side effects compared to many traditional therapies (Ghouri et al. 2014). However, it is important to note that probiotics may not exhibit the same level of efficacy as pharmacological agents. The use of specific probiotics strains are potentially beneficial in GI disorders such as irritable bowel disease (IBS) and colitis (Niu and Xiao 2020; Xie et al. 2023), possibly through increased intestinal SCFA production and improved intestinal barrier function (LeBlanc et al. 2017).

Gastrointestinal symptoms (GIS) are widely reported during prolonged endurance exercise, with 27% of recreational runners reporting moderate symptoms, including belching, bloating, flatulence, abdominal cramping, side stitch, nausea, vomiting, diarrhea, and the urge to defecate, during a race (Pugh et al. 2018). These symptoms are detrimental to exercise performance in both recreational and elite runners. The cause of the discomfort is multifactorial and includes a change in blood flow, as blood is shunted from the viscera

Fig. 1. The CONSORT diagram.



to skeletal muscle and the heart. The resultant hypoxia impacts the permeability of the intestinal barrier by reducing epithelial tight junctions (Otte et al. 2001). Loss of epithelial integrity results in translocation of bacterial products, including lipopolysaccharide, and increasing release of systemic inflammatory molecules (Jeukendrup et al. 2000).

As many of the symptoms of exercise-induced GI discomfort resembles that of IBS and colitis, probiotics are being tested for the improvement of exercise-induced GIS. Regular use of probiotics can alter both the abundance and structure of the microbial community and also affect immune responses in healthy as well as trained individuals (Mach and Fuster-Botella 2017). The therapeutic efficacy of probiotic supplementation therefore varies depending on the mechanism of action of bacterial strains or strains included; the single stain probiotic *Bacillus subtilis* DE111 has been shown to reduce the proinflammatory cytokine TNF-α in athletes but did not affect gut permeability (Townsend et al. 2018), while the *Lactobacillus plantarum* strain TWK10 (plant-derived strain isolated from Taiwanese pickled cabbage) showed significant improvement in endurance performance and body composition after a 6-week supplementation period (Huang et al. 2018). *Lacticaseibacillus rhamnosus GG* supplementation has also been shown to cause a significant reduction in the duration of GI symptom episodes (Kekkonen et al. 2007) and *Lactobacillus acidiphilus* (LAFTI®L10) improved immunity in fatigued athletes and increased IFN-γ production (Clancy et al. 2006). Multi-cocktail probiotic supplementation has also been used in endurance athletes and a cocktail of two *Lactobacillus* strains was shown to reduce oxidative stress induced by intense exercise (Martarelli et al. 2011).

The 3-strain cocktail containing two *L. plantarum* strains and one *Pediococcus acidilactici* strain demonstrated improvement in IBS-related quality of life in a placebo-controlled, double-blind, multicentric clinical trial of patients with IBS (Lorenzo-Zúñiga et al. 2014). These specific strains survive gut passage and adhere to intestinal mucus in vitro (Lorén et al. 2017). This cocktail has also been shown to increase SCFA production (D'Argenio and Mazzacca 1999) and reduce inflammation in two preclinical models of intestinal inflammation (Lorén et al. 2017). Due to the characteristics of these bacterial strains and improvement in symptoms associated with of intestinal discomfort, similar to that seen in exercise-induced GI discomfort, we hypothesized that 4 weeks of supplementation with the cocktail of *L. plantarum* and *P. acidilactici* will be safe to use and would improve exercise-induced GIS and associated biochemical molecules in runners with GIS.

2. Materials and methods

2.1. Experimental design

This was a randomized placebo-controlled, double-blinded, cross-over study. Study design is indicated by the CONSORT flow diagram in Fig. 1. Forty-five runners were screened and 25 men and women with self-reported GI complaints that occur during distance running were enrolled in the study. The University of Memphis Institutional Review Board approved all procedures. The study was registered with ClinicalTrials.gov (NCT05425329). Recruitment and sample collection occurred between August 2020 and June 2021. All subjects provided written informed consent prior to participation. Eight participants discontinued the study after enrollment, but prior to data collection. Data from one participant were removed due to disease diagnosis. Data from 16 participants were included for analysis. Data were collected at the Centre for Nutraceuticals and Dietary Supplement Research at the University of Memphis.

Inclusion criteria for participation was as follows: healthy men and women between 18 and 50 years of age; who have run for more than 2 consecutive years; run at least 15 miles a week; run three times or more per week; have completed a

run of 90 min or more at least once per month for the past 6 months; and frequently experience GI discomfort during or after running (a self-reported rating of at least 4 on a scale of 0–9 indicating "moderate problems" for one of the six lower abdominal symptoms recorded). Exclusion criteria includes: being pregnant or breastfeeding; using NSAIDs, probiotics or supplements that might alter the gut microbiome within 1 month before enrollment, following a low FODMAP (fermentable oligo, di-, monosaccharide, and polyols) diet, have a history of drug or alcohol abuse in the past year; have had GI surgery in the past year or have a history of severe heart, liver, kidney, neurological, oncological, psychiatric, coeliac or inflammatory bowel disease, acute pancreatitis, or are immunosuppressed.

Participants were recruited via social media posts, flyers, and word of mouth. After screening for eligibility, subjects provided informed consent and enrolled in the study. Participants were randomized to probiotic or placebo using a simple randomization technique. The primary investigator generated the random allocation and assigned participants to either treatment A or B. To minimize bias both participants and study investigators were blinded to treatment content. Anthropometric data (weight and height) were collected, and a urine pregnancy test was performed on all female participants. Prior to supplementation all participants performed in a VO_{2max} test to determine running parameters to be used during the in-house treadmill run tests. For the VO_{2max} test, participants ran in a temperature-controlled room (~27 °C) and heart rate and respiratory variables was measured using a metabolic cart (Parvo Medics TrueOne 2400, UT) and a POLAR heart rate monitor. Participants had eight laboratory visits over a 3-month period; at the start and end of the 1-month probiotic and placebo supplementation period, which is separated by a 1-month washout period. At each timepoint, participants came to the laboratory for a fasted blood draw and distribution of fecal sample collection supplies. Fecal samples were self-collected during the 24 h after blood collection, but prior to the treadmill run test. A breakfast bar (fat: 6 g, carbohydrates: 43 g, dietary fiber: 5 g, and protein: 10 g) was provided to be consumed prior to the treadmill run. Participants returned to the laboratory the following day to return fecal samples (stored at −80 °C until analysis) and to perform the treadmill run test where participants ran at approximately 65%–70% of their VO_{2max} for a maximum of 90 min. Rating of perceived exertion and GI discomfort were recorded every 10 min during the run and the run was terminated with the onset of fatigue (a rating of 20 on the Borg exertion scale) or GI symptoms become too severe to continue (a rating of 5–9 on the GI discomfort scale which indicate "severe symptoms that are substantial enough to interfere with the exercise workload"). In addition, at the end of 1 month of the probiotic and placebo supplementation period, venous blood was also collected within 5 min after the treadmill run concluded.

Participants were instructed to maintain their habitual diet, lifestyle, and training routine during the study period. Participants recorded their food and beverage intake for 3 days prior to each data collection point and nutrient analysis was performed using The Food Processor v11. GIS were recorded weekly using the clinical rating scale for GIS for patients with IBS and peptic ulcer disease (GSRS) (Svedlund et al. 1988).

2.2. Study supplement

The probiotic supplement (I3.1, Kaneka Corporation, AB-Biotics) contained a cocktail of *P. acidilactici,* and two *L. plantarum* strains at a ratio of 1:1:1 and was consumed at a dose of 3.0×10^9 cfu/capsule/day. The placebo capsules contained maltodextrin and were indistinguishable from the probiotic product. Participants were instructed to consume one capsule (either placebo or probiotic) per day for the duration of the 4-week supplementation period.

2.3. Sample collection

At baseline and after 1 month of placebo or probiotic treatment, participants reported to the laboratory in the morning after an overnight fast. Blood was collected by venipuncture in serum separator (Greiner Bio-One Serum Clot activator) and plasma (Greiner Bio-One K_2EDTA) and white blood cell (WBC) numbers were measured using a Hematology analyzer (VetScanHM2, Abaxis). Whole blood was centrifuged, and serum/plasma immediately harvested and stored at −80 °C for batch biochemical analysis. An additional blood sample was also collected immediately after the treadmill run after one month of supplementation and processed as mentioned. For blood SCFA analysis, plasma was harvested by centrifugation from whole blood and immediately frozen using liquid nitrogen. Samples were stored at −80 °C. Frozen samples were shipped to Metabolon Inc. where SCFA were analyzed using GC-MS.

2.4. Fecal collection for fecal SCFA and microbiome analysis

Fecal samples were self-collected by participants during the 24 h prior to the treadmill run test. For DNA analysis, fecal samples were collected using the OmniGENE-Gut system (OM-200, DNAGENOTEK) and for fecal SCFA analysis, samples were collected using OmniMET-Gut (ME-200, DNAGENOTEK) system. Collected fecal samples were stable at room temperature until delivery to the laboratory (<24 h), at which time the samples were stored at −80 °C.

2.5 Blood chemistry and biochemical analysis

General health markers were analyzed using a chemistry analyzer (Diasys Respons910 vet chemistry analyzer). Commercially available kits were used for the measurement of lipopolysaccharide (LPS)-binding protein (LBP, HycultBiotech, #HK315-02), zonulin (Alpco, 30-ZONSHU), intestinal fatty acid binding protein (intestinal fatty acid binding protein (iFABP), HycultBiotech, #HK406-02), calprotectin (EagleBiosiences, CAL35-K01), C reactive protein (CRP, Eagle Biosciences, CRP31-KO1), and insulin (Eagle Bioscieces, INS31-K01) following manufacturers' instructions.

Cytokine levels were measured with magnetic bead assays (Sigma Millipore) for the analytes IL-1β, IL-6, IL-8, IL-10, MCP-1 and TNF-α. Bead color and fluorescence intensity were analyzed using a Luminex Magpix analyzer and xPONENT Acquisition and Analysis Software.

2.6. Fecal and plasma SCFA analysis

Acetic acid (C2), propionic acid (C3), isobutyric acid (C4), butyric acid (C4), 2-methyl-butyric acid (C5), isovaleric acid (C5), valeric acid (C5), and caproic acid (hexanoic acid, C6) by were measured LC-MS/MS (Metabolon method TAM135: "LC-MS/MS method for the quantitation of SCFA (C2 to C6) in human feces" and TAM186: "LC-MS/MS method for the quantitation of SCFA (C2 to C6) in stool collection tubes (DNA Genotek)").

2.7. Microbiome analysis

For microbiome analysis genomic DNA was extracted using the QIAamp PowerFecal Pro DNA Kit (#51804, Qiagen) according to the manufacturer's instructions. DNA was extracted by mechanical perturbation using a bead-beater (BioSpec Minibeadbeater 16) for 3 min. DNA quantity and quality were assessed by absorbance ratio at 260/280 nm using a Nanodrop (Fisher Scientific). DNA samples were shipped to Heflin Center for Genomic Science (University of Alabama, Birmingham) where library generation and 16 srRNA sequencing were performed according to Kumar et al. (Kumar et al. 2014, 2016). Briefly, amplicon libraries were prepared by PCR amplification of the V4 region of the 16S rRNA gene. PCR products were sequenced using NextGen sequencing Illumina MiSeq platform. The quality of the raw data was assessed using FASTQC. Low quality data were filtered out using the FASTX toolset. The QIIME suite were utilized for clustering reads into operational taxonomic units (uclust), taxa assignment (RDP classifier using the Greengenes 16S rDNA database (Cole et al. 2007; Wang et al. 2007; McDonald et al. 2012) and as necessary, alignment and phylogenetic inference using PyNAST (Caporaso et al. 2010) and Fasttree (Price et al. 2010). The automated pipeline, QWRAP, plus DADA2 (Callahan et al. 2016) was used to provide a robust error model supporting sample filtering and clustering.

2.8. Gastrointestinal symptom assessment

Symptoms associated with GI discomfort were monitored weekly by a self-reported clinical rating scale of gastrointestinal symptoms (GSRS) questionnaire (Svedlund et al. 1988). A cumulative weekly GSRS score was determined by summation of all 15 topics included in the questionnaire, each rated 0–3 ("none" to "severe"). GIS were also recorded during and immediately after the treadmill run using a questionnaire developed by Pfeiffer et al. (Pfeiffer et al. 2012). The questionnaire had three sections; upper abdominal problems (reflux/heartburn, belching, bloating, stomach pain/cramps, vomiting, and nausea), lower abdominal problems (intestinal/lower abdominal cramps, side ache/stitch, flatulence, urge to defecate, diarrhea, and intestinal bleeding), and systemic problems (dizziness, headache, muscle cramp, urge to urinate, and low blood sugar). Each question was assessed on a 10-point scale, ranging from 0 or "no problem at all" to 9 or "the worst it has ever been".

2.9. Statistical analysis

Data are mean ± SD. Statistical analysis were performed with GraphPad Prism version 9.1.2. Datasets were tested for homogeneity or variance by using the D'Agostino-Pearson omnibus test. Log10 transformations were performed in case of nonhomogeneity. Statistical significance for all fasting clinical and biochemical parameters were determined using a repeated measures mixed-effect model with time, treatment, and time × treatment interaction as fixed effects and Sidak's multiple comparison test. A Wilcoxon paired tests were used to analyze post run biochemical and symptom data. Statistical significance was established at $p < 0.05$. Sample size determined based in previous studies measuring similar outcomes.

For microbiome analysis, alpha diversity was analyzed using Shannon and Chao indexes. Bray Curtis dissimilarity and UniFrac were performed to determine beta diversity. Samples were grouped and significant differences between groups determined by performing a PERMANOVA test on each of the beta diversity indices. A Kruskal–Wallis test was performed to identify taxonomic differences between treatment groups. Statistical tests regarding the microbiome were performed using tools within the QIIME package. Processed data were also imported to MicrobiomeAnalyst for visualization and analysis (Dhariwal et al. 2017; Chong et al. 2020).

Table 1. Characteristics and habitual diet composition of participants.

Characteristics	Baseline ($n = 16$)
Age, years	32.7 ± 8
Male	8/16 (50%)
Female	8/16 (50%)
Weight, kg	70.7 ± 12.2
Body mass index, kg/m^2	24.0 ± 1.9
Resting HR, bpm	70.0 ± 10.8
Resting SBP, mmHg	130.8 ± 10.8
Resting DBP, mmHg	81.0 ± 11.3
Habitual diet	
Fat, g/day	65.1 ± 23.4
Protein, g/day	84.1 ± 36.2
Carbohydrate, g/day	200.6 ± 68.9
Total Fiber, g/day	20.7 ± 8.9
Self-reported lower GIS score (cumulative score/person)	12.2 ± 6.71

Note: Baseline data of 16 participants. Values are mean ± SD. HR, heart rate; SBP, systolic blood pressure; DBP, diastolic blood pressure.

3. Results

Eight male and eight female runners participated in this study. General health and anthropometric parameters of the cohort are described in Table 1. Habitual diet was recorded using a 3-day food log prior to each lab visit, and habitual diet is indicated by mean daily macronutrient consumption (Table 1). A cumulative GIS score was also calculated from the six lower abdominal symptoms that participants self-reported to experience during or after running (Table 1). The average self-reported GIS score was 12.2 ± 6.71 (range 4–30).

Biochemical markers for general health parameters were measured in serum or plasma collected from whole blood after an overnight fast. Markers of liver (aspartate aminotrans-

Table 2. General health parameters measured in fasting blood before and after 1 month of placebo or probiotic supplementation.

	Placebo		Probiotic						
Parameter (mg/dL)	Baseline	Month 1	Baseline	Month 1	p^1	p^2	p^3	p^{Pl}	p^{Pro}
ALT, U/L	29.0 ± 11.8	29.1 ± 12.4	29.3 ± 11.6	28.8 ± 12.9	0.21	0.77	0.90	0.53	0.67
AST, U/L	37.3 ± 12.7	36.2 ± 8.7	39.7 ± 13.3	34.5 ± 8.0	0.03	0.91	0.27	0.65	0.05
Tbili, mg/dL	1.1 ± 0.5	1.0 ± 0.6	1.0 ± 0.4	1.0 ± 0.4	0.20	0.91	0.87	0.48	0.65
Creatinine, mg/dL	1.5 ± 0.3	1.5 ± 0.3	1.4 ± 0.4	1.4 ± 0.3	0.80	0.41	0.37	0.87	0.67
BUN, mg/dL	17.5 ± 4.3	17.2 ± 4.9	16.7 ± 4.5	15.9 ± 1.9	0.44	0.45	0.50	1.0	0.52
BUN/creatine ratio	11.8 ± 2.3	11.6 ± 2.5	12.2 ± 3.4	11.7 ± 2.8	0.62	0.74	0.87	0.96	0.88
Cholesterol, mg/dL	222 ± 34.8	221 ± 39.5	218 ± 47.0	218 ± 33.7	0.45	0.59	1.0	0.83	0.84
Trig, mg/dL	101 ± 29.6	90 ± 28.3	99 ± 29.5	83 ± 24.2	0.02	0.56	0.57	0.34	0.09
[a]Insulin, U/L	4.7 ± 3.7	4.8 ± 3.5	4.7 ± 3.2	4.0 ± 3.0	0.36	0.69	0.30	0.99	0.32
[a]CRP, mg/L	1.9 ± 2.9	2.3 ± 1.7	2.4 ± 3.0	2.0 ± 1.7	0.11	0.87	0.27	0.11	0.92
WBC ($\times 10^9$/L)	5.9 ± 1.6	6.6 ± 2.2	5.6 ± 1.2	6.0 ± 1.7	0.18	0.50	0.78	0.43	0.68
% Lymphocytes	38.0 ± 12.6	37.7 ± 11.9	34.0 ± 14.3	38.6 ± 7.9	0.29	0.48	0.59	0.92	0.46
% Monocytes	5.5 ± 8.5	3.2 ± 1.5	9.4 ± 15.4	3.9 ± 2.4	0.11	0.40	0.51	0.74	0.21
% Granulocytes	56.6 ± 7.8	59.1 ± 12.1	59.1 ± 12.1	56.5 ± 6.3	0.52	0.90	0.81	0.95	0.78

Note: Values are mean ± SD. Significance determined using linear mixed model analysis for time (p^1), supplementation (p^2), and time*supplementation interaction (p^3). Sidak's post hoc analysis for placebo indicated by p^{PL} and probioticindicated by p^{Pro}. ALT, alanine aminotransferase; AST, aspartate amino transferase; Tbili, total bilirubin; BUN, blood urea nitrogen; Trig, triglycerides, CRP, C-reactive protein, WBC, white blood cells.
[a]Log10 transformations were performed prior to significance determination.

ferase (AST), alanine aminotransferase (ALT), and total bilirubin) and kidney (creatine and blood urea nitrogen) function, lipid levels and insulin, and C-reactive protein were all within normal range with no significant time × supplementation interaction ($p > 0.05$, Table 2). AST did have a significant time effect ($p = 0.03$) and Sidak's post hoc analysis showed a significant decrease during the probiotic supplementation period ($p = 0.05$), not seen with the placebo treatment ($p = 0.65$). Triglycerides also showed a significant time effect ($p = 0.02$), with post hoc analysis showing a trend towards a decrease in triglycerides ($p = 0.09$) with probiotic supplementation, while no change were observed during placebo treatment ($p = 0.34$). WBC count was monitored at each lab visit, and no changes were observed in either total cell number or cellular composition ($p > 0.05$, Table 2).

Zonulin, LBP, iFABP, and calprotectin are biochemical markers associated with intestinal injury and permeability. Zonulin, LBP, and iFABP were measured from blood collected after an overnight fast, while calprotectin was measured in feces at baseline and after 4 weeks of supplementation. No significant time × supplementation interaction was detected for iFABP (Fig. 2A, $p = 0.11$), LBP (Fig. 2B, $p = 0.53$), zonulin (Fig. 2C, $p = 0.91$), and fecal calprotectin (Fig. 2D, $p = 0.25$).

As various probiotic supplements are known to alter immune function, serum cytokine levels were monitored as an indicator of changes within the immune system. Serum levels of IL-10, IL-1β, IL-6, IL-8, MCP-1, and TNF-α were measured after an overnight fast at baseline and after 4 weeks of supplementation (Table 3). There was substantial variation in levels amongst individuals for all cytokines measured, but within-participant levels of most cytokines were remarkably stable. As shown in Table 3, no significant time × supplementation interaction was detected for IL-10 ($p = 0.61$), IL-1β ($p = 0.17$), IL-6 ($p = 0.81$), IL-8 ($p = 0.17$), MCP-1 ($p = 0.41$), and TNF-α ($p = 0.77$).

To determine if the probiotic treatment resulted in changes in the intestinal microbial diversity and composition, DNA collected from fecal samples at baseline and after 4 weeks of supplementation were subjected to 16S rDNA sequencing. The most abundant phyla detected within the participant cohort were Firmicutes (61.5 ± 15.04%) and Bacteroidetes (34.1 ± 16%), with Actinobacteria (1.9 ± 2.6%), Cyanobacteria (0.2 ± 0.4%), Proteobacteria (1.6 ± 0.9%), Tenericutes (0.1 ± 0.15%), and Verrucomicrobia (0.6 ± 0.128%) present at lower amounts. A heatmap indicating prevalence of the core microbiome (genus level) identified in the participant populations is shown in Fig. 3A, with the 20 most abundant genera indicated in Table 4. Table 4 specifies 80.6% of the total bacterial cohort identified with the 19.4% comprised of the >200 genera not included on this list. No probiotic-induced changes were detected in the abundance of each of the top 20 genera (Fig. 3B). Kruskal–Wallis analysis showed nominally significant differences between groups (placebo and probiotic treatment at baseline and after 4 weeks of supplementation) for *Hydrogenoanaerobacterium* ($p = 0.004$), *Pediococcus* ($p = 0.03$), *Prevotella* ($p = 0.04$), *Lachnospiraceae UCG-009* ($p = 0.05$). However, these differences did not survive Bonferroni and FDR post hoc multiplicity correction (Supplemental Table 1).

Alpha diversity indices were used to assess within sample changes induced by the probiotic treatment (Fig. 3C). No significant differences were observed for Chao ($p = 0.83$), Shannon ($p = 0.78$), and Simpson ($p = 0.28$) indices. Bray–Curtis dissimilarity measurements also revealed that the β-diversity was not altered between placebo and probiotic treatment (PERMANOVA F value: 0.3; R-squared: 0.016; $p < 1$). Data are visualized on the principal component analysis plot (Fig. 3D).

Fig. 2. Probiotic treatment did not alter gut-associated biochemical markers of intestinal injury and permeability. Line graphs of fasting plasma intestinal fatty acid binding protein (iFABP) (A), lipopolysaccharide binding protein (LBP), Zonulin (C) and fecal calprotectin (D) levels at baseline and after 4 weeks of either placebo or probiotic supplementation. Each line represents a person at fasting before and after treatment with placebo or probiotic. iFABP, LBP, and calprotectin were Log10 transformed. Significance determined using mixed-effect model with Sidak multiple comparison test. $N = 16$/group.

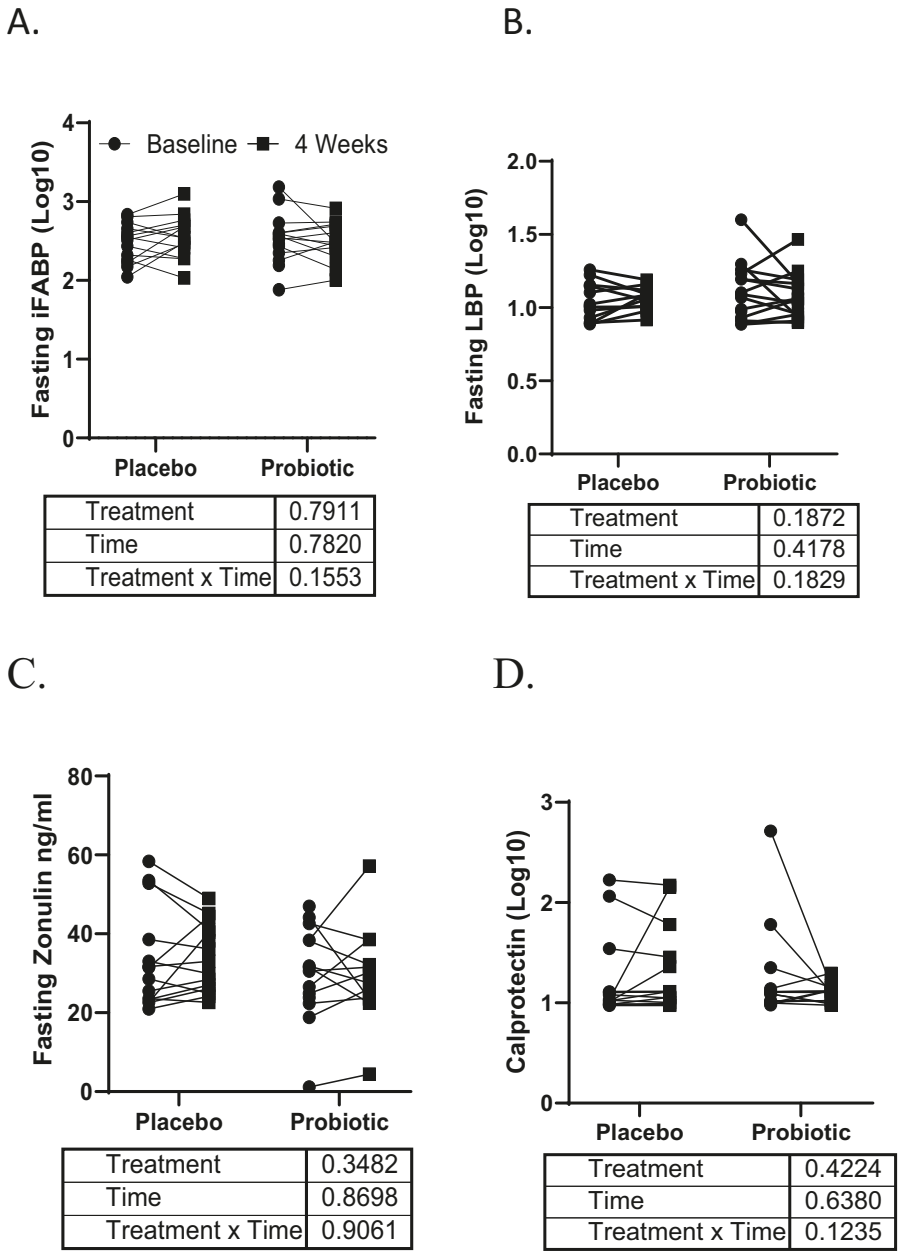


Table 3. Cytokine levels measured in serum form blood collected after an overnight fast before and after 1 month of placebo or probiotic supplementation.

Parameter (pg/mL)	Placebo		Probiotic		p^1	p^2	p^3
	Baseline	Month 1	Baseline	Month 1			
[a]IL-10	34.87 ± 73.6	35.7 ± 82	31.97 ± 69.7	35.6 ± 82.8	0.52	0.81	0.61
[a]IL-1β	40.4 ± 120.1	36.9 ± 116.4	37.3 ± 115.3	43.2 ± 125.8	0.83	0.87	0.17
[a]IL-6	194.5 ± 310.5	185.0 ± 303.8	204.3 ± 345	209.7 ± 324	0.15	0.95	0.81
[a]IL-8	106.0 ± 167.3	103.8 ± 177.2	108 ± 198.2	114.8 ± 210	0.13	0.83	0.17
MCP-1	659.7 ± 459	739.3 ± 443.5	680 ± 421.8	590.17 ± 319.6	0.51	0.55	0.41
[a]TNF-α	24.2 ± 22.6	23.8 ± 19.4	24.2 ± 19.9	22.3 ± 20.1	0.48	0.93	0.77

Note: Values are mean ± SD. Significance determined using linear mixed model analysis for time (p^1), supplementation (p^2), and time × supplementation interaction (p^3).
[a]Log10 transformations were performed prior to significance determination.

Fig. 3. Gut bacterial diversity was not altered by probiotic treatment. (A) Heatmap demonstrating core microbiome at genus level of the endurance runners participating in the current study. (B) Relative abundance of the top 20 genera as measured before (0) and after 4 weeks (4) of placebo (Pla) and probiotic (Pro) supplementation. (C) Box plots showing α-diversity as measured using Shannon and Chao indexes before (0) and after (4) placebo (Pla) and probiotic (Pro) supplementation. (D) Principal component analysis (PCoA) plot showing the dissimilarity between groups. Each point represents one sample with the color indicating the treatment group and timepoint. Ellipses represent the multivariate T-distribution of each group.

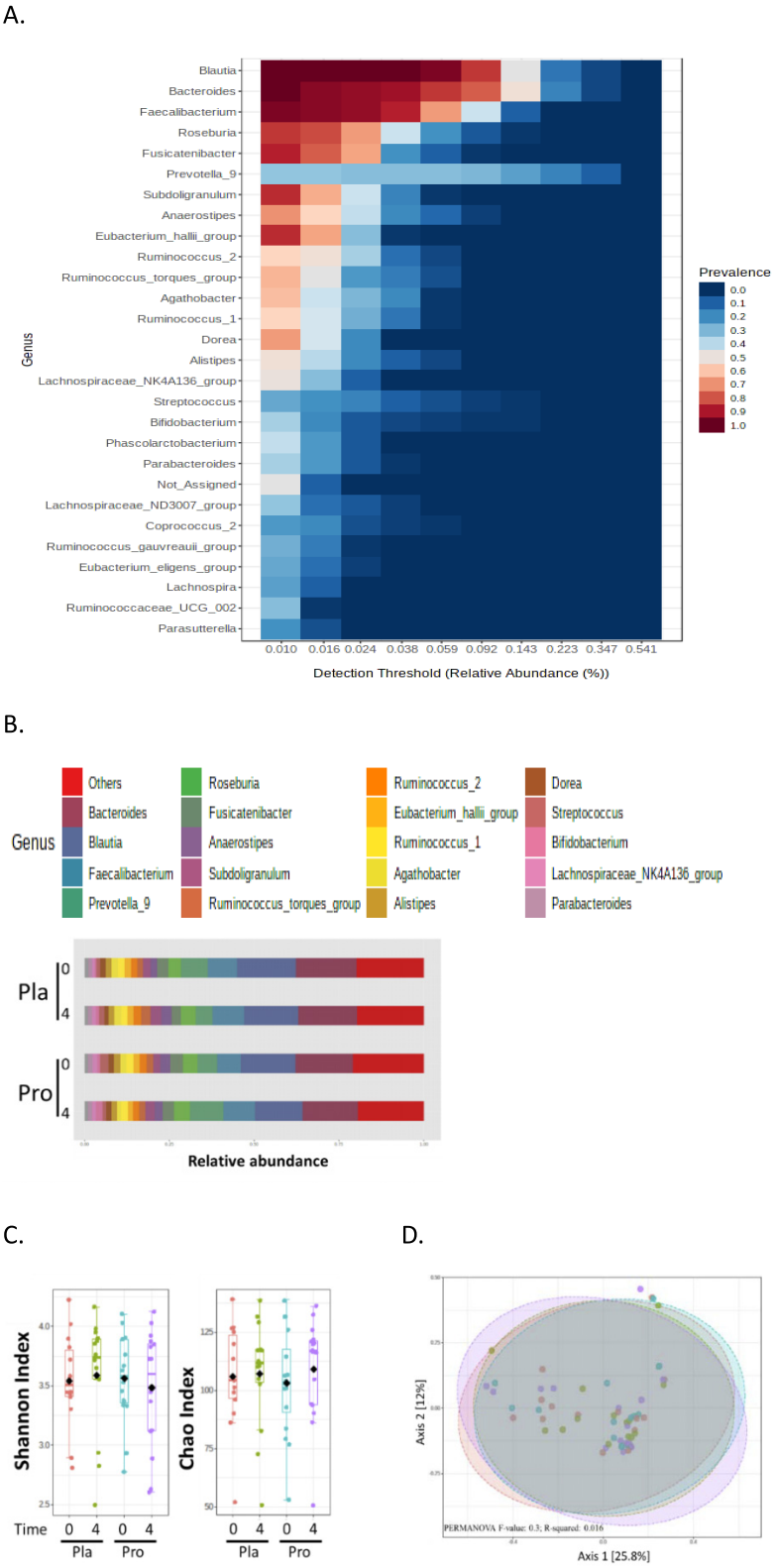


Table 4. Top 20 genera present in the study populations.

Taxa	% Abundance
Bacteroides	17.29
Blautia	15.93
Faecalibacterium	8.57
Prevotella_9	7.10
Roseburia	4.11
Fusicatenibacter	3.3
Anaerostipes	2.74
Subdoligranulum	2.42
Ruminococcus_torques_group	2.06
Ruminococcus_2	1.93
Eubacterium_hallii_group	1.93
Agathobacter	1.89
Ruminococcus_1	1.88
Alistipes	1.70
Dorea	1.50
Streptococcus	1.45
Lachnospiraceae_NK4A136_group	1.13
Bifidobacterium	1.12
Parabacteroides	1.09
Lachnospiraceae_ND3007_group	1.06

To further determine the effect of the probiotic treatment on functional microbiome output, microbial metabolic products, SCFA and branched chain fatty acids (BCFA), were measured in both fecal and plasma samples. No significant time × supplementation interaction was detected for fecal 2-methylbutiric acid ($p = 0.14$), acetic acid ($p = 0.38$), butyric acid ($p = 0.62$), hexanoic acid ($p = 0.24$), isobutyric acid ($p = 0.19$), isovaleric acid ($p = 0.30$), propionic acid ($p = 0.10$), and valeric acid ($p = 0.93$). Plasma SCFA and BCFA also showed no significant time × supplementation interaction (2-methylbutiric acid, $p = 0.12$; acetic acid, $p = 0.42$; butyric acid, $p = 0.11$, hexanoic acid, $p = 0.57$; isobutyric acid, $p = 0.07$; propionic acid, 0.22; valeric acid, $p = 0.45$), however, isobutyric acid ($p = 0.07$) and isovaleric acid ($p = 0.08$) showed a trend towards a significant time × supplementation interaction (Table 5). Post hoc analysis showed no difference for placebo and probiotic treatment for isobutyric acid, while isovaleric acid showed a significant change with probiotic supplementation only ($p = 0.01$). It is worth pointing out that the baseline isovaleric acid level was much higher than that of the placebo baseline measurement.

The effect of probiotic treatment on markers of intestinal injury (zonulin, iFABP, and LBP), inflammation (cytokines), and GIS were also determined after an exhaustive treadmill run. Study participants performed a treadmill run test at baseline and after 4 weeks of either placebo treatment or probiotic supplementation. Run was terminated at exhaustion or when GIS appeared. Blood was collected within 5 min of concluding the treadmill run test. Running resulted in a significant increase in LBP ($p < 0.0001$), IL-10 ($p < 0.0001$), IL-1β ($p = 0.02$). IL-8 ($p = 0.002$), MCP-1 ($p < 0.0001$) and TNF-α ($p = 0.0001$), and a decrease in serum levels of zonulin ($p = 0.004$), but no change in serum iFABP ($p = 0.21$). No significant difference between placebo and probiotic supplementation were detected for iFABP ($p = 0.23$, Fig. 4A), LBP ($p = 0.41$, Fig. 4B), and zonulin ($p = 0.73$, Fig. 4C) or cytokine measured (IL-10, IL-1β, IL-8, MCP-1, TNF-α, and IL-6, $p > 0.05$, Fig. 5).

Time to run termination also was monitored during the treadmill run test. No significant change in time- to-exhaustion was detected with placebo (run time at baseline: 59.75 ± 21.11 min, run time at end: 54.89 ± 18.03 min) or probiotic (run time at baseline 62.58 ± 23.10, run time at end: 62.49 ± 22.29) treatment ($p = 0.39$, Fig. 6).

During the supplementation period, GIS were monitored weekly through a self-reported clinical rating scale for gastrointestinal symptoms (GSRS) developed for patients with IBS and peptic ulcer disease (Svedlund et al. 1988). Fifteen items were monitored using a rating scale of 0–3. Weekly cumulative scores of all 15 items were compiled and compared between the placebo and the probiotic treatment for each week (Supplemental Fig. 1). No significant differences between placebo and probiotic supplementation were detected during week 1 ($p = 0.58$), week 2 ($p = 0.42$), week 3 ($p = 0.89$), and week 4 ($p = 0.37$). The most common symptoms experienced by the participants included abdominal distention, loose stool, and defecation. Exercise-associated GIS (upper and lower abdominal symptoms) were also assessed immediately following the treadmill run test. Serious symptoms (GIS score > 4) were rare and only detected for "intestinal/lower abdominal cramping", "side ache/stitch", and "urge to defecate" for 1–2 participants per run. A cumulative abdominal symptom score was calculated for each participant by adding all categories for both upper and lower symptoms. There was no significant difference in the mean abdominal symptom score for the upper (2.6 ± 3.3 vs. 2.4 ± 2.2, $p = 0.62$) and lower (4.1 ± 5.2 vs. 3.7 ± 3.6, $p = 0.5$) symptoms recorded after 1 month of placebo or probiotic supplementation (Table 6). Of note, the cumulative lower abdominal symptom score collected during the in-house treadmill run tests did not reach the level of the self-reported GIS scores collected during the screening process. We also assessed the distribution frequency of the cumulative GIS scores (including both upper and lower abdominal scores) after the treadmill run test and demonstrate that GIS were absent or minor (cumulative score ≤ 8) for approximately 90% of participants after both placebo or probiotic treatment. (Supplemental Fig. 2). Spearman correlation tests were performed to determine if post-run upper and lower GIS correlated with markers of gut injury and permeability. Trends towards significant associations with GIS were detected for zonulin (upper GIS, $p = 0.07$, $r = -0.4$; lower GIS, $p = 0.05$, $r = 0.4$) and iFABP (upper GIS, $p = 0.3$, $r = -0.21$; lower GIS, $p = 0.06$, $r = 0.4$), but not LBP (upper GIS, $p = 0.56$, $r = 0.11$. lower GIS, $p = 0.32$, $r = -0.20$).

4. Discussion

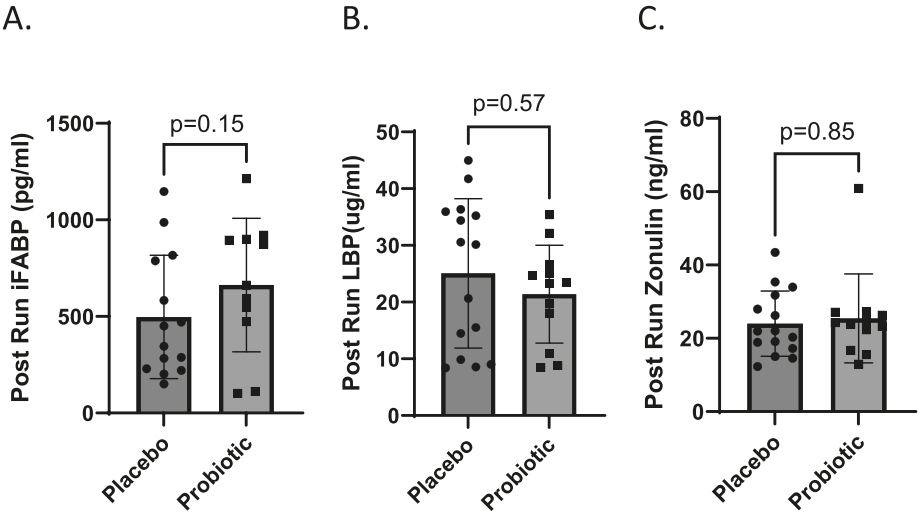
The therapeutic potential of probiotic supplementation has been studied in various disease states, particularly in regard to GI disorders (Ford et al. 2014). Several probiotic strains have shown favorable mechanistic effects on intestinal epithelial integrity (Karczewski et al. 2010) and demonstrate

Table 5. Short chain fatty acids levels in plasma and feces before and after 1 month of placebo or probiotic supplementation

	Placebo		Probiotic						
Parameter	Baseline	Month 1	Baseline	Month 1	p^1	p^2	p^3	p^{Pl}	p^{Pro}
Feces									
[b]2-Methylbutiric acid[#]	0.4 ± 0.22	0.3 ± 0.3	0.4 ± 0.2	0.4 ± 0.2	0.77	0.80	0.14	0.38	0.63
[a]Acetic acid[#]	8.6 ± 4.1	11.7 ± 8.3	10.1 ± 7.3	8.7 ± 5.1	0.84	0.71	0.38	0.69	0.86
[a]Butyric acid[#]	4.7 ± 2.8	5.1 ± 3.3	5.0 ± 3.5	4.4 ± 2.3	0.99	0.78	0.62	0.93	0.92
[a]Hexanoic acid[#]	0.3 ± 0.3	0.5 ± 0.5	0.6 ± 0.6	0.5 ± 0.8	0.95	0.79	0.24	0.66	0.62
[b]Isobutyric acid[#]	0.5 ± 0.3	0.4 ± 0.3	0.4 ± 0.2	0.5 ± 0.2	0.34	0.68	0.19	0.21	0.96
[b]Isovaleric acid[#]	0.5 ± 0.3	0.5 ± 0.3	0.5 ± 0.2	0.5 ± 0.5	0.54	0.70	0.30	0.42	0.93
[a]Propionic acid[#]	4.1 ± 2.1	5.6 ± 6.8	4.0 ± 2.4	4.3 ± 3.1	0.60	0.58	0.10	0.92	0.92
[a]Valeric acid[#]	0.7 ± 0.3	0.9 ± 0.8	0.7 ± 0.3	0.8 ± 0.4	0.45	0.98	0.93	0.86	0.80
Plasma									
[a]2-Methylbutyric acid[*]	27.1 ± 9.7	24.3 ± 7.7	26.6 ± 10.9	36.5 ± 31.4	0.85	0.32	0.12	0.37	0.56
[a]Acetic acid[#]	3.9 ± 2.4	3.6 ± 1.7	7.0 ± 8.3	6.6 ± 10.2	0.30	0.04	0.42	0.98	0.37
[a]Butyric acid[*]	44.5 ± 39.6	51.7 ± 33.6	66.4 ± 43.8	54.8 ± 41.6	0.77	0.28	0.11	0.58	0.34
Hexanoic acid[*]	41.4 ± 15.6	46.4 ± 14.1	49.7 ± 21.3	55.0 ± 32.0	0.24	0.23	0.57	0.38	0.89
[b]Isobutyric acid[*]	35.2 ± 5.14	32.6 ± 7.10	32.6 ± 4.57	34.56 ± 6.33	0.81	0.84	0.07	0.25	0.46
[a]sovaleric acid[*]	28.8 ± 18.7	27.1 ± 20.0	50.2 ± 37.5	32.8 ± 14.1	0.02	0.11	0.08	0.88	0.01
[a]Propionic acid[*]	88.7 ± 38.5	91.8 ± 32.0	125.1 ± 70.0	114.0 ± 77.3	0.41	0.11	0.22	0.95	0.28
[a]Valeric acid[*]	6.1 ± 1.8	8.9 ± 6.8	7.8 ± 4	12.2 ± 17.6	0.18	0.47	0.45	0.25	0.89

Note: Values are mean ± SD. Significance determined using linear mixed model analysis for time (p^1), supplementation (p^2), and time × supplementation interaction (p^3). Plasma: [*]ng/mL, [#]ug/mL. Feces: [*]ug/g, [#]mg/g.
[a]Log10 transformations were performed prior to significance determination.
[b]Two outliers removed.

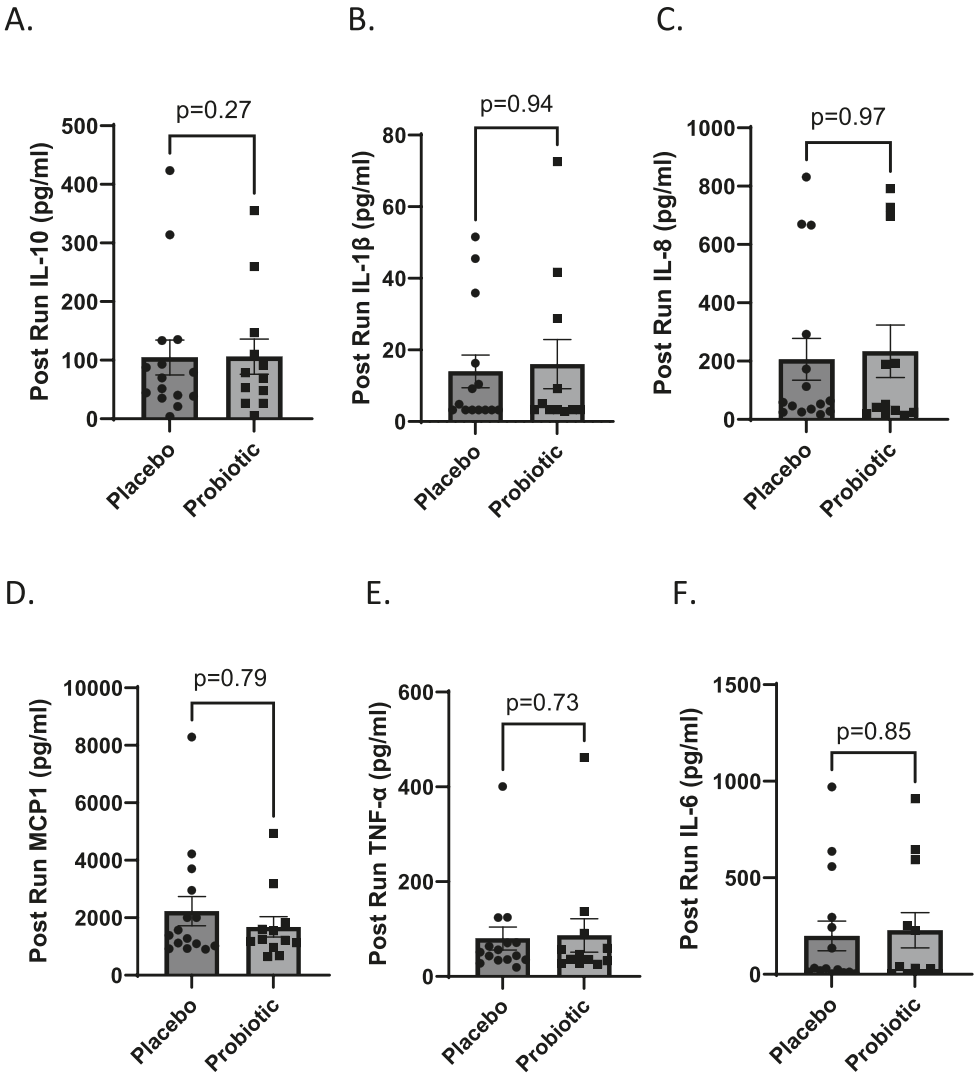
Fig. 4. Probiotic supplementation did not alter gut-associated biochemical markers of intestinal injury and permeability after an exhaustive treadmill run. Bar charts of plasma intestinal fatty acid binding protein (iFABP) (A), lipopolysaccharide bind protein (LBP) (B) and zonulin (C) collected immediately after an exhaustive treadmill run. Significance determined using Wilcoxon nonparametric test.



improvements in populations presenting with compromised intestinal barrier (Bron et al. 2017). Probiotics supplementation can also affect microbial diversity by altering the balance of organisms in the GI tract and reducing colonization of pathogenic bacteria, or by producing soluble molecules that can be absorbed and influence distal tissues in the host (Wieërs et al. 2019).

The current study evaluated the safety and efficacy of a probiotic cocktail for the improvement of exercise-induced GIS and circulatory markers of GI dysregulations and immune activation in healthy male and female endurance runners that had frequently experienced moderate to severe GIS during running. We also evaluated the changes in the intestinal microbiome. The probiotic cocktail used in this study is a combination of three probiotic strains, one *P. acidilatici* and two *L. plantarum* strains. This cocktail has shown a positive impact on IBS-related quality of life in patients with IBS (Lorenzo-Zúñiga et al. 2014), as well as digestive symptoms both in IBS and lactose-intolerant patients (Barraza-Ortiz et al. 2021; Cano-Contreras et al. 2022). Based on these results we hypoth-

Fig. 5. Probiotic supplementation did not alter systemic cytokine levels after an exhaustive treadmill run. Bar charts of plasma levels of IL-10 (A), IL-1β (B), IL-8 (C), MCP1 (D), TNF-α (E), and IL-6 (F) collected immediately after an exhaustive run. Significance determined using Wilcoxon nonparametric test.



esized it may also ameliorate exercise-induced GIS in runners as there are overlapping symptom manifestations, although pathophysiology may differ. Symptoms are often also more prevalent during endurance events in individuals with a reported history of GIS (Peters et al. 1999).

In the current study, supplementation of the probiotic for 4 weeks did not result in in harmful changes in general health parameters including liver and kidney function, demonstrating that a daily dose of 3×10^9 CFU/dose is safe to use in a healthy population. Use of this probiotic also did not alter circulatory biochemical markers associated with intestinal dysfunction or cytokine levels in blood collected after an overnight fast. A post hoc analysis did identify a reduction in serum AST as marginally significant ($p = 0.05$) in the probiotic group, while no changes were observed in placebo. It is possible that this significant result is due to a multiplicity due to the number of outcomes that was analyzed.

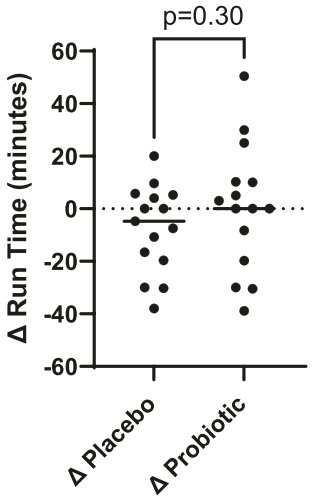
As probiotics are defined as live microorganisms, which when administered in adequate amounts confer a health benefit on the host (Food and Agriculture Organization of the United Nations and World Health Organization 2006), we evaluated the effect of the probiotic on the luminal gut microbial content. Although many studies have demonstrated effects of probiotics on the microbiome in the context of a disease state (Wieërs et al. 2019), less is known about the effect of probiotic supplementation in a healthy population. A recent systematic review reported that there is no convincing evidence for consistent effects of probiotics on fecal microbiota in healthy adults (Kristensen et al. 2016). In the current study we also observed no changes between treatment groups in the overall composition, α-diversity and compositional dissimilarity (β-diversity). The most abundant genera within the study cohort were *Bacteroides*, *Blautia*, *Faecalibacterium*, and *Prevotella*. The presence of the probiotic strains namely *P. acidilatici* and *L. plantarum* were also not signif-

Table 6. Mean upper and lower abdominal symptom score recoded immediately after an exhaustive run.

	Placebo		Probiotic			
Parameter (mg/dL)	Baseline	Month 1	Baseline	Month 1	p^{Base}	p^{End}
Upper abdominal symptoms	4.5 ± 6.0	2.6 ± 3.3	3.0 ± 3.0	2.4 ± 2.2	0.61	0.62
Lower abdominal symptoms	5.6 ± 7.6	4.1 ± 5.2	4.3 ± 3.8	3.7 ± 3.6	0.93	0.50

Note: Values are mean ± SD for all symptom categories measured. Significance difference between placebo and probiotic treatment determined using Wilcoxon matched-pair test. p^{Base} indicates significant difference between the placebo and probiotic groups at baseline and after 1 month of supplementation (p^{End}). Scoring scale: 0 "no problem at all", 1 "very, very minor problems", 2 "very minor problems", 3 "minor problems", 4 "moderate problems", 5 "serious problems", 6 "severe problems", 7 "very severe problems", 8 "very, very severe problems", and 9 "the worst it has ever been".

Fig. 6. Probiotic supplementation did not alter run time in an exhaustive treadmill run test. Data indicate change in run time between baseline and end of the supplementation period. Significance determined using Wilcoxon nonparametric test.



icantly increased after the supplementation period. Interestingly, the *Pediococcus* genus showed some increase only with probiotic supplementation, although the sequencing of short reads (V4 fragment) precludes unambiguous identification down to the strain level. Microbial-derived SCFA and BCFA were also measured in the feces and circulation, but no probiotic-induced changes were observed in feces or plasma.

To determine if supplementation of the probiotic strains alter the functional GI response to exertional-heat stress, markers of intestinal dysfunction, immune activation and GIS were monitored after a treadmill run test. The run occurred in a temperature-controlled facility where the temperature was maintained at 27–28 °C to increase heat-stress as hyperthermia is a main contributing factor for exercise-induced GI injury and permeability (van Wijck et al. 2012). Effect of exertional-heat stress on intestinal damage and permeability is commonly assessed by iFABP, a protein that is exclusive to epithelial mucosa of intestinal wall. Heat and duration of exercise are major contributing factors to the stress on the intestinal integrity and the resulting release of iFABP. Exercise-induced increase in iFABP is also greater in the dehydrated state, although it has no effect on endotoxemia and cytokinemia (Costa et al. 2020). In the current study we compared levels of markers associated with gut permeability and epithelial injury collected after an overnight fast with levels after an exertional exercise. Although it has previously been shown that iFABP is increased with exertional exercise, we did not detect an increase in the current study (Ferrario et al. 2014; Santos-Marcos et al. 2019). As epithelial injury is dependent on the level of exertion and temperature (Jandhyala et al. 2015), this result might suggest that the runners in our study was not sufficiently exerted during the run. No difference was detected in post run iFABP between the placebo and probiotic group.

LBP is an acute phase protein that can be used as a surrogate measure of internal exposure to bacterial lipopolysaccharide and therefore functions as marker of gut permeability. Consistent with previous studies, there was a significant increase in LBP with running, however no significant difference was detected between the placebo and probiotic treatment. Zonulin levels were also monitored and levels in the plasma was decreased after running. The validity of serum zonulin as a marker of intestinal permeability has been questioned recently as it does not correlate with lactulose/rhamnose ratio (gold standard for measuring gut permeability) in healthy adults (Tatucu-Babet et al. 2020). The cytokines IL-10, IL1β, IL-8, MCP-a, and TNF-α concentration were also increased with run, but no differences were observed in the cytokine response between the placebo versus probiotic treatment. It is worth noting that many factors influence these biochemical markers e.g., environmental temperature during exercise, hydration levels, dietary habits, and carbohydrate consumption during exercise. To reduce confounding effects of diet in particular, participants were requested to consume their habitual meals in the days leading up to the running bout to reduce dietary effects between sampling points.

It was previously demonstrated that 4 weeks of supplementation with a multistrain probiotic increased running time to fatigue in the heat (Shing et al. 2014). However, in the current study, supplementation of *P. acidilatici* and *L. plantarum* did not result in a change in run time to exhaustion. Supplementation with a probiotic cocktail containing *Lactobacillus acidophilus* CUL60, *L. acidophilus* CUL21, *Bifidobacterium bifidum* CUL20, and *Bifidobacterium animalis* subsp. Lactis CUL34 was associated with lower incidence and severity of GIS in marathon runners (Pugh et al. 2019), however in the current study, we did not detect an improvement in GIS dur-

ing the supplementation period or altered GIS severity and upper/lower abdominal symptoms during the treadmill run test. However, this result is confounded by the fact that the GIS recorded during the treadmill run tests did not reach the level of "moderate" to "severe" GIS as reported during entry into the study, suggesting that the probiotic effect might be blunted by a lack of measurable functional outcome. The lack of changes in GIS to exhaustive run might be due to the average duration of the run (e.g., around 1 h in our study vs. 4 h in the marathon study) or the fact that the temperature were not as high has been used in previous studies (e.g., ~27 °C in our study vs. ~35 °C in a study using a cocktail of various bifidobacteria, lactobacilli, and streptococci) (Shing et al. 2014). Therefore, increasing duration and temperature might be required to observe the probiotic effect on GIS.

An additional consideration is the fact that this study was conducted during the COVID-19 epidemic. Even though all possible precautions were taken to reduce risk of infection, participants remained cautious to avoid reducing immunity. Notably, the GIS score collected after the in-house treadmill run did not reach moderate symptom level for most participants suggesting that exhaustion level was not sufficient to induce GIS.

In conclusion, the probiotic containing *P. acidilatici* and two *L. plantarum* strains appeared to be safe to use in a healthy population. No significant differences were observed with the probiotic under the test conditions in the current study. Future studies should use longer running time and/or exercising at higher temperatures to assess probiotic effect on time-to-exertion, muscle, or liver damage and changes in serum iFABP in response to exercise.

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Data availability

Data generated during this study are not publicly available due to confidentiality agreement with research collaborators but are available from the corresponding author upon reasonable request.

Author information

Author ORCIDs

Zoey C. Kearns https://orcid.org/0009-0000-9711-8600
Maxime R. Paquette https://orcid.org/0000-0002-6671-4570
Anyou Wang https://orcid.org/0000-0002-4981-3606
Marie van der Merwe https://orcid.org/0000-0001-5683-4790

Author contributions

Conceptualization: MRP, RJB, MvdM
Data curation: SL, TL, AM, ZCK, MvdM
Formal analysis: SL, TL, AM, AW, MvdM
Funding acquisition: MvdM, RJB
Investigation: SL, TL, ZCK, MRP, MvdM
Methodology: AM, MvdM
Project administration: SL, MvdM
Writing – original draft: SL, MvdM
Writing – review & editing: SL, MRP, RJB, MvdM

Competing interests

The authors declare there are no competing interest.

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Supplementary material

Supplementary data are available with the article at https://doi.org/10.1139/apnm-2023-0449.

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