

Supplemental Information

Gut microbiome remodeling and metabolomic profile improves in response to intermittent fasting with protein pacing versus continuous caloric restriction

Alex E. Mohr^{1,2}, Karen L. Sweazea^{1,2,3}, Devin A. Bowes², Paniz Jasbi^{4,5}, Corrie M. Whisner^{1,2}, Dorothy D. Sears¹, Rosa Krajmalnik-Brown², Yan Jin⁶, Haiwei Gu^{1,6}, Judith Klein-Seetharaman^{1,4}, Karen M. Arciero⁷, Eric Gumprecht⁸, Paul J. Arciero^{7,9*}

¹College of Health Solutions, Arizona State University, Phoenix, AZ, USA

²Biodesign Institute Center for Health Through Microbiomes, Arizona State University, Tempe, AZ, USA

³Center for Evolution and Medicine, College of Liberal Arts and Sciences, Arizona State University, Tempe, AZ, USA

⁴School of Molecular Sciences, Arizona State University, Tempe, AZ, USA

⁵Systems Precision Engineering and Advanced Research (SPEAR), Theriome Inc., Phoenix, AZ, USA

⁶Center of Translational Science, Florida International University, Port St. Lucie, FL, USA

⁷Human Nutrition and Metabolism Laboratory, Department of Health and Human Physiological Sciences, Skidmore College, Saratoga Springs, NY, USA

⁸Isagenix International, LLC, Gilbert, AZ, USA

⁹School of Health and Rehabilitation Sciences, Department of Sports Medicine and Nutrition, University of Pittsburgh, Pittsburgh, PA, USA

***Corresponding Author: parciero@skidmore.edu**

Page S-4, Table S1. Dietary regimens of the intermittent fasting protein pacing (IF-P) and continuous calorie restriction (CR) diet groups.

Page S-5, Table S2. Nested permutational analysis of variance model parameters and term results for Bray-Curtis dissimilarity matrix of diet groups, intermittent fasting protein pacing (IF-P) and continuous calorie restriction (CR) and time.

Page S-6, Table S3. Plasma cytokine concentrations between intermittent fasting protein pacing (IF-P) and continuous calorie restriction (CR) diet groups at baseline and weeks four and eight.

Page S-7, Table S4. Significance, fold change (FC), and area under the curve (AUC) details of metabolite markers of intermittent fasting protein pacing (IF-P) and continuous calorie restriction (CR).

Page S-8, Table S5. Variance and difference between intermittent fasting protein pacing (IF-P) and continuous calorie restriction (CR) for the eight latent factors extracted from multi-omics factor analysis.

Page S-9, Table S6. Baseline characteristics of high and low response subgroup study participants.

Page S-10, Table S7. Permutational analysis of variance model parameters and term results for Bray-Curtis dissimilarity matrix of high and low response group and time.

Page S-11, Fig S1. (a) CONSORT diagram describing enrollment, allocation, and data analysis of the parent clinical study. (b) Daily consumption of macronutrients measured by mass (g) at baseline, week four, and week eight between the intermittent fasting protein pacing (IF-P) and continuous calorie restriction (CR) groups. There were no differences between groups at baseline for all dietary intake variables (two-sided Student's t-test $p < 0.05$). (c) Targeted gas chromatography-mass spectrometry (GC-MS) measurement of fecal short-chain fatty acids (SCFAs) over the study duration. No significant effects were observed for time or interaction (linear mixed effects models, two-sided $p > 0.05$). For all panels, IF-P: $n = 20$, CR: $n = 19$. Source data are provided as a Source Data file.

Page S-12, Fig S2. Cytokines and the microbiome in intermittent fasting protein pacing (IF-P) participants. (a) Grid-fused least absolute shrinkage and selection operator (GFLASSO) regression identified several genera with correlative relationships with our cytokine panel. Two-sided spearman correlations of (b) IL-4 and (c) IL-13 with microbial genera using intervention samples (weeks four and eight). For all panels, IF-P: $n = 20$, CR: $n = 19$. Source data are provided as a Source Data file.

Page S-13, Fig S3. Analysis of plasma metabolome. (a) As assessed by the Canberra distance metric, no differences between intermittent fasting protein pacing (IF-P) and continuous calorie restriction (CR) were detected over the intervention in the metabolome (two-sided Wilcoxon rank sum test). (b) Five metabolites with a VIP > 1.0 (2,3-dihydroxybenzoic acid, malonic acid, protocatechuic acid, agmatine, and myo-inositol) were retained to construct an enhanced orthogonal projection to latent structures discriminant analysis (OPLS-DA) model. In contrast, model fit was assessed with 100-fold leave-one-out cross-validation (LOOCV). (c) A refined OPLS-DA model overview showing predictive and explanatory capacity ($Q^2 = 0.460$, $p < 0.001$; $R^2 = 0.506$, $p < 0.001$). (d) Receiver operating characteristic analysis of the OPLS-DA model (AUC = 0.929, 95% CI: 0.868-0.973, sensitivity = 0.8, specificity = 0.9). For all panels, IF-P: $n = 20$, CR: $n = 19$. Source data are provided as a Source Data file.

Page S-14, Fig. S4. (a) Short-chain fatty acid (SCFA) analysis from high and low weight loss responders did not differ significantly (two-sided Wilcoxon rank-sum test, $p_{\text{adj}} \geq 0.10$). Values from the four principle SCFAs generated from targeted gas chromatography-mass spectrometry (GC-MS) are displayed as $\log_2$

fold change (FC). **(b)** Change in the fecal metabolome was not significantly different between weight loss response classification when comparing log2FC values (two-sided Wilcoxon rank-sum test, $p_{\text{adj}} > 0.10$). For visual interpretability, changes are displayed at the chemical sub-class level. For all panels, High: $n = 5$, Low: $n = 5$. Source data are provided as a Source Data file.

Page S-15, Fig. S5. **(a)** Bray Curtis dissimilarity of functional pathways derived from metagenomic analysis with **(b)** top PERMANOVA model coefficients (analysis: pathway ~ time). **(c)** Top PERMANOVA model coefficients (analysis: pathway ~ time) from fecal metabolome, including the subclass, amino acids, peptides, and analoges. **(d)** Alluvial plot displaying the variation in abundance of the most abundant chemical subclasses (>1% relative abundance) over time. For visual clarity, the less abundant metabolite classes (<1% relative abundance) are not displayed. **(e)** Heatmap of the fecal metabolites captured by pathway analysis from the case study assessment. Source data are provided as a Source Data file.

Table S1. Dietary regimens of the intermittent fasting protein pacing (IF-P) and continuous calorie restriction (CR) diet groups.

Variable	IF-P		CR	
	Men (1800 kcals/day)	Women (1450 kcals/day)	Men (1500 kcals/day)	Women (1200 kcals/day)
Breakfast (0600–0800)	Liquid meal replacement shake; 400-450 kcals, 36 g protein , 40-43 g carbohydrate, 12-15 g fat; Caffeine beverage; Antioxidant & Adaptogen mix; 20 kcals	Liquid meal replacement shake; 350-400 kcals, 30 g protein , 35-40 g carbohydrate, 10-12 g fat; Caffeine beverage; Antioxidant & Adaptogen mix; 20 kcals	Oatmeal, fruit, milk, honey, egg, whole grain toast, coffee; 400 kcals, 20 g protein , 55 g carbohydrate, 11 g fat	Oatmeal, fruit, milk, honey, egg, coffee; 350 kcals, 15 g protein , 50 g carbohydrate, 10 g fat
Lunch (1100–1300)	Choice of liquid meal replacement shake or Fresh vegetables with choice of fish/poultry/beef/plant-based protein; 400 kcals, 36 g protein , 40 g carbohydrate, 12 g fat	Choice of liquid meal replacement shake or Fresh vegetables with choice of fish/poultry/beef/plant-based protein; 350 kcals, 30 g protein , 35 g carbohydrate, 10 g fat	Whole grain pita, poultry/fish/beef/plant-based protein, tomato, lettuce, mayonnaise, mustard, apple, skim milk; 400 kcals, 20 g protein , 60 g carbohydrate, 10 g fat	Whole grain pita, poultry/fish/beef/plant-based protein, tomato, lettuce, mayonnaise, mustard, apple, skim milk; 350 kcals, 15 g protein , 50 carbohydrate, 10 g fat
Mid-Afternoon snack (1400–1600)	Greek yogurt, fruit; 200 kcals, 20 g protein , 15 g carbohydrate, 5 g fat		Yogurt, fruit; 200 kcals, 10 g protein , 30 g carbohydrate, 5 g fat	
Dinner (1700–1900)	Fish/Poultry/Beef, fresh vegetables, chopped nuts, dried fruit, olive oil, milk; 500 kcals, 36 g protein , 50 g carbohydrate, 18 g fat	Fish/Poultry/Beef, fresh vegetables, chopped nuts, dried fruit, olive oil, milk; 450 kcals, 30 g protein ; 50 g carbohydrate; 15 g fat	Whole grain, fish/beef/poultry/plant-based protein, vegetables, nuts, dried fruit, olive oil, milk; 500 kcals, 25 g protein , 80 g carbohydrate, 12 g fat	Whole grain, fish/beef/poultry/plant-based protein, vegetables, nuts, dried fruit, olive oil, milk; 450 kcals, 20 g protein , 75 g carbohydrate, 8 g fat
Evening snack (2100–2200)	Protein shake/bar snack; 250 kcals, 30 g protein , 20 g carbohydrate, 8 g fat	Protein shake/bar snack; 225 kcals, 25 g protein ; 20 g carbohydrate; 8 g fat	Milk, crackers; 225 kcals, 15 g protein , 30 g carbohydrate, 4 g fat	Milk, crackers; 225 kcals, 10 g protein , 30 g carbohydrate, 5 g fat

Intermittent Fasting Day Intake for IF-P

Antioxidant plant-based powder	4/day	160 kcal total
Plant-based herbal adaptogen powder	2/day	40 kcal total
Collagen bone broth	2/day	45 kcal total
Low-glycemic protein crackers	1/day	100 kcal total
Electrolyte beverage	1/day	20 kcal total
Optional foods:	1/day	Dark chocolate square; antioxidant/caffeine beverage; ½ nut bar; fresh vegetable/fruit, nut/seed mix (all < 50 kcal total for IF1-P and 150-200 kcal total for IF2-P)

Table S2. Nested permutational analysis of variance model parameters and term results for Bray-Curtis dissimilarity matrix of diet groups, intermittent fasting protein pacing (IF-P) and continuous calorie restriction (CR) and time.

	DF	SS	R²	F	p
Participant	38	22.664	0.749	7.056	0.001
Time*Group	2	0.542	0.018	3.206	0.001
Time*Group (nested: participant)	37	3.724	0.123	1.191	0.003
Residuals	39	3.297	0.109		
Total	116	30.227	1.000		

Abbreviations: DF, degrees of freedom; SS, sum of squares.

Table S3. Plasma cytokine concentrations between intermittent fasting protein pacing (IF-P) and continuous calorie restriction (CR) diet groups at baseline and weeks four and eight.

Variable	Baseline		Week 4		Week 8	
	IF-P (n = 20)	CR (n = 19)	IF-P (n = 20)	CR (n = 19)	IF-P (n = 20)	CR (n = 19)
GM-CSF (pg/mL)	80.34±13.93	85.81±26.56	82.23±13.92	84.21±26.95	81.14±12.79	77.35±22.46
IFN γ (pg/mL)	8.04±0.77	9.56±1.13	8.64±1.03	9.51±1.04	7.64±0.65	9.41±0.87
IL-1 β (pg/mL)	4.05±0.36	4.21±0.49	4.11±0.35	4.16±0.43	4.02±0.30	3.96±0.41
IL-2 (pg/mL)	6.04±0.55	7.18±0.92	6.31±0.66	6.67±0.79	5.45±0.43	6.67±0.73
IL-4 (pg/mL)	53.15±14.59	73.26±22.05	72.22±17.27 ^a	84.19±24.07	79.64±16.92 ^a	76.96±20.81
IL-5 (pg/mL)	5.72±3.66	2.41±0.29	5.85±3.63	2.33±0.32	6.05±3.84	2.21±0.24
IL-6 (pg/mL)	3.99±0.98	5.33±1.24	4.86±1.12 ^a	5.21±1.30	5.43±1.08 ^a	4.96±1.23
IL-8 (pg/mL)	14.84±4.63	14.88±3.51	18.72±5.22 ^a	15.47±3.54	20.80±5.04 ^a	13.55±3.09
IL-10 (pg/mL)	14.79±1.76	17.85±2.73	17.03±2.07	19.09±2.59	17.80±2.20	18.57±2.73
IL-12p70 (pg/mL)	7.31±0.59	9.56±1.01	6.82±0.53	8.67±0.93	6.42±0.49	9.16±1.01
IL-13 (pg/mL)	14.83±2.80	17.17±2.84	18.06±3.14 ^a	17.08±2.91	17.94±2.87 ^a	16.68±3.21
IL-17A (pg/mL)	18.51±1.25	19.44±2.23	17.73±1.36	18.47±1.99	16.69±1.19	18.55±1.88
IL-23 (pg/mL)	1442.30±276.38	1491.48±210.08	1379.28±265.49	1502.74±217.47	1347.88±264.22	1443.68±194.26
TNF α (pg/mL)	8.28±0.73	9.94±0.70	8.81±0.70	9.62±0.74	8.31±0.58	9.47±0.65

Data are reported as mean $\pm$ SEM. Statistical analysis was conducted using linear mixed effect modeling. All p-values were calculated using two-sided tests. Significant increases compared to baseline values: ^ap.adj < 0.1

Table S4. Significance, log fold-change (logFC), and area under the curve (AUC) details of metabolite markers of intermittent fasting protein pacing (IF-P) and continuous calorie restriction (CR).

Metabolite	p*	p.adj*	logFC**	AUC
2,3-Dihydroxybenzoic acid	1.6e-07	2.2e-05	-0.519	0.816
Malonic acid	3.3e-06	2.3e-04	0.464	0.808
Choline	1.6e-05	7.2e-04	-0.249	0.819
Agmatine	6.9e-05	0.002	-0.278	0.799
Protocatechuic acid	1.9e-04	0.005	-0.334	0.738
Myo-inositol	0.002	0.036	-0.309	0.718
Dulcitol	0.002	0.037	-0.305	0.630
Oxaloacetic acid	0.002	0.037	-0.213	0.760
Xylitol	0.002	0.037	-0.187	0.722
N-Acetylglutamine	0.003	0.037	-0.220	0.669
Asparagine	0.003	0.037	-0.171	0.729
Sorbitol	0.005	0.063	-0.271	0.606
Cytidine	0.006	0.069	-0.206	0.662
Acetylcarnitine	0.008	0.081	0.185	0.582
Urate	0.009	0.089	-0.138	0.731
Kynurenine	0.015	0.119	-0.171	0.639
9-Octadecynoic acid	0.015	0.119	0.1563	0.651
Decanoylcarnitine	0.021	0.150	-0.201	0.589
Pyroglutamic acid	0.024	0.150	-0.164	0.644
5-Hydroxyindoleacetic acid	0.024	0.150	-0.158	0.642
Tryptophan	0.022	0.150	-0.139	0.595
Asymmetric dimethylarginine	0.021	0.150	-0.119	0.629
D-Mannitol	0.026	0.159	-0.287	0.613
L-(+)-Arabinose	0.031	0.179	-0.199	0.618
D-Galacturonic acid	0.033	0.185	-0.168	0.615
2-Hydroxyglutarate	0.036	0.186	-0.152	0.611
Dimethylglycine	0.036	0.187	-0.145	0.623
Alanine	0.037	0.187	-0.114	0.639
Sarcosine	0.038	0.187	-0.113	0.631
Indole-3-lactic acid	0.049	0.210	-0.122	0.599
Palmitic acid	0.049	0.210	0.120	0.608
Valeric acid	0.049	0.210	0.126	0.596

*Derived from age-, sex-, and time-adjusted general linear model (GLM) with Benjamini–Hochberg multiple testing correction. All p-values were calculated using two-sided tests.

**Analyzed as IF-P/CR.

Table S5. Variance and difference between intermittent fasting protein pacing (IF-P) and continuous calorie restriction (CR) for the eight latent factors extracted from multi-omics factor analysis.

Factor	R²*	p*	p.adj*
Factor 1	11.98	4e-05	3.2e-04
Factor 2	8.78	0.61	0.697
Factor 3	7.71	0.063	0.169
Factor 4	6.92	0.830	0.830
Factor 5	6.71	0.093	0.186
Factor 6	5.28	0.002	0.007
Factor 7	5.26	0.150	0.240
Factor 8	4.27	0.510	0.679

*Two-sided Wilcoxon rank sum test performed between IF-P and CR groups for each factor.

Table S6. Baseline characteristics of subgroup study participants.

Variable	“Low” (n=5)	“High” (n=5)	p*
Age, mean $\pm$ SD	50.8 $\pm$ 2.3	47.2 $\pm$ 8.9	0.249
Sex, % (n)			
Men	40 (2)	40 (2)	
Women	60 (3)	60 (3)	
Race/ethnicity, % (n)			
White	100 (5)	80 (4)	
Asian	0 (0)	20 (1)	
Height (cm)	168.1 $\pm$ 16.8	175.1 $\pm$ 4.9	0.602
Weight (kg)	81.9 $\pm$ 18.1	108.9 $\pm$ 30.8	0.117
Body fat (%)	36.3 $\pm$ 6.4	41.6 $\pm$ 7.6	0.251
Body mass index (kg/m ²)	28.8 $\pm$ 2.8	35.6 $\pm$ 10.2	0.117
Waist circumference (cm)	96.0 $\pm$ 11.3	115.3 $\pm$ 21.0	0.076
Physical Activity (kcal/day)	366 $\pm$ 357	371 $\pm$ 214	0.754
Diet, mean $\pm$ SD			
Kcals	2536 $\pm$ 595	2640 $\pm$ 556	0.754
Carbohydrates (g)	252.5 $\pm$ 54.4	267.9 $\pm$ 74.2	0.917
Sugar (g)	76.1 $\pm$ 40.7	107.9 $\pm$ 46.9	0.347
Fiber (g)	19.7 $\pm$ 3.9	21.8 $\pm$ 12.5	0.602
Protein (g)	108.4 $\pm$ 22.9	103.2 $\pm$ 40.6	0.251
Fat (g)	102.9 $\pm$ 35.0	123.3 $\pm$ 25.9	0.175
Sodium (mg)	3696.9 $\pm$ 759.9	3704.7 $\pm$ 1675.5	0.917

*Two-sided Mann-Whitney U test, alpha = 0.05.

Table S7. Permutational analysis of variance model parameters and term results for Bray-Curtis dissimilarity matrix of high and low response group and time.

	DF	SS	R²	F	p
Participant	9	3.237	0.711	3.615	0.001
Time*Group	2	0.519	0.114	2.609	0.001
Residuals	8	0.796	0.175		
Total	19	4.553	1.000		

Abbreviations: DF, degrees of freedom; SS, sum of squares.

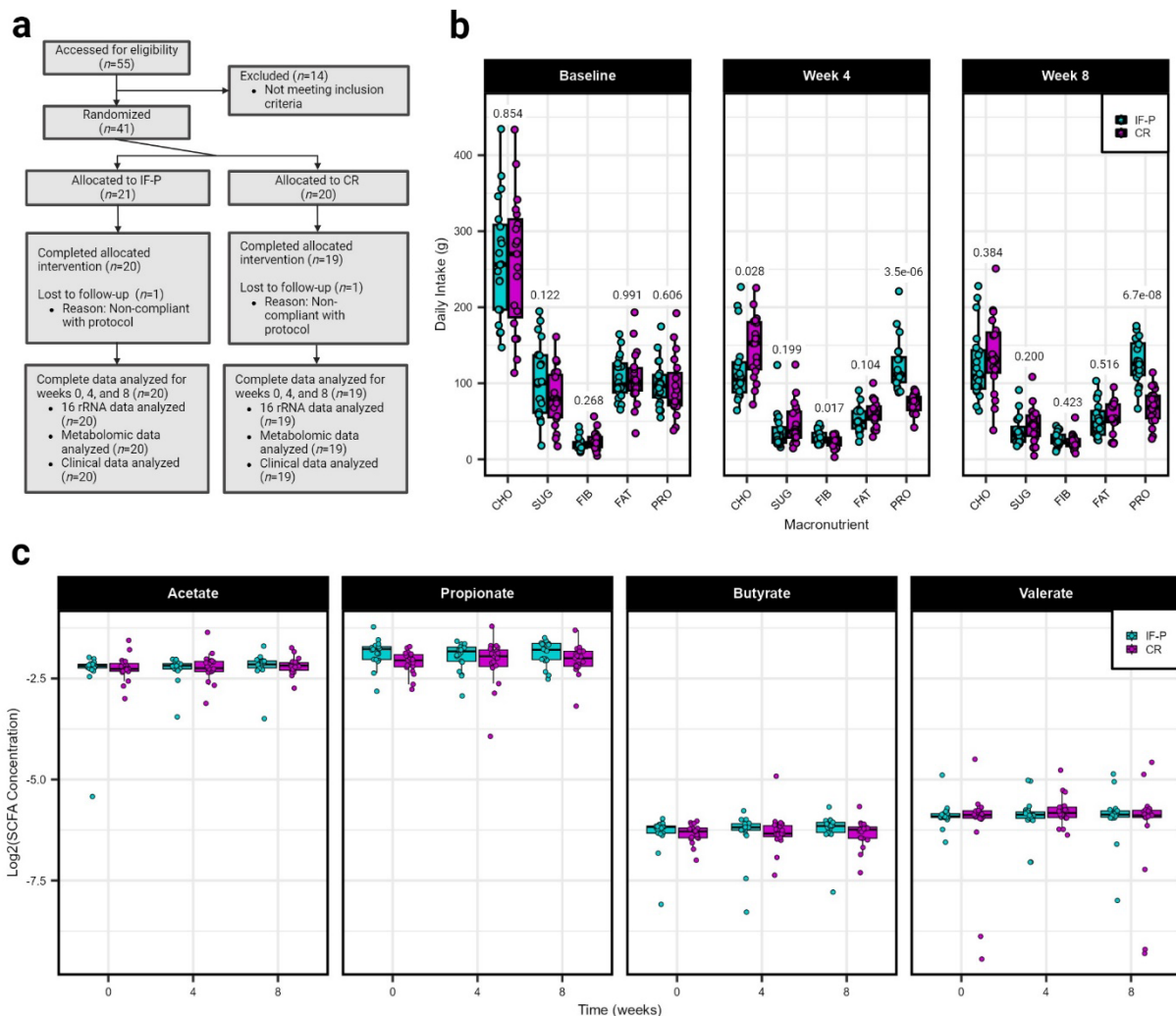


Fig S1. (a) CONSORT diagram describing enrollment, allocation, and data analysis of the parent clinical study. **(b)** Daily consumption of macronutrients measured by mass (g) at baseline, week four, and week eight between the intermittent fasting protein pacing (IF-P) and continuous calorie restriction (CR) groups. There were no differences between groups at baseline for all dietary intake variables (two-sided Student's t-test $p < 0.05$). **(c)** Targeted gas chromatography-mass spectrometry (GC-MS) measurement of fecal short-chain fatty acids (SCFAs) over the study duration. No significant effects were observed for time or interaction (linear mixed effects models, two-sided $p > 0.05$). For all panels, IF-P: $n = 20$, CR: $n = 19$. Source data are provided as a Source Data file.

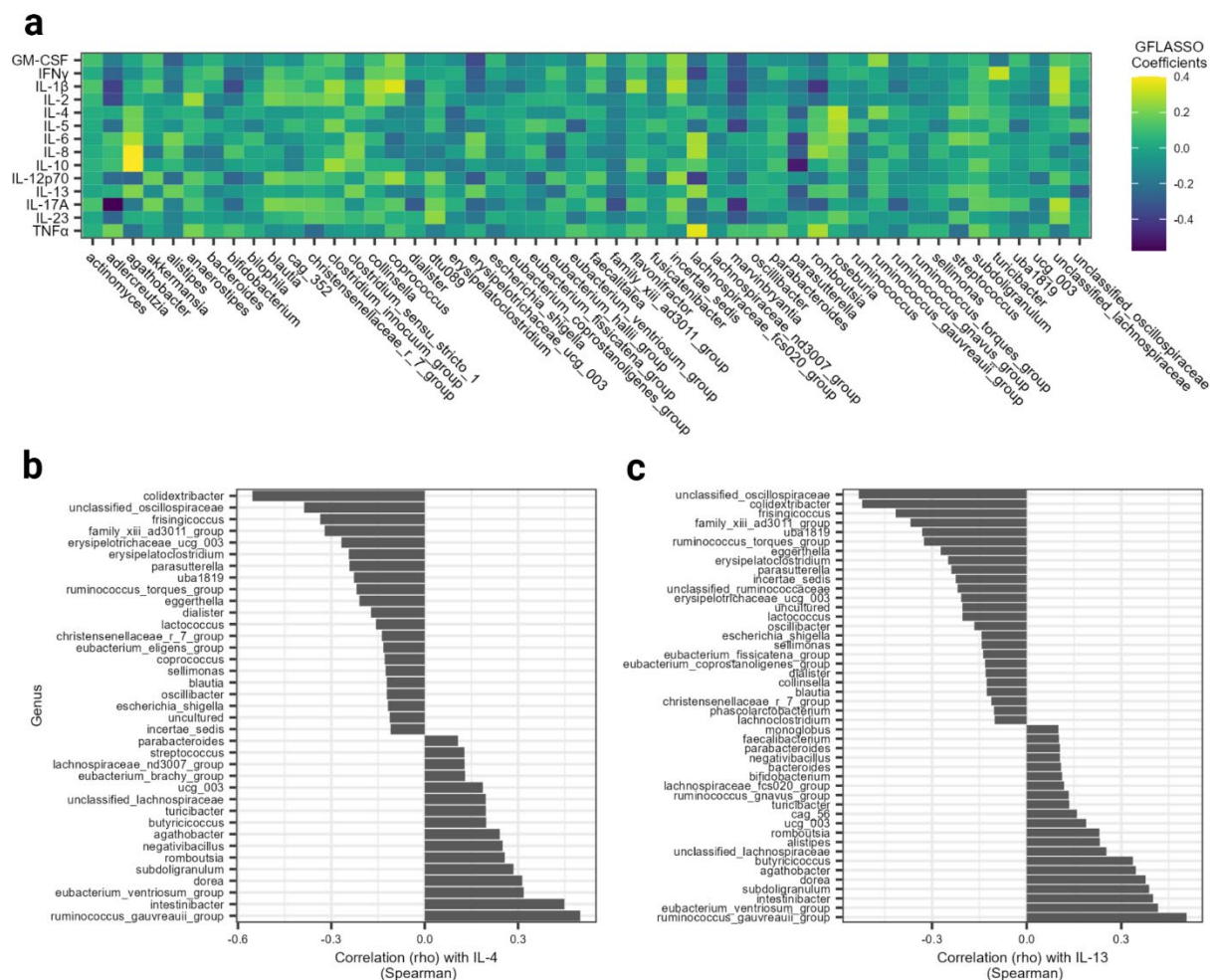


Fig S2. Cytokines and the microbiome in intermittent fasting protein pacing (IF-P) participants. **(a)** Grid-fused least absolute shrinkage and selection operator (GFLASSO) regression identified several genera with correlative relationships with our cytokine panel. Two-sided spearman correlations of **(b)** IL-4 and **(c)** IL-13 with microbial genera using intervention samples (weeks four and eight). For all panels, IF-P: $n = 20$, CR: $n = 19$. Source data are provided as a Source Data file.

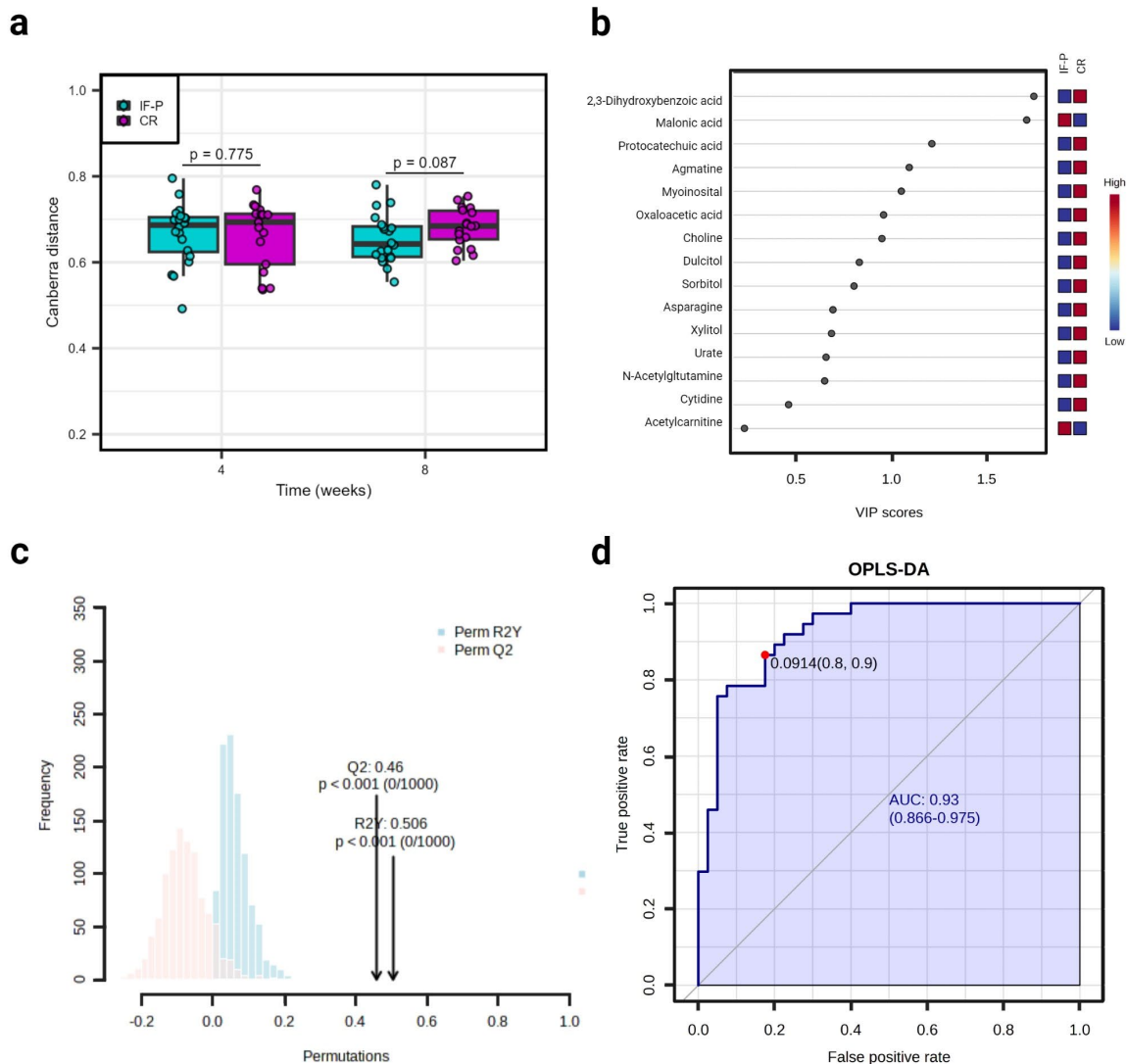


Fig S3. Analysis of plasma metabolome. **(a)** As assessed by the Canberra distance metric, no differences between intermittent fasting protein pacing (IF-P) and continuous calorie restriction (CR) were detected over the intervention in the metabolome (two-sided Wilcoxon rank sum test). **(b)** Five metabolites with a VIP > 1.0 (2,3-dihydroxybenzoic acid, malonic acid, protocatechuic acid, agmatine, and myo-inositol) were retained to construct an enhanced orthogonal projection to latent structures discriminant analysis (OPLS-DA) model. In contrast, model fit was assessed with 100-fold leave-one-out cross-validation (LOOCV). **(c)** A refined OPLS-DA model overview showing predictive and explanatory capacity ($Q^2 = 0.460$, $p < 0.001$; $R^2 = 0.506$, $p < 0.001$). **(d)** Receiver operating characteristic analysis of the OPLS-DA model (AUC = 0.929, 95% CI: 0.868-0.973, sensitivity = 0.8, specificity = 0.9). For all panels, IF-P: $n = 20$, CR: $n = 19$. Source data are provided as a Source Data file.

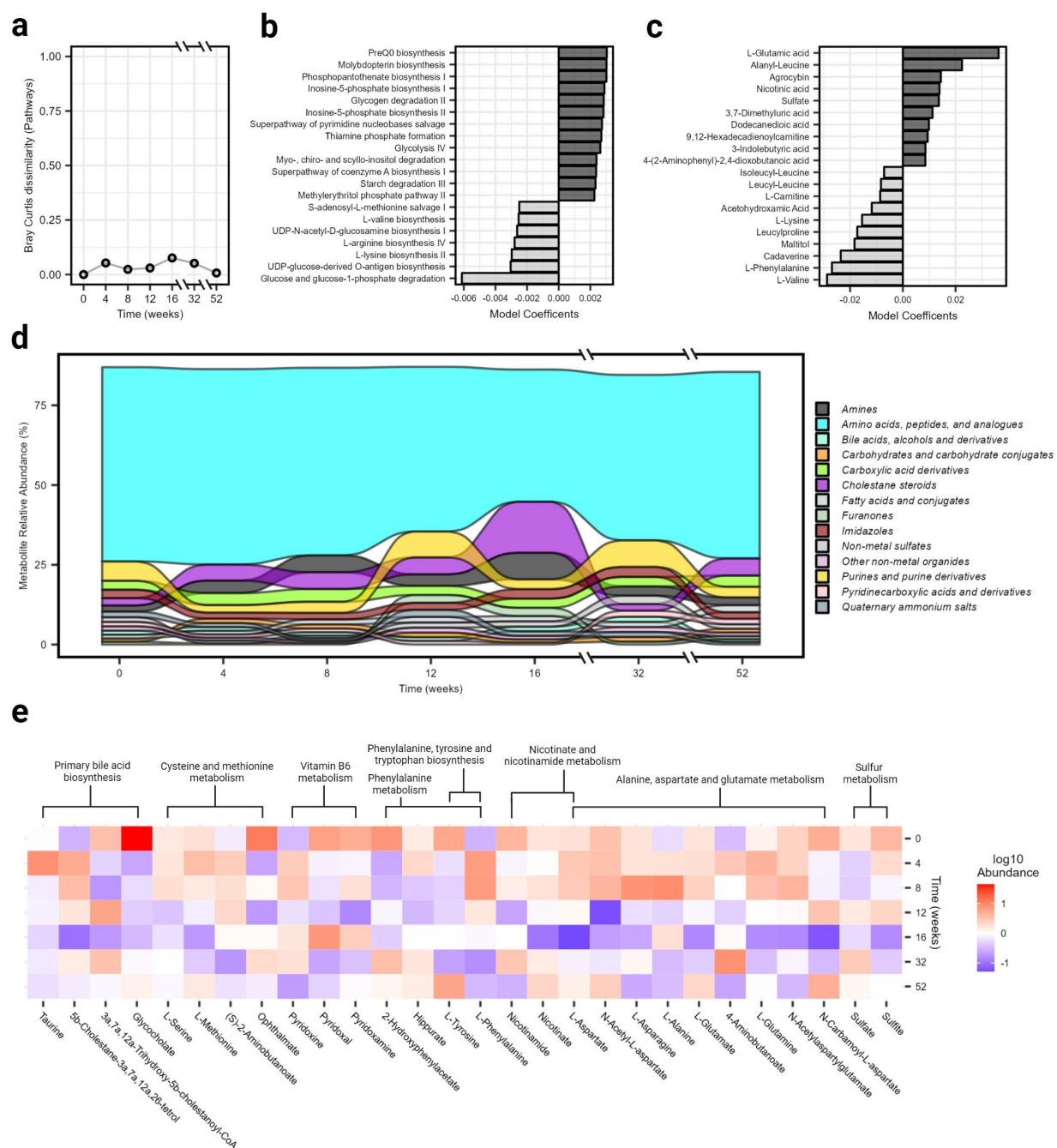


Fig. S5. (a) Bray-Curtis dissimilarity of functional pathways derived from metagenomic analysis with (b) top PERMANOVA model coefficients (analysis: pathway ~ time). (c) Top PERMANOVA model coefficients (analysis: pathway ~ time) from fecal metabolome, including the subclass, amino acids, peptides, and analogues. (d) Alluvial plot displaying the variation in abundance of the most abundant chemical subclasses (>1% relative abundance) over time. For visual clarity, the less abundant metabolite classes (<1% relative abundance) are not displayed. (e) Heatmap of the fecal metabolites captured by pathway analysis from the case study assessment. Source data are provided as a Source Data file.