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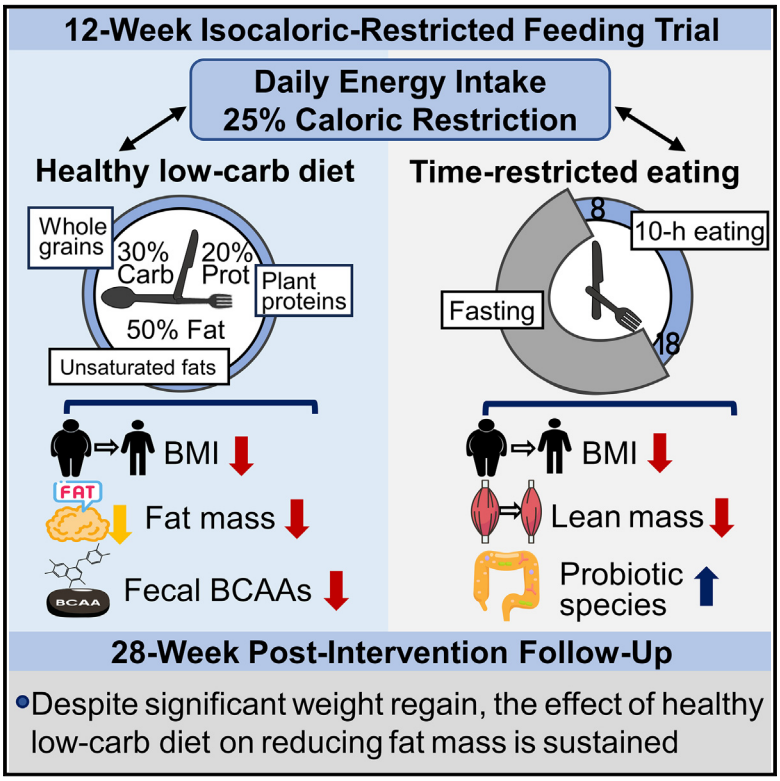
Effects of healthy low-carbohydrate diet and time-restricted eating on weight and gut microbiome in adults with overweight or obesity: Feeding RCT

Graphical abstract



Highlights

- HLCD and 10-h TRE are effective in weight management beyond caloric restriction (CR)
- HLCD leads to greater fat mass loss while TRE yields more lean mass loss beyond CR
- The effect of HLCD on reducing fat mass is sustained 28 weeks after intervention
- HLCD and TRE lead to profound alterations in the gut microbiome and metabolome

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In brief

Through a 12-week isocaloric-restricted feeding trial with 28-week post-intervention follow-up, Li et al. demonstrate the efficacy of HLCD and TRE in weight management beyond caloric restriction among adults with overweight or obesity. They also identify that both HLCD and TRE yield profound alterations in the gut microbiome and metabolome.

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Effects of healthy low-carbohydrate diet and time-restricted eating on weight and gut microbiome in adults with overweight or obesity: Feeding RCT

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SUMMARY

The effect of a healthy low-carbohydrate diet (HLCD) and time-restricted eating (TRE), alone or in combination, on body weight and gut microbiome beyond caloric restriction remains unclear. In this 12-week two-by-two factorial randomized trial with a 28-week follow-up among 96 participants with overweight or obesity, isocaloric-restricted feeding yields significant weight loss, ranging from 2.57 to 4.11 kg across different groups. Beyond caloric restriction, HLCD and TRE lead to additional reduction in body mass index. HLCD results in additional fat mass loss while TRE yields more lean mass loss. Additionally, HLCD leads to decreased fecal branched-chain amino acids, and TRE tends to yield an increased abundance of probiotic species involved in synthesizing short-chain fatty acids. Moreover, the effect of HLCD on reducing fat mass is sustained during the post-intervention follow-up. Overall, HLCD and TRE are effective in weight management and yield profound gut microbiome and metabolome alteration beyond caloric restriction. This study was registered at ChiCTR.org.cn (ChiCTR2200056363).

INTRODUCTION

Obesity has been a global health challenge,[1] with the prevalence of overweight and obesity rising rapidly in China, where central obesity prevails with elevated cardiovascular risk.[2] While moderate calorie restriction is recommended for people with obesity,[3] the additional cardiometabolic benefits of dietary patterns in conjunction with calorie restriction remain unclear.

Although low-carbohydrate diet (LCD) has shown favorable effects on short-term weight loss and improvement of cardiometabolic biomarkers,[4,5] most previous clinical trials on LCD neglected the quality and largely concentrated on the quantity of macronutrients.[6–9] Evidence, especially from feeding trials, is scarce on the cardiometabolic effect of a relatively healthy LCD (HLCD) emphasizing high-quality carbohydrates (i.e., whole grains), plant protein, and unsaturated fats.[10,11] In addition, time-restricted eating (TRE) has been linked to weight loss and cardiometabolic health.[12,13] However, findings regarding whether TRE can confer additional health benefits beyond daily calorie restriction remain inconsistent,[13–16] with sparse evidence from feeding trials.[17] Moreover, the potentially superior metabolic benefit of combining HLCD and TRE is currently unknown. Furthermore, the long-term sustainability of weight loss and metabolic benefits after stopping the HLCD and TRE intervention remains uncertain.[18]

Diet is also a pivotal external factor affecting the gut microbiome, and the important effect of diet-induced alterations in the microbiome on human physiology and disease processes has attracted widespread attention.[6,19] Although evidence suggests that the effect of LCD on the gut microbiome might be mixed, encompassing both harmful and beneficial effects,[20–23] the effect of HLCD emphasizing high-quality carbohydrates, fatty acids, and plant protein on the gut microbiome remains unclear, especially from isocaloric-restricted feeding trials. Furthermore, intermittent fasting may also affect multiple gut functions, but evidence regarding the effect of TRE on the human gut

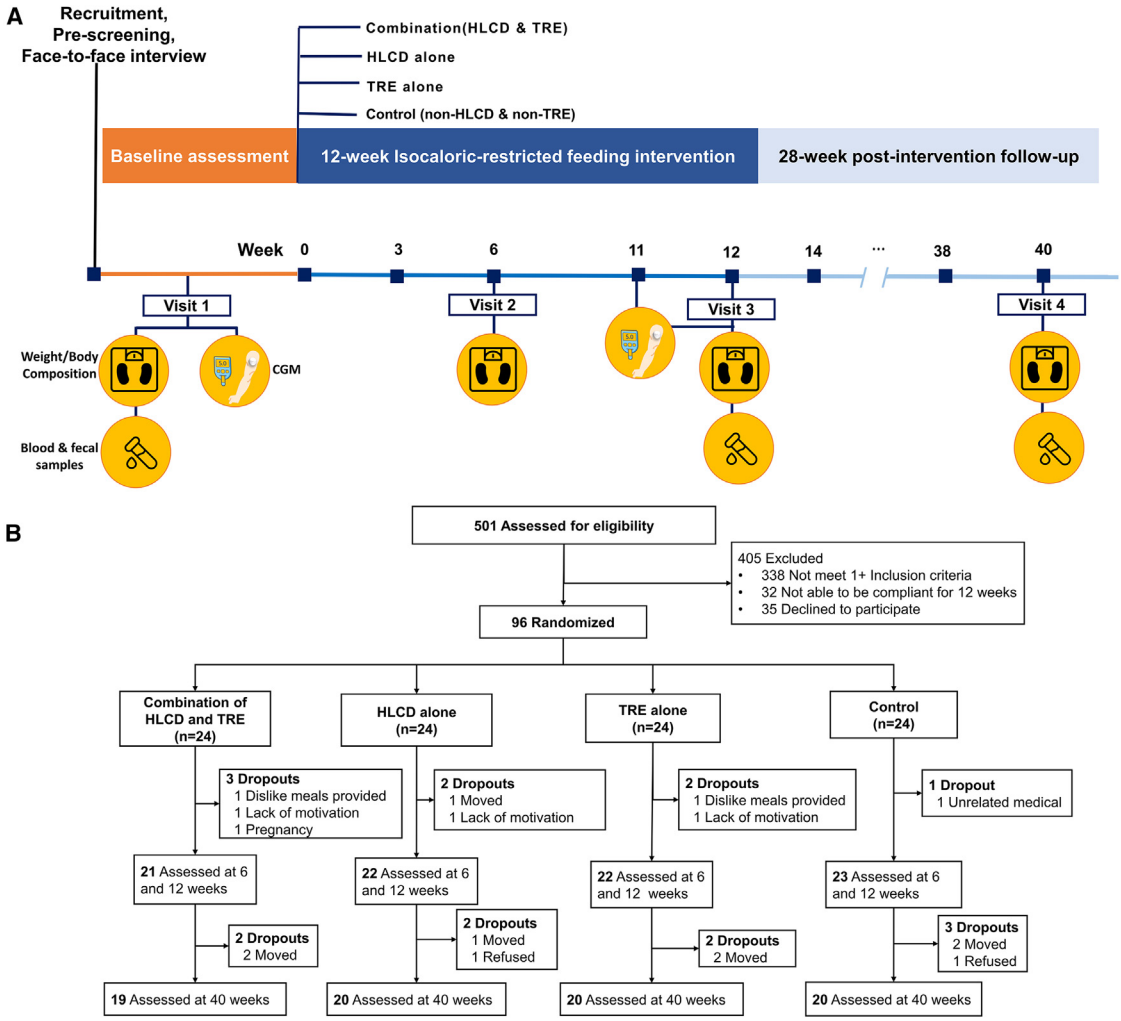

Figure 1. Study design and flow diagram

(A) Study design. Before baseline, participants were screened with a face-to-face interview. At clinical visit 1, questionnaires, dietary records, and blood and fecal samples were collected, and 40 participants (10 participants/each group) were randomly selected to wear CGM for 2 weeks. At week 6, the visit 2 was conducted among all participants to assess body weight and body composition. Two weeks before the end of the intervention (approximately at the start of week 11), participants who received CGM measurement at baseline repeated the CGM measurement for 2 weeks. At the end of week 12 (visit 3), all participants repeated the assessments at visit 1. At week 40, the post-intervention visit (visit 4) was conducted, and questionnaires, dietary records, and blood and fecal samples were collected.

(B) Flow diagram of the trial. Abbreviations: CGM, continuous glucose monitor; HLCD, healthy low-carbohydrate diet; TRE, time-restricted eating.

microbiome in clinical trials is scarce.[16,24,25] Furthermore, aside from the short-term alteration of the gut microbiome, whether the changes could be sustained for a relatively long time after stopping diet intervention still needs exploration.

The LCD and TRE (LEAN-TIME) study, a 12-week randomized controlled isocaloric-restricted feeding trial followed by a 28-week post-intervention follow-up, was designed to address these knowledge gaps. LEAN-TIME assessed the effects of HLCD and TRE, either alone or in combination, on body weight, cardiometabolic biomarkers, gut microbiome, and fecal metabolome among adults with overweight or obesity. The trial also explored whether and to what extent the changes observed during the intervention could be sustained after the dietary intervention ended.

RESULTS

To investigate the effect of HLCD and TRE alone or in combination on body weight, composition, cardiometabolic markers, and gut microbiome among adults with overweight or obesity, we conducted a 12-week two-by-two factorial randomized controlled feeding trial with a 28-week follow-up from March 2022 to April 2023 (ChiCTR.org.cn: ChiCTR2200056363). Our study design is summarized in Figure 1A; there are two phases in this study, including a 12-week dietary intervention period and a 28-week post-intervention follow-up period. In this two-by-two factorial design study involving two factors (HLCD and TRE) with two levels (yes or no), a total of 96 participants were randomly assigned across four treatment groups (24 persons

in each group): combination of HLCD and TRE group, HLCD alone group, TRE alone group, or control group (neither HLCD nor TRE). During the 12-week dietary intervention period, the isocaloric-restricted diets on five workdays (1,600 kcal/day for men and 1,300 kcal for women, approximately 25% caloric restriction) and dietary advice on the weekend were provided. The participants in the combination group were provided with HLCD and instructed to follow the 10-h TRE. The HLCD was a relatively healthy type of LCD consisting of approximately 30%, 50%, and 20% of total energy from carbohydrates, fats, and proteins, emphasizing the consumption of unsaturated fatty acids, plant proteins, and high-quality carbohydrates including whole grains, fresh vegetables, and fruits. The 10-h TRE required participants to consume the provided meals within a 10-h window each day. The participants in the HLCD alone group were provided with HLCD and instructed to follow their usual eating regimens. The participants in the TRE alone group were provided with control diets and instructed to follow the 10-h TRE. The control diet was the traditional Chinese diet designed according to Dietary Guidelines for Chinese Resident,[26] characterized by approximately 50%, 34%, and 16% of energy from carbohydrates, fat, and protein. The participants in the control group were provided with control diets and instructed to follow their usual eating regimens. More detailed nutrient and food composition of HLCD and control diet are presented in Table S1. The primary outcomes were changes in body weight, body composition, and fasting glucose during the 12-week feeding trial. The secondary outcomes were the changes in other cardiometabolic biomarkers, gut microbiome, fecal metabolome, and adverse events during the 12-week feeding trial; and the changes in body weight, body composition, cardiometabolic biomarkers, gut microbiome, and fecal metabolome from baseline to post-intervention visit.

Participant characteristics and adherence to the intervention

As shown in Figure 1B, during the 12-week feeding trial, all dropouts occurred within the first 6 weeks of the intervention ($n = 8$). A total of 88 participants (91.7%) completed the 12-week intervention phase, with 21, 22, 22, and 23 completers in the combination of HLCD with TRE, HLCD alone, TRE alone, and control groups, respectively. Tables 1 and S2 display the baseline characteristics between the dietary groups. The mean (SD) age of the participants was 36.3 (7.4) years, with an average body mass index (BMI) of 26.9 (2.3) kg/m^2, and cardiometabolic parameters were relatively favorable. There were no significant differences between the groups in all baseline characteristics. In addition, there was no significant difference in general baseline characteristics between completers and non-completers in the dietary intervention (Table S3). Additionally, all participants in each group showed favorable adherence to the respective dietary patterns, and the mean compliance rates (SD) for the HLCD and non-HLCD groups were 90.5% (12.6%) and 91.8% (11.7%), respectively, with no significant between-group differences observed. The average eating window, defined as the duration from the start to the end of eating each day, was 10.47 (0.38) h for the TRE group, meeting the requirements for a 10-h TRE[27]; meanwhile, the non-TRE group had an average eating window of 11.76 (0.45) h. Table S4 shows the daily physical activity and sedentary time of participants, and no significant between-group differences were detected during the 12-week feeding trial period. Only mild adverse events were detected, such as dizziness, weakness, constipation, and diarrhea during the trial, with no significant difference across groups (Table S5).

Both HLCD and TRE reduced BMI, but HLCD exerted more fat mass loss while TRE led to more lean mass loss

During the 12-week 25% isocaloric-restricted dietary intervention, the average weight loss was about 5% (3.53 kg). The weight loss after the 12-week intervention was 2.57 kg (95% confidence interval [CI]: 1.66, 3.48) for the control group, 3.70 kg (95% CI: 2.76, 4.63) for the HLCD alone group, 3.78 kg (95% CI: 2.85, 4.72) for the TRE alone group, and 4.11 kg (95% CI: 3.16, 5.06) for the combination group (Table 2). However, due to the nonsignificant interaction terms between two interventions ($P_{HLCD*TRE} > 0.05$) and between interventions and time ($P_{HLCD*TRE*Time}$, $P_{HLCD*Time}$, and $P_{TRE*Time} > 0.05$), the main effect of each intervention across 12-week intervention period was evaluated and represented by changes across 12 weeks (Table 3). For the HLCD arm, participants in the HLCD group had significantly decreased BMI, and the change across 12 weeks was −1.32 kg/m^2 (95% CI: −1.52, −1.12) in HLCD vs. −1.00 kg/m^2 (95% CI: −1.20, −0.80) in non-HLCD (difference, −0.32 kg/m^2 [95% CI: −0.60, −0.04], $p = 0.03$). Moreover, there was a greater reduction in fat mass in HLCD (−3.37 kg [95% CI: −3.93, −2.80]) vs. non-HLCD (−2.44 kg [95% CI: −2.99, −1.89]) (difference, −0.93 kg [95% CI: −1.70, −0.15], $p = 0.02$). For the TRE arm, the change in BMI was −1.33 kg/m^2 (95% CI: −1.54, −1.13) in TRE vs. −0.99 kg/m^2 (95% CI: −1.19, −0.79) in non-TRE (difference, −0.34 kg/m^2 [95% CI: −0.62, −0.06], $p = 0.02$). Moreover, TRE led to more reduction in soft lean mass and hip circumference than the non-TRE group (all $p < 0.05$), with mean changes of −0.58 kg (95% CI: −0.92, −0.23) vs. −0.09 kg (95% CI: −0.43, 0.26) for soft lean mass and −4.22 cm (95% CI: −5.08, −3.37) vs. −2.98 cm (95% CI: −3.82, −2.15) for hip circumference, respectively. Furthermore, although the interaction between HLCD and TRE was not significant, the exploratory analysis that compared the effects of weight loss between combination and control groups might yield some valuable insights. We found that the combination of HLCD and TRE led to more weight loss than the control group (neither HLCD nor TRE) at the end of the intervention (difference, −1.54 kg [95% CI: −2.85, −0.24], $p = 0.02$), which might potentially provide some exploratory research evidence for subsequent research.

Moreover, after a 12-week caloric restriction, all participants had significantly improved blood pressure, total cholesterol, and liver and kidney function indicators compared to baseline. Although the fasting glucose was not significantly changed among all participants after the 12-week intervention, we found significant decreases in all-day and daytime mean glucose levels from continuous glucose monitoring among all participants compared with baseline. Since there were no significant interactions between the HLCD and TRE groups in all metabolic markers except for aspartate aminotransferase (all $P_{interaction} > 0.05$) (Table S6), the analyses were based on the two dietary

Table 1. Baseline characteristics of participants

	Control (n = 24)[a]	HLCD alone (n = 24)[a]	TRE alone (n = 24)[a]	HLCD & TRE (n = 24)[a]	p [e]
Male, no. (%)	15 (62.50)	15 (62.5)	16 (66.67)	14 (58.33)	0.95
Age, years	37.50 ± 9.44	34.13 ± 5.89	35.63 ± 6.67	38.13 ± 6.67	0.22
Education, no. (%)	–	–	–	–	0.42
0–12 years	1 (4.17)	0 (0.00)	0 (0.00)	1 (4.17)	–
≥13 years	23 (95.83)	24 (100.00)	24 (100.00)	23 (95.83)	–
Current smoker, no. (%)	2 (8.33)	1 (4.17)	1 (4.17)	3 (12.50)	0.65
Alcohol drinker, no. (%)	6 (25.00)	4 (16.67)	3 (12.50)	6 (25.00)	0.61
Physical activity, no. (%)[b]	–	–	–	–	0.65
Low	17 (70.83)	14 (58.33)	17 (70.83)	14 (58.33)	–
Moderate	7 (29.17)	10 (41.67)	7 (29.17)	10 (41.67)	–
Antihypertensive drugs, no. (%)	1 (4.17)	1 (4.17)	0 (0.00)	0 (0.00)	0.42
Sedentary time, hours	7.02 ± 3.07	6.31 ± 2.73	6.00 ± 2.66	5.95 ± 2.54	0.55
Dietary intake[c]					
Eating window, hours	12.00 ± 1.38	11.79 ± 1.39	11.85 ± 1.37	11.89 ± 1.45	0.73
Energy, male, kcal/day	1,941 ± 318	1,823 ± 302	1,886 ± 368	1,944 ± 273	0.70
Energy, female, kcal/day	1,525 ± 339	1,516 ± 317	1,632 ± 292	1,614 ± 282	0.80
Carbohydrate, %	51.45 ± 5.69	47.79 ± 9.53	47.22 ± 7.15	47.78 ± 6.40	0.18
Protein, %	16.69 ± 1.73	16.34 ± 2.59	17.75 ± 4.37	17.35 ± 2.87	0.37
Total fat, %	31.91 ± 5.48	35.98 ± 7.94	34.92 ± 5.42	34.4 ± 3.80	0.11
Cholesterol, mg/day	534.1 ± 157.7	503.6 ± 226.0	527.9 ± 212.0	496.7 ± 162.0	0.88
Fiber, g/1,000 kcal	7.02 ± 1.87	6.82 ± 2.64	7.20 ± 3.05	7.35 ± 2.02	0.89
Anthropometrics					
Weight, kg	74.62 ± 11.06	75.78 ± 11.47	76.75 ± 9.05	74.15 ± 7.78	0.80
BMI, kg/m^2	26.68 ± 2.21	26.94 ± 2.58	26.78 ± 2.03	27.26 ± 2.31	0.83
FM, kg	23.20 ± 6.12	24.01 ± 5.58	22.70 ± 5.32	23.28 ± 5.27	0.88
FAT%, %	30.94 ± 5.92	31.74 ± 5.93	29.80 ± 6.85	31.54 ± 6.80	0.72
SLM, kg	48.51 ± 7.76	48.79 ± 8.72	50.96 ± 8.83	47.98 ± 7.71	0.62
SMM, kg	28.71 ± 4.94	28.99 ± 5.64	30.33 ± 5.70	28.46 ± 5.04	0.63
Hip circumference, cm	100.63 ± 5.71	100.31 ± 5.95	101.88 ± 4.98	101.01 ± 5.35	0.78
Waist circumference, cm	89.33 ± 7.84	89.23 ± 9.12	89.33 ± 6.57	89.87 ± 6.93	0.99
Blood pressure					
SBP, mmHg	126.68 ± 15.48	122.64 ± 14.79	125.78 ± 14.52	120.38 ± 13.4	0.42
DBP, mmHg	78.63 ± 10.72	77.26 ± 12.29	76.11 ± 10.03	74.57 ± 9.71	0.61
Fasting glucose, mmol/L	5.02 ± 0.60	5.20 ± 0.47	5.05 ± 0.43	5.16 ± 0.76	0.65
Blood lipids					
TG, mmol/L	1.30 (0.95, 2.15)	1.23 (0.83, 1.23)	1.32 (0.88, 1.70)	1.70 (1.20, 1.70)	0.35
HDL-C, mmol/L	1.30 ± 0.27	1.25 ± 0.28	1.21 ± 0.28	1.22 ± 0.27	0.70
LDL-C, mmol/L	2.83 ± 0.65	2.74 ± 0.66	2.75 ± 0.72	3.03 ± 0.51	0.37
TC, mmol/L	5.33 ± 0.94	5.01 ± 0.88	5.02 ± 0.95	5.46 ± 0.66	0.19
Renal function					
BUN, mmol/L	5.79 ± 1.48	5.78 ± 1.06	6.14 ± 1.31	6.00 ± 1.17	0.71
Cr, μmol/L	67.24 ± 15.55	71.56 ± 16.86	68.48 ± 14.1	63.14 ± 15.18	0.31
UA, μmol/L	403.54 ± 113.33	417.54 ± 102.09	389.13 ± 99.54	400.00 ± 101.62	0.82
Liver function					
AKP, U/L	73.58 ± 18.28	76.00 ± 22.78	75.25 ± 17.53	73.33 ± 15.52	0.95
GGT, U/L	21.50 (13.50, 33.50)	25.5 (15.00, 35.50)	21.5 (14.5, 36.00)	31.00 (19.50, 40.50)	0.50
ALT, U/L	29.05 (15.15, 51.35)	26.40 (15.85, 34.75)	24.65 (18.65, 29.40)	35.95 (19.45, 48.45)	0.34
AST, U/L	27.48 ± 8.52	24.20 ± 7.09	25.13 ± 8.7	26.8 ± 6.50	0.44

(*Continued on next page*)

Table 1. *Continued*

	Control (n = 24)[a]	HLCD alone (n = 24)[a]	TRE alone (n = 24)[a]	HLCD & TRE (n = 24)[a]	p [e]
CGM[d]					
All-day average glucose, mmol/L	5.47 ± 0.71	5.09 ± 0.52	5.81 ± 0.49	5.88 ± 1.03	0.13
All-day average CV, %	22.56 ± 5.9	18.47 ± 3.59	18.58 ± 4.11	22.21 ± 4.32	0.13
Daytime average glucose, mmol/L	5.82 ± 0.7	5.32 ± 0.5	6.07 ± 0.49	6.23 ± 1.12	0.11
Daytime average CV, %	20.66 ± 5.31	17.6 ± 3.12	18.05 ± 4.6	20.65 ± 4.77	0.35
Nighttime average glucose, mmol/L	4.44 ± 0.78	4.38 ± 0.64	5.03 ± 0.5	4.85 ± 0.82	0.19
Nighttime average CV, %	9.91 ± 5.89	7.86 ± 2.84	8.86 ± 2.82	10.58 ± 7.08	0.71

Abbreviations: AKP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; BUN, blood urea nitrogen; CGM, continuous glucose monitoring; Cr, creatinine; CV, coefficient of variation; DBP, diastolic blood pressure; FAT%, fat percentage; FM, fat mass; GGT, gamma-glutamyl transpeptidase; HDL-C, high-density lipoprotein cholesterol; HLCD, healthy low-carbohydrate diet; LDL-C, low-density lipoprotein cholesterol; SBP, systolic blood pressure; SLM, soft lean mass; SMM, skeletal muscle mass; TC, total cholesterol; TG, triglyceride; TRE, time-restricted eating; UA, uric acid.

[a]Data were mean ± standard deviation or median (interquartile range) for continuous variables with normal distribution or skewed distribution and n (%) for categorical variables.

[b]Physical activity was calculated based on the International Physical Activity Questionnaire.

[c]Dietary intake data were calculated from the 3-day dietary record.

[d]A total of 35 participants had valid CGM records at baseline, 9, 8, 8, and 10 in control, HLCD alone, TRE alone, and HLCD & TRE groups, respectively.

[e]The p values were assessed by chi-square test for categorical variables, ANOVA, or Kruskal-Wallis H test for continuous variables with normal distribution or skewed distribution.

intervention arms, and changes in metabolic markers were shown in Table S7. Furthermore, to assess the robustness of the results, we also conducted the subgroup analyses stratified by gender and BMI and found no significant heterogeneity in any clinical indices between groups (Tables S8–S11, all $P_{\text{heterogeneity}} > 0.05$). In addition, the primary results were not significantly changed when using multiple imputed data (Table S12).

Both HLCD and TRE altered the compositions and functions of the gut microbiome

To investigate the gut microbiome alterations, we conducted metagenomic sequencing on fecal samples collected. At baseline, no significant differences between groups in alpha and beta diversity were observed (Figures S1A–S1C). For adaptations of the gut microbiome during the intervention, a significant difference between baseline and endpoint (week 12) in beta diversity was found in the HLCD and TRE groups ($P_{\text{PERMANOVA}} < 0.05$, Figures 2A and 2B), while not in the non-HLCD or non-TRE groups. Although the gut microbial α-diversity (i.e., Shannon index) showed no significant shifts over the intervention, we found that the Firmicutes/Bacteroidetes ratio (F/B ratio) was significantly decreased after the 12-week HLCD intervention ($p = 0.03$) (Figure S1D). However, no significant differences in α-diversity, β-diversity, or F/B ratio were observed between the HLCD and non-HLCD groups or between the TRE and non-TRE groups.

Microbiome taxonomic profiles were significantly altered during this caloric-restricted feeding trial, and the patterns of alteration between groups differed. Cladograms with taxonomic labels for lineage having a significant change are provided in Figure S2. Linear discriminant analysis identified 9, 8, 11, and 6 species-level alterations during a 12-week feeding trial among participants randomized to HLCD, non-HLCD, TRE, and non-TRE groups, respectively (Figures 2C and 2D). Regarding between-group species-level differences, species like *Ruminococcus callidus* was significantly decreased in the HLCD compared to the isocaloric-restricted non-HLCD group; and TRE intervention increased probiotic species *Parabacteroides distasonis*, *Bacteroides intestinalis*, *Parabacteroides goldsteinii*, and *Escherichia coli*, compared to the non-TRE group (all $p < 0.05$ before false discovery rate [FDR] correction). However, these differences were not significant after FDR correction. This lack of significance may be due to limited statistical power, as well as the fact that all groups were isocaloric restricted, potentially obscuring the effects of the dietary patterns on the gut microbiome (Table S13; Figures S3 and S4).

We also profiled the functional potential of the gut microbiome and examined functional differences between the HLCD or TRE intervention and respective control groups. There was no significant difference in the gut microbial function between groups at baseline or the end of the dietary intervention. However, significant shifts of functional profiles from baseline to the end of the intervention were observed in the HLCD, non-HLCD, and TRE groups ($P_{\text{PERMANOVA}} < 0.01$) (Figure S5). To determine the microbial features underlying these effects, top loadings of significant comparison before and after dietary intervention were shown, and several top loadings in the HLCD group were related to purine metabolism and fatty acid biosynthesis (Figure 2E; Table S14). Top loadings for the TRE group included pathways related to polyamine metabolism (Figure 2F; Table S15). Collectively, these results suggest that dietary intervention can impact the functional potential of the human microbiome in intestinal digestion, with the most prominent effects on the gut microbiome observed with HLCD. As for the between-group differences in individual functions of the gut microbiome, for the HLCD arm, HLCD intervention mainly affected pathways involving carbohydrate degradation (e.g., GLUCUROCAT-PWY and UDPNAGSYN-PWY) and amino acid biosynthesis compared with the non-HLCD group. For the TRE arm, TRE

Table 2. Changes in anthropometric indicators among 4 treatment groups according to factorial design during the 12-week feeding trial

	Control (n = 23)[a]	HLCD alone (n = 22)[a]	TRE alone (n = 22)[a]	HLCD & TRE (n = 21)[a]	P_{HLCD}[c]	P_{TRE}[c]	P_{Time}[c]	$P_{HLCD*Time}$[c]	$P_{TRE*Time}$[c]	$P_{HLCD*TRE}$[c]	$P_{HLCD*TRE*Time}$[c]
Weight, kg	–	–	–	–	0.07	0.02	<0.001	0.91	0.29	0.34	0.93
Change at week 6[b]	−1.78 (−2.53, −1.02)	−2.91 (−3.69, −2.14)	−3.25 (−4.03, −2.47)	−3.63 (−4.42, −2.84)	–	–	–	–	–	–	–
Change at week 12[b]	−2.57 (−3.48, −1.66)	−3.70 (−4.63, −2.76)	−3.78 (−4.72, −2.85)	−4.11 (−5.06, −3.16)	–	–	–	–	–	–	–
BMI, kg/m^2	–	–	–	–	0.03	0.02	<0.001	0.95	0.30	0.43	0.94
Change at week 6[b]	−0.63 (−0.89, −0.37)	−1.06 (−1.33, −0.79)	−1.14 (−1.41, −0.87)	−1.34 (−1.61, −1.06)	–	–	–	–	–	–	–
Change at week 12[b]	−0.91 (−1.23, −0.59)	−1.34 (−1.67, −1.02)	−1.32 (−1.65, −0.99)	−1.53 (−1.86, −1.20)	–	–	–	–	–	–	–
FM, kg	–	–	–	–	0.02	0.24	0.40	0.28	0.96	0.58	0.31
Change at week 6[b]	−2.17 (−2.91, −1.44)	−3.31 (−4.06, −2.55)	−3.03 (−3.79, −2.28)	−3.38 (−4.15, −2.62)	–	–	–	–	–	–	–
Change at week 12[b]	−2.03 (−2.95, −1.12)	−3.18 (−4.12, −2.24)	−2.53 (−3.46, −1.59)	−3.59 (−4.54, −2.63)	–	–	–	–	–	–	–
FAT%	–	–	–	–	0.03	0.62	0.06	0.32	0.96	0.93	0.21
Change at week 6[b]	−2.32 (−3.20, −1.43)	−3.45 (−4.37, −2.53)	−2.92 (−3.81, −2.03)	−3.33 (−4.25, −2.41)	–	–	–	–	–	–	–
Change at week 12[b]	−1.92 (−3.10, −0.74)	−2.92 (−4.14, −1.71)	−1.86 (−3.05, −0.66)	−3.42 (−4.65, −2.18)	–	–	–	–	–	–	–
SLM, kg	–	–	–	–	0.40	0.04	<0.001	0.21	0.36	0.48	0.26
Change at week 6[b]	0.34 (−0.14, 0.82)	0.35 (−0.14, 0.85)	−0.29 (−0.77, 0.20)	−0.28 (−0.78, 0.22)	–	–	–	–	–	–	–
Change at week 12[b]	−0.54 (−1.15, 0.07)	−0.49 (−1.12, 0.13)	−1.23 (−1.84, −0.61)	−0.51 (−1.15, 0.12)	–	–	–	–	–	–	–
SMM, kg	–	–	–	–	0.63	0.02	<0.001	0.18	0.37	0.39	0.22
Change at week 6[b]	0.26 (−0.03, 0.54)	0.19 (−0.10, 0.48)	−0.18 (−0.46, 0.11)	−0.22 (−0.51, 0.08)	–	–	–	–	–	–	–
Change at week 12[b]	−0.36 (−0.73, 0.01)	−0.40 (−0.79, −0.02)	−0.85 (−1.22, −0.47)	−0.43 (−0.81, −0.04)	–	–	–	–	–	–	–
Hip circumference, cm	–	–	–	–	0.32	0.04	<0.001	0.67	0.85	0.51	0.05
Change at week 6[b]	−1.54 (−2.80, −0.28)	−2.20 (−3.49, −0.90)	−2.91 (−4.20, −1.63)	−3.19 (−4.50, −1.88)	–	–	–	–	–	–	–
Change at week 12[b]	−4.23 (−5.55, −2.90)	−3.97 (−5.33, −2.61)	−4.55 (−5.91, −3.19)	−6.24 (−7.62, −4.86)	–	–	–	–	–	–	–

(*Continued on next page*)

Table 2. *Continued*

	Control (n = 23)[a]	HLCD alone (n = 22)[a]	TRE alone (n = 22)[a]	HLCD & TRE (n = 21)[a]	P_{HLCD}[c]	P_{TRE}[c]	P_{Time}[c]	$P_{HLCD*Time}$[c]	$P_{TRE*Time}$[c]	$P_{HLCD*TRE}$[c]	$P_{HLCD*TRE*Time}$[c]
Waist circumference, cm	–	–	–	–	0.64	0.17	<0.001	0.93	0.51	0.69	0.44
Change at week 6[b]	−1.86 (−3.33, −0.39)	−2.73 (−4.25, −1.22)	−3.54 (−5.06, −2.01)	−3.37 (−4.90, −1.84)	–	–	–	–	–	–	–
Change at week 12[b]	−4.02 (−5.54, −2.51)	−4.37 (−5.93, −2.81)	−4.83 (−6.40, −3.26)	−5.09 (−6.67, −3.51)	–	–	–	–	–	–	–

Abbreviations: BMI, body mass index; FAT%, fat percentage; FM, fat mass; HLCD, healthy low-carbohydrate diet; SLM, soft lean mass; SMM, skeletal muscle mass; TRE, time-restricted eating.
[a]Twenty-four participants were randomized to each treatment group at baseline. After 1, 2, 2, and 3 participants dropped out during the 12-week trial, the sample size (n) refers to the number of completers in each treatment group.
[b]Changes in variables from baseline were presented as the least-squares adjusted means (95% confidence interval) from the mixed-effects linear model according to the repeated measures analysis.
[c]The effects of interventions, time, and their interactions on variable changes were tested using mixed-effects linear models with unstructured variance structure, which adjusted for age, gender, and corresponding baseline measures. Results for week 6 were the adjusted changes between week 6 follow-up outcomes and baseline outcomes. Similarly, week 12 results reflected the adjusted change between week 12 follow-up outcomes and baseline.

intervention mainly affected pathways involving amino acid biosynthesis, carbohydrate degradation, lipid biosynthesis, and energy metabolism compared with the non-TRE group (Table S16).

HLCD reduced fecal levels of BCAAs and increased unsaturated fatty acids, while TRE tended to increase fecal levels of DCA and IAA

To explore how the 12-week feeding trials affected the metabolism of the commensal gut microbiome and the human host, targeted metabolomics was performed in the current study. In total, we measured 217 metabolites in feces, including 40 amino acids, 42 fatty acids, 19 carbohydrates, 34 bile acids, 6 indoles, 10 short-chain fatty acids, and other categories of metabolites including benzoic acids, organic acids, and phenols. There were no significant between-group differences in the composition of fecal metabolites at baseline, but a significant difference was observed between the HLCD and non-HLCD groups at the end of the intervention ($P_{\text{principal coordinate [PC] 2 paired Wilcoxon}}$ = 0.004, Figures S6A and S6B). Significant shifts in the composition of fecal metabolites were observed in the HLCD and non-TRE groups during 12-week feeding trials (all $P_{\text{PC2 paired Wilcoxon}} < 0.05$), and no significant shifts were detected in (Figure S6C).

Individual metabolite analysis identified 34, 31, 4, and 10 significantly changed fecal metabolites after a 12-week feeding trial among participants in the HLCD, non-HLCD, TRE, and non-TRE groups, respectively ($P_{FDR} < 0.05$) (Tables S17 and S18). Compared with baseline, 12-week HLCD intervention significantly decreased the fecal levels of amino acids especially aromatic amino acids, branched-chain amino acids (BCAAs), indoles, carbohydrates, and others such as phthalic acid and alpha-hydroxybutyric acid, whereas increased the levels of unsaturated fatty acids, such as oleic acid, ricinoleic acid, alpha-linolenic acid, and palmitoleic acid. However, these metabolites were not significantly changed among participants in the non-HLCD group (Figure 3A; Table S17). Compared with the non-HLCD group, a greater decrease in the fecal concentrations of BCAAs (including leucine, isoleucine, and valine), indoles, and phthalic acid, and a greater increase in unsaturated fatty acids was observed among participants in the HLCD group (all $P_{FDR} < 0.05$, Figure 3B). Figure 3C shows Spearman's correlations between the changes in identified fecal metabolites related to HLCD intervention and changes in anthropometric indicators. BCAAs were significantly positively associated with body weight, BMI, and fat mass; N-acetylserine and phthalic acid were positively correlated with fat mass; and 9e-tetradecenoic acid was positively associated with hip circumference. In the TRE arm, the fecal metabolite changes in the TRE group were also mainly reflected by the decreased concentrations of amino acids, carbohydrates, and fatty acids, and enriched levels of deoxycholic acid (DCA) and indoleacetic acid (IAA) (all $p < 0.05$ before FDR correction). After FDR correction, there were only four significantly decreased fecal metabolites in the TRE group, including L-asparagine, glyceric acid, alpha-hydroxybutyric acid, and glycylproline (Figure 3A; Table S18). No significant difference was observed in fecal metabolites between the TRE and non-TRE groups. In addition, to integrate the taxonomy, pathways, and metabolite data, the

Table 3. Changes across 12 weeks in anthropometric indicators of each intervention during the feeding trial

–	HLCD[a]				TRE[a]			
	Non-HLCD (n = 45)	HLCD (n = 43)	Mean difference	p^c	Non-TRE (n = 45)	TRE (n = 43)	Mean difference	p^c
Weight, kg[b]	−2.84 (−3.42, −2.26)	−3.59 (−4.18, −3.00)	−0.74 (−1.55, 0.06)	0.07	−2.74 (−3.31, −2.16)	−3.69 (−4.29, −3.10)	−0.95 (−1.76, −0.15)	0.02
BMI, kg/m^2[b]	−1.00 (−1.20, −0.80)	−1.32 (−1.52, −1.12)	−0.32 (−0.60, −0.04)	0.03	−0.99 (−1.19, −0.79)	−1.33 (−1.54, −1.13)	−0.34 (−0.62, −0.06)	0.02
FM, kg[b]	−2.44 (−2.99, −1.89)	−3.37 (−3.93, −2.80)	−0.93 (−1.70, −0.15)	0.02	−2.67 (−3.23, −2.12)	−3.13 (−3.69, −2.57)	−0.46 (−1.23, 0.32)	0.24
FAT%[b]	−2.25 (−2.91, −1.59)	−3.28 (−3.97, −2.59)	−1.03 (−1.95, −0.11)	0.03	−2.65 (−3.33, −1.98)	−2.88 (−3.55, −2.21)	−0.23 (−1.15, 0.69)	0.62
SLM, kg[b]	−0.43 (−0.76, −0.09)	−0.23 (−0.58, 0.12)	0.20 (−0.27, 0.66)	0.40	−0.09 (−0.43, 0.26)	−0.58 (−0.92, −0.23)	−0.49 (−0.95, −0.03)	0.04
SMM, kg[b]	−0.28 (−0.48, −0.08)	−0.21 (−0.43, 0.00)	0.07 (−0.21, 0.35)	0.63	−0.08 (−0.29, 0.13)	−0.42 (−0.62, −0.21)	−0.34 (−0.62, −0.06)	0.02
Hip circumference, cm[b]	−3.31 (−4.15, −2.47)	−3.90 (−4.75, −3.05)	−0.59 (−1.76, 0.58)	0.32	−2.98 (−3.82, −2.15)	−4.22 (−5.08, −3.37)	−1.24 (−2.41, −0.07)	0.04
Waist circumference, cm[b]	−3.56 (−4.57, −2.56)	−3.89 (−4.90, −2.88)	−0.33 (−1.71, 1.06)	0.64	−3.25 (−4.24, −2.25)	−4.21 (−5.23, −3.19)	−0.97 (−2.34, 0.42)	0.17

Abbreviations: BMI, body mass index; FAT%, fat percentage; FM, fat mass; HLCD, healthy low-carbohydrate diet; SLM, soft lean mass; SMM, skeletal muscle mass; TRE, time-restricted eating.
[a]Forty-eight participants were randomized to each intervention group. After 3, 5, 3, and 5 participants dropped out during the 12-week trial, the sample size (n) refers to the number of completers in each intervention group.
[b]Changes in variables from baseline were presented as the least-squares adjusted means (95% confidence interval) from mixed-effects linear models according to the repeated measures analysis. Changes across 12 weeks are a weighted, adjusted average of the weekly effects ([changes at week 6 + changes at week 12]/2). Changes across 12 weeks conceptually reflect the average changes from baseline to follow-up time points since the interaction terms between two interventions and between interventions and time were nonsignificant ($P_{HLCD*TRE}$>0.05, $P_{HLCD*TRE*Time}$, $P_{HLCD*Time}$, and $P_{TRE*Time}$ >0.05).
[c]The between-group differences in variable changes were tested using mixed-effects linear models with unstructured variance structure, which adjusted for age, gender, and corresponding baseline measures.

circosPlot was performed, suggesting further omics studies in the future (Figure 3D). Moreover, correlation analyses were performed on variable changes in the gut microbiome, metabolites, and clinical outcomes in the HLCD intervention group during the intervention period. The widespread correlations among microbiome, metabolites, and clinical outcomes were revealed, which indicated an interconnected relationship (Figure 3E). For example, fecal concentrations of BCAAs were inversely associated with the relative abundance of *Oscillibacter* and were positively correlated with BMI, body fat mass, and serum triglyceride.

The effects of HLCD on reducing fat mass and fecal phthalic acid were sustained during the post-intervention follow-up

To assess the long-term sustainability of the effects of HLCD or TRE on clinical parameters and gut microbiome, a post-intervention visit was conducted 28 weeks after the end of the intervention. A total of 79 (82.3%) individuals completed the post-intervention visit. Most alterations in clinical parameters were significantly rebound during post-intervention follow-up. Despite weight regain, HLCD still significantly decreased the body fat mass and percentage at the post-intervention visit compared with baseline. Specifically, changes in body fat mass were −0.85 kg (95% CI: −1.80, 0.09) in the HLCD group vs. 0.64 kg (95% CI: −0.27, 1.56) in the non-HLCD group, resulting in a difference of −1.50 kg (95% CI: −2.80, −0.19) (p = 0.03). Changes in body fat percentage were −1.33% (95% CI: −2.58, −0.08) in the HLCD group compared to 0.49% (95% CI: −0.68, 1.65) in the non-HLCD group, yielding a difference of −1.82% (95% CI: −3.49, −0.15) (p = 0.03) (Figures 4A and 4B). In the TRE arm, the changes from baseline to week 40 in urea acid were −12.38 μmol/L (95% CI: −29.42, 4.66) in the TRE group compared to −39.15 μmol/L (95% CI: −56.15, −22.15) in the non-TRE group, yielding a difference of 26.77 μmol/L (95% CI: 3.83, 49.71) (p = 0.02). No significant between-group difference was detected in other clinical parameters at the post-intervention follow-up (Tables S19 and S20).

Certain significant impacts of HLCD on gut microbial function and fecal metabolites were also sustained during the follow-up. For example, compared with baseline levels, the functional profiles related to L-arginine biosynthesis (ARGSYN-PWY and ARGSYNBSUB-PWY) were still significantly decreased while NAGLIPASYN-PWY related to lipid IVA biosynthesis was increased in the HLCD group at the post-intervention visit, which was consistent with the direction of changes during dietary intervention (Figure 4C). As for fecal metabolites, compared with the baseline, phthalic acid was significantly decreased and *p*-hydroxyphenylacetic acid was increased in the HLCD group at the post-intervention follow-up (P_{FDR}<0.05, Figure 4D), and the decrease in phthalic acid was significant between HLCD and non-HLCD groups (P_{FDR}<0.05, Figure 4E). In addition, the alterations in gut microbial function and fecal metabolites from baseline to the post-intervention visit in other groups, including the TRE, non-TRE, and non-HLCD groups, are shown in Figures S7 and S8. For example, compared with baseline levels, NAGLIPASYN-PWY related to lipid IVA biosynthesis was increased, while the PWY-3001 related to L-isoleucine

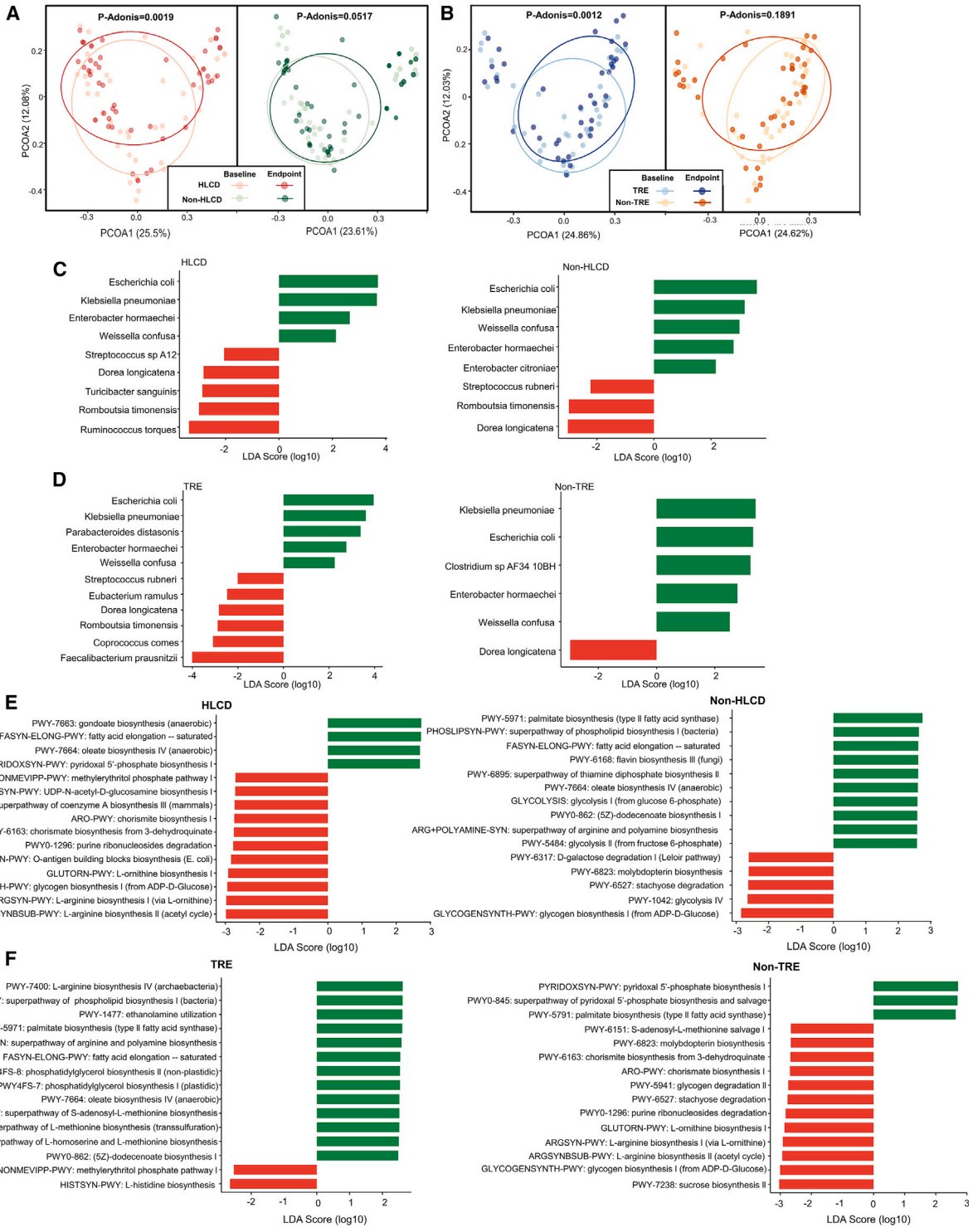

Figure 2. The alterations in the gut microbiome taxonomic and functional profiles during the 12-week feeding trial

(A and B) The change of gut microbial composition in HLCD (left) and non-HLCD (right) groups (A) and TRE (left) and non-TRE (right) groups (B) during the intervention. The intra-group differences in β-diversity during the intervention were calculated using permutational multivariate analysis of variance (PERMANOVA).

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biosynthesis I was decreased in the TRE group at the post-intervention visit. Moreover, the *p*-hydroxyphenylacetic acid was increased in the TRE group at the post-intervention follow-up (P_{FDR}<0.05), but there was no significant difference in changes in *p*-hydroxyphenylacetic acid between the TRE and non-TRE groups.

DISCUSSION

In this 12-week isocaloric-restricted feeding trial among adults with overweight or obesity, both HLCD and TRE achieved additional benefits on BMI reduction beyond caloric restriction, with HLCD resulting in more body fat mass loss and TRE leading to more soft lean mass loss. Furthermore, both HLCD and TRE induced profound alteration in the gut microbiome and microbial-host co-metabolites. HLCD downregulated amino acid biosynthesis and decreased fecal BCAAs. TRE enriched the abundance of probiotic species involved in the synthesis of short-chain fatty acids, and elevated fecal DCA and IAA concentrations. While some improvements observed during the dietary intervention were not sustained, the effects of HLCD on reducing body fat mass and fecal phthalic acid, as well as altering gut microbial functions, were sustained 28 weeks after the intervention ended.

Although several clinical trials, based on dietary advice, have explored the effects of LCD emphasizing high-quality food sources on body weight and metabolic health,[8,28] evidence is still lacking from well-controlled isocaloric feeding trials. In our isocaloric feeding trial with 25% caloric restriction, in addition to the limited carbohydrates consumption (approximately 30% of total energy), the majority of these carbohydrates (approximately 63% of total carbohydrates) in HLCD were derived from high-quality food sources such as whole grains, vegetables, and fruits. In addition, unsaturated fatty acids and plant-based protein were also emphasized in HLCD, with approximately 50% of total protein from plant-based foods and 71% of fatty acids from unsaturated fatty acids. Aligned with previous studies,[29,30] we found that in the context of isocaloric restriction, HLCD exerted an additional effect in reducing BMI and fat mass than the traditional Chinese control diet, which indicated that HLCD might yield additional benefits in body weight and fat mass loss beyond caloric restriction.

Apart from the changes in clinical parameters, the consumption of LCD has been associated with alteration in the gut microbiome. The effects of LCD on the gut microbiome have been suggested to be mixed, with beneficial outcomes (e.g., correcting imbalanced gut microbiome) and adverse effects (e.g., diminished total bacterial levels and lowered α-diversity).[20–23] The complexity of these results may be partly attributed to various characteristics of the LCD intervention, such as the quantity and quality of macronutrients.[6,20–22] Therefore, the current study aimed to investigate the impacts of a moderately carbohydrate-restricted diet (about 30% of calories from carbohydrates) that emphasizes high-quality carbohydrates, unsaturated fatty acids, and plant protein on the human gut microbiome. 12-week HLCD significantly altered gut microbial composition and functional profiles, and significantly decreased the F/B ratio, which was consistent with previous studies.[21] Although there was a significant decrease in the abundance of species and gut microbiome functions related to carbohydrate metabolism due to limited dietary carbohydrate intake, it is worth noting that some specific species or functions were significantly changed after HLCD intervention. Specifically, HLCD significantly decreased the abundance of *Ruminococcus callidus*, which was an obesity-associated genus in Western populations.[31] Evidence also indicated that the abundance of *Ruminococcus* was increased among individuals with prediabetes.[32] Additionally, HLCD significantly downregulated carbohydrate and amino acid metabolism pathways and upregulated the lipid biosynthesis pathway, which reflected the characteristics of HLCD including restricting energy intake, decreasing carbohydrate, and increasing fat intake.

Following the gut microbiome alteration, significant shifts in fecal co-metabolites were observed in HLCD, especially amino acids and fatty acids. Notably, the fecal concentrations of amino acids, particularly, three BCAAs including valine, isoleucine, and leucine, and two aromatic amino acids including phenylalanine and tyrosine were significantly decreased after 12-week HLCD intervention, compared to the control diet (non-HLCD) intervention. Additionally, increased levels of BCAAs and aromatic amino acids are widely considered to be a metabolic hallmark of obesity, insulin resistance, and type 2 diabetes mellitus.[33,34] The reduction of BCAAs in HLCD was positively associated with body weight and fat mass loss. Of note, an 8-week trial in obese participants found that a very LCD (carbohydrate intake <20 g/d) led to significant body weight loss but was unlikely to affect plasma concentrations of BCAAs,[35] whereas the 2-year POUNDS LOST trial among adults with overweight or obesity found that the significant decrease in plasma BCAAs induced by weight-loss diet was associated with reductions in abdominal fat.[36] Interestingly, we found that although the total dietary protein intake in HLCD (approximately 19% of total calories) was higher than isocaloric control diet (approximately 16%), the fecal BCAAs were significantly lower after HLCD than control diet intervention, which suggested that the consumption of HLCD increased the reliance on protein for energy-yielding purposes and therefore reduced the concentrations of BCAAs.[37] Furthermore, mechanistic studies have indicated that the disturbance in BCAA metabolism was involved in obesity, diabetes, and other metabolic disease pathogenesis, including the stimulation of insulin secretion, modulation of energy homeostasis, and the control of appetite.[38–40] Taken together, the current study provided evidence from a human feeding trial for the potential role of BCAAs in the weight-loss effect of HLCD, warranting further

(C and D) Significant taxonomic changes at species levels in HLCD (left) and non-HLCD (right) groups (C) and TRE (left) and non-TRE (right) groups (D) during the intervention, as assessed by the linear discriminant analysis (LDA) effect size (LEfSe) method; $p < 0.05$, LDA > 2.0.

(E and F) Significant functional changes in HLCD (left) and non-HLCD (right) groups (E) and TRE (left) and non-TRE (right) groups (F) during the intervention, as assessed by LEfSe method; $p < 0.05$, LDA > 2.0. The sample size was 43, 44, 43, and 44 for HLCD, non-HLCD, TRE, and non-TRE groups. Abbreviations: HLCD, healthy low-carbohydrate diet; TRE, time-restricted eating.

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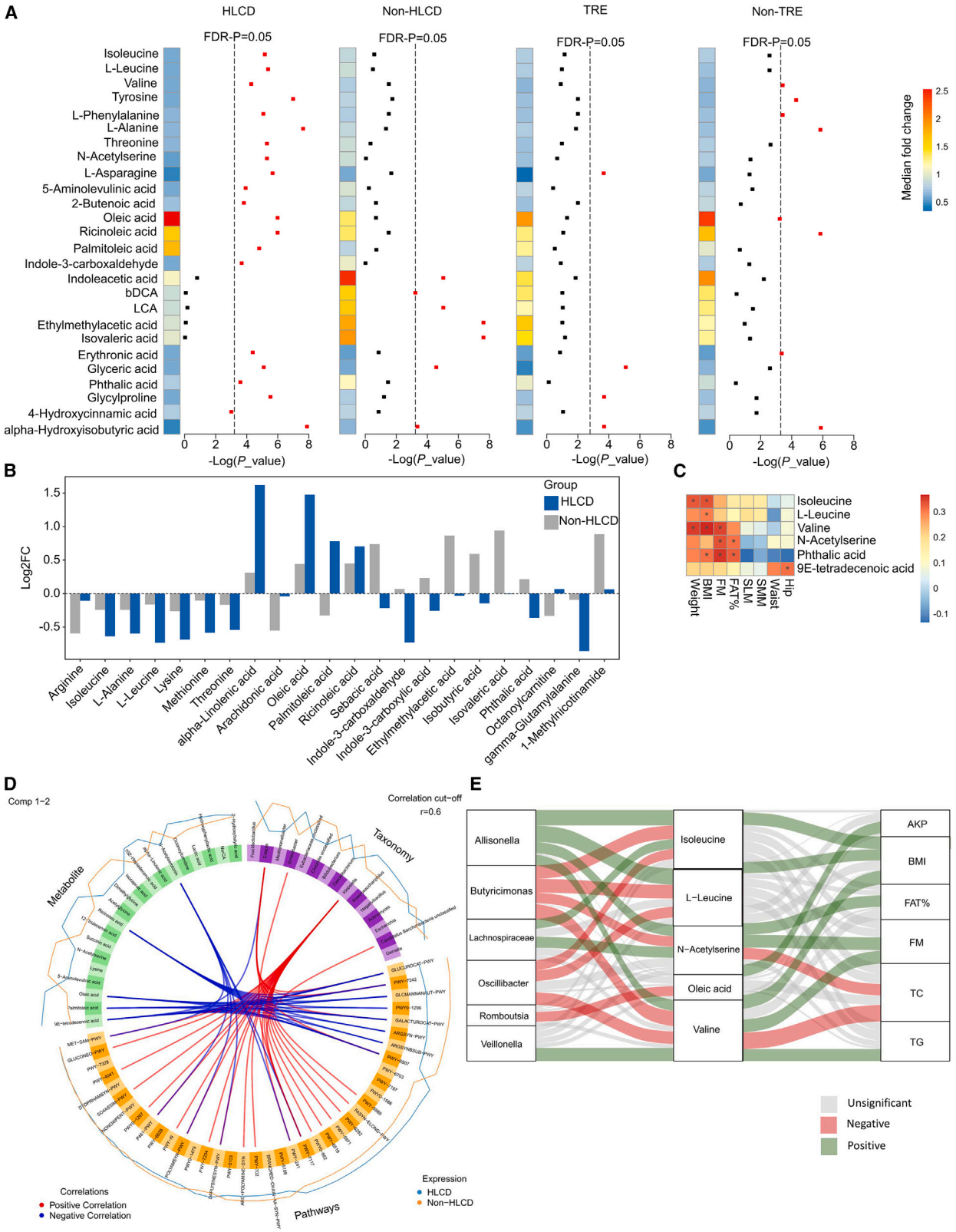

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investigation into their involvement in energy metabolism. Moreover, unsaturated fatty acids have been proven to be associated with multiple cardiometabolic benefits, including improved lipid status, insulin resistance, and inflammation.[41,42] In contrast to previous studies that found decreased linoleic acid and increased saturated fatty acids following LCD intervention,[6,43] our 12-week HLCD significantly increased the concentration of fecal unsaturated fatty acids, including oleic acid, α-linolenic acid, and palmitoleic acid than control diet intervention. The inconsistency was probably due to the emphasis on unsaturated fatty acid consumption in HLCD in the current study.

In addition to the quality and quantity of carbohydrates and other macronutrients, it is crucial to pay attention to the eating window, since consuming food over an extended period can disturb circadian rhythms, contributing to metabolic diseases.[27] In this study, participants with regular 8 work hours in Central China had an 11.9-h eating window at baseline. Considering the regular working hours of participants, 10-h TRE was used, which might better align with the eating habits of office workers and improve adherence. In adherence to previous clinical trials that provided dietary advice,[9,14,27] this 12-week TRE with isocaloric-restricted meals provided significantly reduced body weight and improved metabolic health. Furthermore, we found that 10-h TRE plus caloric restriction intervention exhibited more weight loss than caloric restriction alone in this isocaloric-restricted feeding trial, which provided further evidence on isocaloric feeding trials for the additional benefits of weight loss of TRE.[13,44] However, several clinical trials based on dietary advice among adults with obesity reported that either 12-month 8-h TRE or 14-week 10-h TRE plus caloric restriction resulted in similar levels of weight loss and metabolic improvements as compared to caloric restriction alone.[13–15] The inconsistency was probably due to the different intervention methods, among which feeding might lead to stricter isocaloric restriction and one-on-one supervision might improve adherence. Of note, we also found that TRE could produce more reduction in lean mass than caloric restriction alone, which was in line with the 12-week trials demonstrating more decrease in lean mass in the 8-h TRE group compared with eating throughout the day.[45,46] Given that lean mass loss might result in weakness and be positively correlated with weight regain,[45,47] despite the additional weight loss effect of TRE beyond caloric restriction, the recommendations for TRE might consider lean mass loss, and future research is needed.

TRE has been reported to significantly improve the diversity, composition, and function of the gut microbiome in animal studies.[48,49] Several human trials with relatively short-term intervention and small sample sizes, primarily using 16S RNA sequencing, revealed inconsistent effects of TRE on the gut microbiome.[24,50,51] Our findings are in keeping with previous studies,[24,48,51] which indicated that TRE exhibited a significant increase in the abundance of probiotic species including *E*. coli and species involved in the synthesis of short-chain fatty acids, such as butyrate- or propionic acid-producing species, compared to caloric restriction alone (before FDR adjustment). However, due to daily caloric restriction and decreased consumption of total carbohydrates, the increases in fecal short-chain fatty acids were not significant in the present study. Moreover, we found that TRE mainly affected functional profiles involving amino acid biosynthesis, carbohydrate degradation, lipid biosynthesis, and energy metabolism. Accordingly, changes in fecal metabolite caused by TRE were also mainly reflected by the decreased levels of amino acids, carbohydrates, and fatty acids, which were in agreement with previous animal studies.[52,53] Of note, α-hydroxyisobutyric acid, as produced by gut microbial amino acid metabolism from valine degradation, was significantly decreased after TRE intervention, which was consistent with the previous studies that found the positive association of α-hydroxyisobutyric acid with obesity.[54,55] In addition, TRE also enriched the fecal level of DCA and IAA, which might play key roles in maintaining the homeostasis of the gut and regulating the immune system and also potentially affect the development of obesity, metabolic syndrome, and cardiovascular diseases.[56,57]

Weight regain following initial rapid weight loss has long been a major challenge in dieting for weight management.[18] In our trial, we observed both the weight regain and rebound of cardiometabolic risk factors. It is worth mentioning that the HLCD effectively reduced body fat mass and the benefit sustained over the 28-week post-intervention follow-up, indicating potential long-term health benefits on adipose tissue distribution beyond mere weight loss.[58] Several mechanisms may explain weight regain and cardiometabolic risk rebound, including a

Figure 3. Changes in fecal metabolomics during the 12-week feeding trial

(A) Median fold changes in some representative fecal metabolite concentrations before and after the intervention in the HLCD, non-HLCD, TRE, and non-TRE groups. Paired t test or Wilcoxon signed-rank test *p* values, after adjustment for Benjamini-Hochberg false discovery rate (FDR), were indicated.

(B) Median fold changes in fecal metabolites with significant differences between the HLCD and non-HLCD groups before and after the intervention. The between-group differences were assessed by Wilcoxon test, and *p* values were adjusted using FDR method. The $P_{FDR} < 0.05$ for all metabolites is shown in the figure.

(C) Spearman correlation coefficients between changes in fecal metabolite concentrations and obesity-related parameters in the HLCD group during a 12-week feeding trial, which were calculated by Spearman's rank test. Only fecal metabolites that significantly changed at intervention and with at least one significant correlation were shown.

(D) Circos plot depicting the most significant variables from each omics dataset and their pairwise correlations post-intervention. Variables are shown on the periphery of the circos plot, with external lines indicating their measured levels across groups (HLCD and non-HLCD). In the center, red lines indicate positive correlations between linked variables, while blue lines indicate negative correlations.

(E) Spearman correlations among changes in the gut microbiome, fecal metabolites, and clinical indices in the HLCD group during the 12-week feeding trial. The correlation coefficients were calculated by Spearman's rank test. Red and green lines, respectively, refer to significant negative and positive correlations, and gray lines refer to nonsignificant correlations. The sample size was 43, 45, 43, and 45 for HLCD, non-HLCD, TRE, and non-TRE groups. Abbreviations: AKP, alkaline phosphatase; AST, aspartate aminotransferase; BMI, body mass index; FAT%, fat percentage; FC, fold change; FM, fat mass; HLCD, healthy low-carbohydrate diet; SLM, soft lean mass; SMM, skeletal muscle mass; TC, total cholesterol; TG, triglyceride; TRE, time-restricted eating.

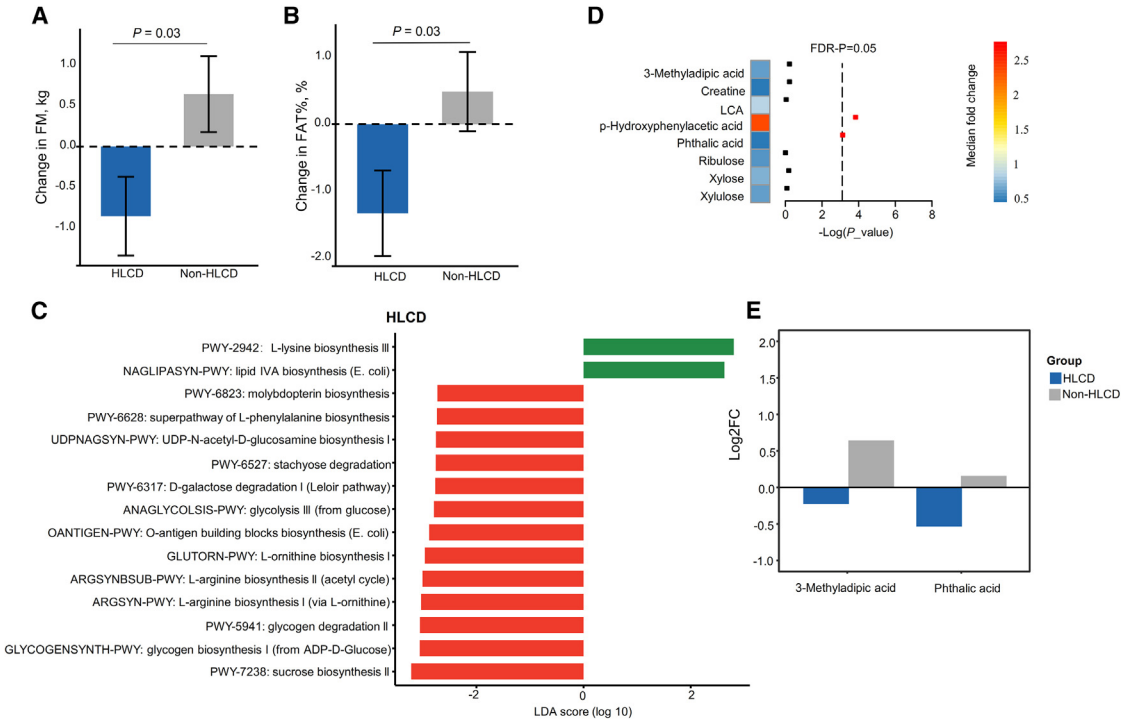

Figure 4. Changes in clinical indicators and gut microbiome from baseline to post-intervention visit

(A and B) Changes in FM and FAT% from baseline to post-intervention visit (week 40 vs. week 0). Data represent mean ± SEM. The *p* values were evaluated by the linear regression model with age, gender, and the corresponding baseline outcome value included as covariates. The sample size was 39 and 40 for HLCD and non-HLCD groups.

(C) Significant functional changes in the HLCD group from baseline to post-intervention visit using the LEfSe method; $p < 0.05$, LDA > 2.0. The top 15 loading pathway changes were shown. The sample size was 33 for the HLCD group.

(D) Median fold changes in fecal metabolite concentrations in the HLCD group from baseline to post-intervention visit. Paired t test or Wilcoxon signed-rank test *p* values, after adjustment for Benjamini-Hochberg FDR, were indicated. The sample size was 33 for HLCD group.

(E) Median fold changes in fecal metabolites with significant differences between HLCD and non-HLCD groups from baseline to post-intervention visit. The between-group differences in fecal metabolites were assessed by Wilcoxon test, and the *p* values were adjusted using FDR method. The $P_{FDR} < 0.05$ for all metabolites is shown in the figure. The sample size was 33 and 36 for HLCD and non-HLCD groups. Abbreviations: BMI, body mass index; FAT%, fat percentage; FC, fold change; FM, fat mass; HLCD, healthy low-carbohydrate diet.

growing understanding of microbial contributions to energy metabolism.[59] Participants who consumed capsules containing fecal material collected during the diet period after achieving maximal weight loss showed less weight regain, suggesting the important role of gut microbiome in weight management.[60] Our study also observed significant alterations in gut microbiome function during the 28-week post-intervention follow-up, particularly pathways related to central intestinal metabolites such as L-arginine biosynthesis. Additionally, higher levels of *p*-hydroxyphenylacetic acid were found in fecal samples from the HLCD group, associated with the growth of small intestinal bacteria and obesity remission.[61] Combined with previous studies, our findings suggest that further research is warranted on the long-term effects of diet-specific gut microbiota on weight management. Of note, the health benefits produced by the 12-week 10-h TRE intervention were not sustained at 28 weeks after the intervention ended. All relapse noted 28 weeks post-intervention suggests that long-term adherence to the HLCD or TRE as a lifestyle change may be needed to sustain meaningful health improvement, but this warrants future study.

Limitations of the study

We acknowledge several limitations in our study. Firstly, most of the participants were overweight office workers (average BMI: approximately 26.9 kg/m^2), while their metabolic parameters were relatively healthy. Therefore, the results of our study might not apply to people at high risk of cardiometabolic diseases. Secondly, the sample size might be insufficient to detect plausible interactions of interventions. Furthermore, due to the heterogeneity of the human gut microbiome, the sample size was also inadequate to detect the between-group differences in the gut microbiome and co-metabolites. Thus, randomized control trials (RCTs) with larger sample sizes are needed to detect the potential interactions, as well as the full picture of the alteration of gut microbiome and metabolites. Thirdly, the current study design focused on the effects of dietary patterns and was unlikely to address the question of which specific food groups or macronutrients play a pivotal role in the health benefits of HLCD. Fourthly, although both HLCD and control diet focused on healthy food sources, the intake of certain nutrients, such as dietary fiber, fell below recommended levels, which was likely

due to caloric restrictions. Finally, the feeding intervention phase was relatively short term, lasting only 12 weeks. Whether greater effects could occur beyond 12 weeks remains uncertain and requires further investigation in well-designed RCTs with a longer duration.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Gang Liu (liugang026@hust.edu.cn).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- The data of gut microbiome and fecal metabolome are available at Mendeley Data: https://data.mendeley.com/datasets/4w7sxpg3yy/1.
- All other data needed to evaluate the conclusions in the paper are present in the paper and/or the supplemental information.
- This paper does not report the original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

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The study was approved by the institutional review boards of the Tongji Medical College, Huazhong University of Science and Technology. All participants provided written informed consent before attendance. The complete clinical trial registration is deposited in the WHO International Clinical Trials Registry Platform and Chinese Clinical Trial Registry (www.chictr.org.cn; registration number: ChiCTR2200056363). This study does not contain reproduced material from other sources.

AUTHOR CONTRIBUTIONS

G.L. designed the study. L.L., Q.T., Ruyi Li, X.L., Y.O., T.G., and X.C. conducted the clinical studies. L.L. and Rui Li performed sample and data analyses. L.L. and Rui Li interpreted the data and wrote the first draft of the manuscript. All authors contributed to the interpretation of the results and critical revision of the manuscript for important intellectual content. All authors approved the final version of the manuscript. G.L. is the guarantor of this work and, as such, has full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

DECLARATION OF INTERESTS

The authors declare no competing interests.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xcrm.2024.101801.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Critical commercial assays		
GGT kit	Nanjing Jiancheng Bioengineering Institute	Cat#: C017-2-1
ALP kit	Nanjing Jiancheng Bioengineering Institute	Cat#: A059-2-2
AST kit	Nanjing Jiancheng Bioengineering Institute	Cat#: C010-2-1
ALT kit	Nanjing Jiancheng Bioengineering Institute	Cat#: C009-2-1
TC kit	Nanjing Jiancheng Bioengineering Institute	Cat#: A111-1-1
TG kit	Nanjing Jiancheng Bioengineering Institute	Cat#: A110-1-1
Glucose kit	Nanjing Jiancheng Bioengineering Institute	Cat#: A154-1-1
HDL-C kit	Nanjing Jiancheng Bioengineering Institute	Cat#: A112-1-1
LDL-C kit	Nanjing Jiancheng Bioengineering Institute	Cat#: A113-1-1
Cr kit	Nanjing Jiancheng Bioengineering Institute	Cat#: C011-2-1
BUN kit	Nanjing Jiancheng Bioengineering Institute	Cat#: C013-2-1
UA kit	Nanjing Jiancheng Bioengineering Institute	Cat#: C012-2-1
Deposited data		
Gut microbiome and fecal metabolome data	This paper	Mendeley Data: https://data.mendeley.com/datasets/4w7sxpg3yy/1
Software and algorithms		
SAS 9.4	SAS	https://www.sas.com/; RRID: SCR_008567
R Project	R Core Team	https://www.r-project.org/; RRID: SCR_001905
Other		
CGM	FreeStyle Libre; Abbott Diabetes Care, Alameda, CA	JM2009-022630

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Human participants

The study was approved by the institutional review boards of the Tongji Medical College, Huazhong University of Science and Technology, following the principles of the Declaration of Helsinki. All relevant ethical regulations were followed during the study. The study was performed from March 2022 to April 2023. As shown in Figure 1B, a total of 501 potential volunteers participated in the screening. After excluding 405 volunteers who did not meet the criteria, 96 healthy overweight or obese volunteers (BMI $\geq$24 kg/m^2) were included in the study. Among them, 62.5% were male. The mean (SD) age of the participants was 36.3 (7.4) years, with an average body mass index (BMI) of 26.9 (2.3) kg/m^2. All participants provided written informed consent before attendance, and the complete clinical trial registration is deposited in the WHO International Clinical Trials Registry Platform and Chinese Clinical Trial Registry (www.chictr.org.cn; registration number: ChiCTR2200056363).

METHOD DETAILS

Study design

This 12-week two-by-two factorial randomized-controlled feeding trial with a 28-week follow-up, as illustrated in Figure 1A. There were two phases in this study, including a 12-week dietary intervention period and a 28-week post-intervention follow-up period. A total of 96 participants were randomly assigned to four treatment groups (24 persons in each group): the combination of HLCD and TRE group, HLCD alone group, TRE alone group, or control group (neither HLCD nor TRE). During the 12-week dietary intervention period, the isocaloric-restricted diets on five workdays and dietary advice on the weekend were provided. During the 28-week post-intervention follow-up, no interventions were exerted.

Detailed inclusion and exclusion criteria

Participants were recruited through email and poster advertisements. The following inclusion criteria were required: (1) aged 20 to 60 years old; (2) overweight/obese (BMI $\geq$24 kg/m^2, defined by Chinese criteria[62]; 3) low or moderate physical activity; (4) written

informed consent obtained. The exclusion criteria included: (1) performed overnight shift work more than once a week; (2) regularly fasted (defined as fasting 15 h per day or having completed twelve 24-h fasts within the past year); (3) on a low-carbohydrate diet or special or prescribed diet for other reasons in the past 3 months; (4) significant changes in body weight in the past 3 months ($\geq$3 kg); (5) had severe digestive disease, gastrointestinal surgery, or impaired nutrient absorption, or history of an eating disorder; (6) currently had acute or chronic diseases (including diabetes or a significant cardiovascular, renal, cardiac, liver, lung, or nervous system disease), as well as cancer, infectious disease, or mental health illnesses; (7) took antibiotics, anti-diabetes medications, steroids, beta-blockers, adrenergic-stimulating agents, or other medications that could affect the study endpoints in the prior 3 months; (8) received any operation treatment in the prior 3 months or a plan to accept surgery in 3 months; (9) regularly performed heavy physical activity; (10) active tobacco abuse or alcohol abuse; (11) pregnancy or breast-feeding; (12) currently participated in other clinical trials. Before recruitment, screening visits were conducted for the participants who signed up.

Randomization grouping and blinding

Randomization stratified by gender was conducted by an independent statistician via the SAS program and assigned alphabetic labels corresponding to the four groups. All clinical investigators who were responsible for enrollment, measurements, or data analyses were blinded to the group allocation. The onsite staff were aware of the participant diet assignment, but they were not involved in the rest of the trial, including later measurement and result analyses. Due to the obvious difference in the meals provided and eating windows, blinding for participants was not feasible.

Dietary interventions

Over the 12-week intervention period, the participants received a weekly 5-day feeding regimen with isocaloric-restricted meals attributed to 1600 kcal/d for men and 1300 kcal/d for women (approximately 25% caloric restriction, according to the Dietary Reference Intakes of energy requirement for Chinese adults with a low level of physical activity [men: 2250 kcal/d; women: 1800 kcal/d]).[26] Trained dietitians weekly updated the 5-day cycle menus for all meals with seasonal vegetables and fruits according to the Chinese Food Composition Table.[63] All participants were requested to finish the provided meals without leaving any leftovers. Participants received dietary advice from trained dietitians and had their meals at home on the weekend.

The participants in the combination of HLCD and TRE group were provided with HLCD and instructed to follow the 10-h TRE. The HLCD was designed as a relatively healthy type of low-carbohydrate diet. It consisted of approximately 30% of total energy from carbohydrates, 50% from fats, and 20% from proteins. Moreover, compared to a traditional low-carbohydrate diet that only focused on carbohydrate restriction, HLCD also emphasized healthy food sources and high-quality macronutrients such as unsaturated fatty acids, plant proteins, and high-quality carbohydrates, including whole grains, fresh vegetables, and fruits. Additionally, 25–35 g of mixed nuts were provided along with HLCD per day, which mainly included walnuts, peanuts, cashews, pistachios, pecans, almonds, and hazelnuts. 10-h TRE required participants to consume the provided meals within 10 h each day. Outside the eating window, only water, and noncaloric beverages were allowed.[64] The participants in HLCD alone group were provided with HLCD and instructed to follow their usual eating regiments. The participants in TRE alone group were provided with control diets and instructed to follow the 10-h TRE. The control diet was the traditional Chinese diet designed according to Dietary Guidelines for Chinese Resident,[26] characterized by approximately 50%, 34%, and 16% of energy from carbohydrates, fat, and protein. The participants in the control group were provided with control diets and instructed to follow their usual eating regimens. More detailed nutrient and food composition of HLCD and control diet are presented in Table S1.

Outcomes and measurements

The primary outcomes were changes in body weight, body composition (body fat mass, percent body fat, soft lean mass, and skeletal muscle mass), and fasting glucose during the 12-week feeding trial. The secondary outcomes were changes in other cardiometabolic biomarkers (including glycemic parameters from CGM, blood pressure, lipid profiles, and liver and renal function), gut microbiome, fecal metabolome, and adverse events during the 12-week feeding trial. Other outcomes included changes in body weight, body composition, cardiometabolic biomarkers, gut microbiome, and fecal metabolome from baseline to post-intervention.

As shown in Figure 1, four clinic visits were conducted in the trial. At visit 1 (before dietary intervention), participants had a fasting blood draw and completed baseline questionnaires, the measurement of anthropometric and biochemical indexes, and urine and feces sample collection. At visit 2 (week 6), anthropometric indexes were measured for all participants. At visit 3 (week 12), all participants completed the endpoint assessment which was similar to the baseline assessment at visit 1. A total of 40 participants (10 participants in each group) were randomly selected to wear continuous glucose monitoring (CGM) for 2 weeks before the beginning and the end of the dietary intervention. At visit 4 (week 40), participants had a fasting blood draw, and completed post-intervention visit questionnaires, the measurement of anthropometric and biochemical indexes, and urine and feces samples collection. During the whole dietary intervention (week 0 - week 12), the ‘‘one-on-one supervision’’ method was used to improve adherence of participants. Every participant was assigned one dedicated staff member. This staff member was responsible for providing corresponding dietary guidance and supervision to this volunteer while offering encouragement and support. Participants were asked to record the time of starting and finishing eating and extra food consumption information by sending photos to the staff members every day. Adverse events were recorded by trained staff members, and no serious adverse events were reported throughout the study.

Blood sampling and assessments of cardiometabolic biomarkers

The overnight fasting blood samples (keep fast at least 8 h) were collected and performed by standard venipuncture. All blood samples were stored at −80°C until laboratory assays. Serum concentrations of glucose, markers of blood lipid, kidney function, and liver function were measured on an automatic analyzer (Hitachi 7600, Hitachi, Japan). All assessments were blinded to group allocation. Blood pressure was measured 3 times using a validated semiautomatic oscillometer (HEM-7000, Omron, Japan) after 5 min of rest between measurements.

Anthropometric indicators measurements

Weight, height, and body composition indicators including body fat mass, percent body fat, soft lean mass, and skeletal muscle mass were measured in light clothing without shoes by a calibrated weighing scale, automatic stadiometer, and body composition analyzer (Inbody-720, Inbody, Korea). Waist circumference was measured halfway between the last rib and the iliac crest and hip circumference at the level of the greater trochanters using an anthropometric tape.

Questionnaires

At the baseline visit, information on demographic properties, lifestyle factors, health status, and medication use was obtained in a face-to-face interview by trained staff with a standard questionnaire. At each visit, the Short Form International Physical Activity Questionnaire[65] was used to assess physical activity levels.

Continuous glucose monitors

Participants wore a continuous glucose monitor (CGM, FreeStyle Libre; Abbott Diabetes Care, Alameda, CA) for 2 weeks both before and after the intervention. The metrics from interstitial glucose CGM, including all-day average glucose, daytime and nighttime average glucose, and coefficient of variation of all-day, daytime, and nighttime glucose, were calculated using the R package "cgmanalysis".

Fecal sample collection

Fecal samples were collected using sterile containers with ice boxes and stored at −40°C within 2 h after collection by trained investigators. Most fecal samples were collected during the working days that provided meals to participants. Frozen fecal samples were transported with dry ice to the central laboratory and stored at −80°C until processing.[66] A complete set of 245 fecal samples was available from 88 participants who completed the 12-week intervention before and after the intervention, and 69 participants who completed the post-intervention visit at week 40.

Measurement of gut metagenome

The standard operating procedure for DNA library preparation and sequencing was described in the previous study.[66] A total of 245 stool samples were collected for high-quality whole-metagenomic sequencing[67] (BGI, Beijing, China), and 242 samples, including 174 samples from 87 participants before and after the intervention, and 68 samples at post-intervention visit were successfully sequenced in the current study.[67] The quality control process of whole-genome shotgun sequencing data was performed by KneadData (version 0.7.2), Trimmomatic (version 0.33), and Bowtie2 (version 2.3.4.3)[68(p2),69]. Human reads and rDNA reads were filtered by mapping the reads to the human reference genome (GRCh37) database and SILVA 128 database. After quality control, an average of 36.9 million (min: 14.8 million, max: 54.1 million) high-quality reads were obtained for each sample.

The taxonomic profiles were determined by MetaPhlan (version 3.0.3),[70] and the microbial functional profiles including MetaCyc pathways and Enzyme Commission gene families were determined by HUMAnN (version 3.0.1).[71] Microbial species with a relative abundance <0.01% in over 90% of all samples were excluded from the downstream analyses. The filtration of microbial pathways was described elsewhere.[72] In brief, the pathways were excluded with a lower median abundance (< median abundance of all identified pathways) or with a relative abundance <0.01% in over 90% of all samples, clustered the remaining pathways at the height of 0.6 using the R function "cutree", and then selected the representative pathways for each cluster. Finally, a total of 205 microbial species and 177 pathways were included in the following analyses.

Measurement of the fecal metabolome

A complete set of 245 fecal samples, including 176 samples from 88 participants before and after the intervention, and 69 samples at post-intervention visit, were included in the measurement of fecal metabolites. The targeted fecal metabolomics profiling was assessed using an ultra-performance liquid chromatography coupled with tandem mass spectrometry (UPLC-MS/MS) system (ACQUITY UPLC-Xevo TQ-S, Waters Corp., Milford, MA, USA). The Q300 Kit provided by Metabo-Profile Corp. (Shanghai, China) was used for targeted metabolomics profiling, which covers up to 310 metabolites across 12 biochemical classes and has been commonly employed in several recent studies.[73,74] Briefly, fecal samples were vortexed with ice-cold methanol containing internal standards, and the supernatant was collected. The sample was then diluted with ice-cold 50% methanol solution and subjected to centrifugation at 4000 × g. The supernatant, mixed with internal standards, was sealed prior to UPLC-MS/MS analysis. The analytical parameters were set as follows: C18 analytical column (2.1 × 100 mm, 1.7 μm); column temperature at 40°C; mobile phase A (water with 0.1% formic acid) and mobile phase B (acetonitrile: isopropanol, 90:10). The entire profiling process was conducted

at Metabo-Profile Corp. (Shanghai, China). Samples were analyzed randomly by generating random numbers to reduce the error introduced by the instrument. Quality control (QC) samples, prepared from pooled samples, were analyzed every 14 samples across the entire sample set to assess instrument stability and consistency in sample processing. The raw data from UPLC-MS/MS were processed using QuanMET software (v2.0, Metabo-Profile, Shanghai, China) for peak integration, calibration, and quantification of each metabolite. Metabolomic features were annotated with MSI level 1 confidence by comparing them to targeted metabolite standards. The detailed information of this method was published elsewhere.[75]

A total of 222 fecal cometabolites were quantified in the targeted metabolomics measurements. After excluding metabolites with missing proportions exceeding 30% or the coefficient of variations >30% in all samples, a total of 217 fecal microbial-host cometabolites were quantified and included in data analysis. The missing values of metabolites included in the analysis were imputed using 1/10 of the minimum detectable value.

Compliance assessment

For the HLCD intervention arm, the compliance was evaluated by dividing the number of consumed meals by the number of provided meals. For the TRE intervention arm, the eating window (eating window = end eating time – start eating time) was used to assess compliance.

QUANTIFICATION AND STATISTICAL ANALYSIS

Sample size and statistical analysis

The trial was designed to provide greater than 90% power at the 0.05 level for each of 5 prespecified comparisons (HLCD alone compared with control, TRE alone compared with control, the combination of HLCD and TRE compared with control, the combination compared with TRE alone, and the combination compared with HLCD alone). We calculated a sample size of 80 subjects with 20/group to detect the effect of dietary interventions on weight loss and improvement of fasting glucose. The estimated minimum clinically important differences and SD were 2.5 kg and 2.4 kg for weight loss, and 0.69 mmol/L and 0.23 mmol/L for fasting glucose.[27,76–78] Considering a 20% dropout, a sample size of 100 was sufficient to assess the effect of dietary interventions throughout the study period. However, due to the relatively favorable health status of the population at the research implementation site, a total of 96 participants meeting the inclusion and exclusion criteria were ultimately included in this study. This sample size was deemed relatively sufficient for the study's objectives, allowing for a 15% dropout rate.

Analyses were based on the intention-to-treat principle and the analysis dataset included all randomized patients with their available data. Baseline characteristics were presented as numbers (percentage) for categorical variables and means ± SDs or medians (interquartile range) for continuous variables with normal distribution or skewed distribution, respectively. Baseline characteristics between groups were compared using T-test or $\chi 2$ tests when appropriate. The within-group differences were tested using the Paired t-test or Wilcoxon signed-rank test. For between-group differences, the changes in continuous variables, which were calculated by subtracting the baseline values from the values at 6 or 12 weeks were used as outcome variables for subsequent analysis. The factorial design allowed the investigator to study the effect of each intervention, as well as the effects of interactions between interventions. For the changes in blood pressure and blood metabolic indicators, the between-group differences were evaluated by the linear regression model with age, gender, and the corresponding baseline outcome value included as covariates. The assumption of no effect modification between two interventions was examined by including indicators of interventions and the interaction term between interventions in the regression model. If the interaction term was significant ($P_{interaction} < 0.05$), the between-group differences among 4 treatment groups were tested. If not significant, the main effects of the two interventions were tested next. For the repeated-measured continuous outcomes such as anthropometric indicators, the linear mixed-effects models with unstructured variance structure were applied with changes from baseline as the dependent variable. The models included indicators for two interventions and time, the interaction term between two interventions, and interaction terms between interventions and time as independent variables with the same covariates as above. The interaction terms between interventions and time were tested. If significant ($p < 0.05$), between-group differences at each time point were tested. If not significant, the changes across 12 weeks were allowed to represent the consistent averaging effect of the intervention across the trial, which conceptually captured the average changes from baseline to follow-up time points: ([changes at week6+changes at week12]/2). Simultaneously, the assumption of no effect modification between two interventions was tested using the same method mentioned above. In addition, although the interactions between the two dietary interventions were not statistically significant, the comparisons between the four treatment groups might be also important, which might provide some exploratory findings. Therefore, the exploration analysis of the comparison between 4 groups was conducted. Subgroup analyses were conducted according to gender and BMI. The missing data were imputed by multiple imputations ($n = 5$) with the use of the Markov chain Monte Carlo method.

The alpha-diversity of the gut microbiome was assessed through the calculation of the Shannon index, utilizing species-level relative abundance data. The beta-diversity between gut microbiome taxonomic (species-level) and functional profiles among samples was determined using Bray-Curtis distance. The results were then visualized through principal coordinate analysis (PCoA). The differences in microbial taxonomic (species-level) and functional profiles during intervention and follow-up were calculated using permutational multivariate analysis of variance (PERMANOVA) with a permutation of 9999 times via the "vegan" R package. We

used LEfSe to identify microbial species and functional pathways that differed between timepoints during the intervention and follow-up. LEfSe determines significance using a Wilcoxon test statistic ($p < 0.05$) and then assesses the difference between groups (log fold change [FC] > 2).[79] The comparisons of microbial species and functional pathways changed between groups were estimated by linear regression adjusting for BMI change. The assumptions of homoscedasticity and normality were examined and determined to be unviolated before the analysis.

For the fecal metabolome, to examine the overall distribution of fecal metabolites before and after intervention in each group, the principal component analysis was performed with all fecal metabolites included. The Euclidean dissimilarity matrix was calculated, and the classical metric multidimensional scaling was calculated to obtain different PCs. The Wilcoxon signed-rank test and Wilcoxon test were used to check differences of PCs including PC1 and PC2 between different times within groups and between different dietary intervention groups, respectively. In the univariate analysis, since the fecal metabolites were measured at baseline and end of the intervention during the 12-week intervention, the paired t-test or Wilcoxon signed-rank test for variables with normal distribution or skewed distribution was used to examine the significant differences in the levels of fecal metabolites before and after the intervention in each group. For the significantly changed fecal metabolites in each group, the Wilcoxon test was further performed to compare the changes in values between the dietary groups. Correlations between variable changes from baseline to end of the intervention (week 12) in fecal metabolites, genus relative abundance, and clinical indexes were calculated by Spearman's rank test.

In addition, the integration of the different Omic datasets was performed with the mixOmics script using the Data Integration Analysis for Biomarker discovery using Latent Components (DIABLO) framework using R (v. 4.4.0). This is a multi-omics integrative method that seeks common information across different data types through the selection of a subset of molecular features while discriminating between multiple phenotypes. For this integration we used the 87 samples with taxonomy (species and genus levels), pathways, and metabolites data. The relative abundance of microbial species and functional pathways was transformed using the arc-sin square root transformation, the fecal levels of metabolite were transformed using inverse normal transformation. The circosPlot was built based on a similarity matrix. A cutoff of 0.6 was included to visualize correlation coefficients above this threshold in the multi-omics signature.

For clinical data, the statistical analyses were performed using SAS version 9.4 (SAS Institute Inc). For metabolomics and metagenomics data, the analyses were performed in R version 4.1.0 unless otherwise stated. The main R packages used were 'ape' and 'vegan' for PCoA and PERMANOVA analyses. A two-sided p value < 0.05 was considered statistically significant for the generalized linear mixed models or general linear models. For concern on multiple testing, the p values were adjusted using the Benjamini-Hochberg false discovery rate (P_{FDR}), and a $P_{FDR} < 0.05$ was considered statistically significant for paired t-test or Wilcoxon signed-rank or Wilcoxon test in the univariate analysis of individual microbial species, functional pathways, and fecal metabolites.