The association of fried meat consumption with the gut microbiota and fecal metabolites and its impact on glucose homoeostasis, intestinal endotoxin levels and systemic inflammation: A randomized controlled-feeding trial

Running Title: Fried meat and glucose homoeostasis

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Supplemental Text

Mechanism for random allocation sequence and concealment

Before this feeding-trial, a professional statistician generated the random sequence number, and determined the numbers for the intervention and control group by orders. Then, a researcher sealed each number in a non-transparent envelope, and gave these envelopes to the other researcher who was in charge of recruitment. This researcher gave each participant an envelope if they met the inclusion criteria, and recorded the number in the envelope for the participants. This procedure was respectively conducted in men and women.

Sample collection

On every sampling day, blood samples were collected by venepuncture after a 12-hour fast. Plasma and serum were centrifuged from the blood samples and immediately stored at −80°C for further analysis. For the collection of fecal samples, the sampling kits were provided to the participants, which included sterile sampling rods, sterile cryotubes, disposable gloves and masks. Then, the researchers taught the participants how to collect stools using the sampling kits. On the night before the sampling day, each participant was provided a thermos flask filled with dry ice to ensure that the participants could immediately put the sterile cryotube below -20°C for storage on the early morning of the sampling day. Most of the participants provided their stools on the morning of sampling day. However, a few participants did not have a bowel movement on the morning of the sampling day, we allowed these participants to provide a stool sample the next morning.

Fecal DNA Extraction and 16S Gene amplicon Sequencing

Total bacterial DNA was extracted using Cetyltriethylammonium bromide (CTAB) and Sodium dodecyl sulfonate (SDS) methods. The integrity, concentration, and purity of DNA were assessed using the 1% agarose gel electrophoresis and NanoDrop 2000 UV-vis spectrophotometer (Thermo Scientific, Wilmington, USA). The extracted DNA was then stored at -20 °C.

The 16S rRNA gene amplification procedure was divided into two PCR steps, in the first PCR reaction, the V3-V4 hypervariable region of the 16S rRNA gene was amplified from genomic DNA using primers 341F(CCTACGGGNGGCWGCAG) and 805R(GACTACHVGGGTATCTAATCC). Amplification was performed in 96-well microtiter plates with a reaction mixture consisting of 15 μL of Phusion® High-Fidelity PCR Master Mix (New England Biolabs), 0.2 μM of forward and reverse primers, and about 10 ng template DNA. Reactions were run according to the following cycling program: initial denaturation at 98°C for 1 min, followed by 30 cycles of denaturation at 98°C for 10 s, annealing at 50°C for 30 s, elongation at 72°C for 30 s, with a final extension at 72°C for 5 min. Subsequently, the amplified products were separated and identified by 2% agarose gel electrophoresis and ethidium bromide staining. The amplification products were purified with Qiagen Gel Extraction Kit (Qiagen, Germany) according to the manufacturer's instructions. Equimolar amounts of the amplification products were pooled together in a single tube. Sequencing libraries were generated using TruSeq® DNA PCR-Free Sample Preparation Kit (Illumina, USA) following the manufacturer's recommendations and index codes were added. The concentration of the pooled libraries was determined by the Qubit@ 2.0 Fluorometer (Thermo Scientific) and Agilent Bioanalyzer 2100

system. Amplicon sequencing was performed on the Illumina NovaSeq platform (Illumina Inc., CA, USA). Automated cluster generation and 2×250 bp paired-end sequencing with dual-index reads were performed.

16S rRNA gene sequence data processing

Fastq-files were demultiplexed by the NovaSeq6000. The sequence was trimmed for amplification primers, diversity spacers, and sequencing adapters, merge-paired and quality filtered by using Quantitative Insights Into Microbial Ecology (Qiime) software (Version 1.9.1). Uparse (Uparse v7.0.1001) was used for OTU clustering equaling or above 97%. Taxonomy of the OTUs was assigned and sequences were aligned with the RDP classifier. The OTUs were analyzed by species annotation using the Mothur method and SSUrRNA database of SILVA132 (set threshold value 0.8~1). Finally, OTUs abundance information was normalized using a standard of sequence number corresponding to the sample with the least sequences. α-diversity (Observed-OTUs, Shannon index, Simpson index, Chao1 index, Goods-coverage index， PD_whole_tree index) and β-diversity (Unweight UniFrac distances and Weight UniFrac distances) measures were calculated based on the rarefied OTU count using Qiime software (Version 1.9.1). The microbiota-predicted pathways were identified by using Tax4Fun prediction to annotate the function of the microbiota based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database.

Measurement of gut microbial-Host cometabolites

All the standards were obtained from Sigma-Aldrich (St. Louis, MO, USA), Steraloids Inc. (Newport, RI, USA) and TRC Chemicals (Toronto, ON, Canada). All the standards were accurately weighed and prepared in water, methanol, sodium

hydroxide solution, or hydrochloric acid solution to obtain the individual stock solution at a concentration of 5.0 mg/mL. The appropriate amount of each stock solution was mixed to create stock calibration solutions. Feces samples were thawed on ice-bath to diminish degradation. About 10mg of each sample was weighted and transferred to a new 1.5mL tube. Then 25μL of water was added and the sample was homogenated with zirconium oxide beads for 3 minutes. 185μL of ACN/Methanol (8/2) was added to extract the metabolites. The sample was centrifuged at 18000g for 20 minutes. Then the supernatant was transferred to a 96-well plate. The following procedures were performed on a Biomek 4000 workstation (Biomek 4000, Beckman Coulter, Inc., Brea, California, USA). 20μL of freshly prepared derivative reagents was added to each well. The plate was sealed and the derivatization was carried out at 30°C for 60 min. After derivatization, 350μL of ice-cold and 50% methanol solution was added to dilute the sample. Then the plate was stored at -20°C for 20 minutes and followed by 4000g centrifugation at 4 °C for 30 minutes. 135μL of supernatant was transferred to a new 96-well plate with 15μL internal standards in each well. Serial dilutions of derivatized stock standards were added to the left wells. Finally, the plate was sealed for analysis.

Ultra-performance liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS) system (ACQUITY UPLC-Xevo TQ-S, Waters Corp., Milford, MA, USA) was used to quantitate the microbial metabolite. The extraction solvents are stored in a -20°C freezer overnight and added to the samples immediately after the samples were thawed. The ice-salt bath was used to keep the samples at a low temperature and minimize sample degradation during sample preparation. All the

prepared samples should be analyzed within 48 hours after sample extraction and derivatization. Three types of quality control samples i.e., test mixtures, internal standards, and pooled biological samples were routinely used in the metabolomics platform.

The raw data files generated by UPLC-MS/MS were processed using the QuanMET software (v2.0, Metabo-Profile, Shanghai, China) to perform peak integration, calibration, and quantitation for each metabolite. Mass spectrometry-based quantitative metabolomics refers to the determination of the concentration of a substance in an unknown sample by comparing the unknown to a set of standard samples of known concentration (i.e., calibration curve).

Sub-group analysis

To ensure compliance with this trial, participants who habitually consumed fried food more than 1 time per week were recruited. The average frequency of fried food intake among the recruited participants was 3.28±2.37 times per week, which was relatively high. It has the possibility that the health effect of the intervention may be due to the effect of stopping a high amount of fried food intake in the control group rather than fried food intake in the intervention group when we analyzed the total sample. Therefore, a sub-group analysis was performed. We identified the participants who habitually consumed fried food 1 time per week before they participated in the trial because previous observational studies frequently selected the participants who consumed fried food 1 time per week as the reference group (6-8). Therefore, the consumption of fried food 1 time per week could be regarded as a relatively low frequency of fried food. The differences for the main and secondary outcomes

between the two groups in this sub-sample were analyzed.

Gastric gavage of cometabolites in mice

Specific pathogenfree(SPF) male C57BL mice (n=72/6 mice per individual cages) were given a high fat diet (HFD) and selected metabolites through gastric gavage every two days for 8 weeks. Mice were arranged to be free access to distilled water and a chow diet for a one-week adaptation. After that, mice were randomly assigned into 6 groups as initial weight, and then fed a chow diet with phosphate buffer saline (PBS) (Control+PBS, AIN-93M Purified Diet), a high-fat diet with PBS (HFD+PBS, AIN-93M, purified diet composing 45% fat energy-supply ratio with PBS), a high-fat diet with carnitine (HFD+carnitine, AIN-93M, purified diet composing 45% fat energy-supply ratio with carnitine), a high-fat diet with valeric acid (HFD+valeric acid, AIN-93M, purified diet composing 45% fat energy-supply ratio with valeric acid), a high-fat diet with 3-indolepropionic acid (IPA) (HFD+IPA, AIN-93M, purified diet composing 45% fat energy-supply ratio with IPA), a high-fat diet with methylglutaric acid (MGA) (HFD+MGA, AIN-93M, purified diet composing 45% fat energy-supply ratio with MGA), respectively.

The composition of the chow diet and high-fat diet is shown as the following table:

Component (g)	Control	High fat
Casein	14	14
L-cystine	0.18	0.18
Cornstarch	46.57	26.07
Dextrinized cornstarch	15.5	15.5
Sucrose	10	10
Soybean oil	5	5
Lard	4	4
Mixed minerals	0.0	20.5
Mixed vitamins	3.5	3.5

Cellulose	1	1
Choline	0.25	0.25
Energy from macronutrients (kcal%)		
Carbohydrate	14.1	10.3
Protein	76.9	44.9
Fat	9.0	44.8

The HFD-cometabolites were given a small dose of cometabolites through gavage (300mg/kg for carnitine; 200mg/kg for valeric acids; 30mg/kg for IPA; 5mg/kg for MGA) every 2 days, and the other groups were PBS (5 mg/kg) based on previous studies (1-4). Dietary food intakes and the body weights of mice were routinely monitored. An OGTT test was conducted. Mice fasted for 6 hours with water provided ad libitum from 7:00 am on the experimental day. At the same time, the animal was transported from the rodent housing facility to the laboratory for acclimating to the laboratory environment. Blood was sampled from the tail vein at 0, 15, 30, 60, and 120 minutes after oral gavage of glucose (2 mg/g body weight). Blood glucose concentrations (mmol/L) were measured serially using a glucometer (Accu-Chek, Roche, Indianapolis, IN) (5). One day after the completion of the OGTT test, the mice fasted for 8 hours to blood collection. Mice were anesthetized with chloral hydrate. And serum was divided into several tubes for further measurement after being centrifuged at 3000r/15min, 4 ℃. Mouse serum insulin was measured using an ELISA kit (Cusabio, College Park, MD, USA). The study design and methods were approved by the ethics committee of Harbin Medical University.

The effect of these identified metabolites on glucose homoeostasis in mice is presented in **Supplementary Figure 9**. The HFD+carnitine intervention increased weight gain and glucose responses whereas HFD+valeric acid or IPA decreased these

measures relative to the HFD+PBS (all $P<0.05$). The fasting insulin level was also significantly lower in the HFD+valeric acid or HFD+IPA group than in the HFD+PBS.

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Supplementary Table 1. The recipes during the intervention

Day Meal	Breakfast	Lunch		Dinner	Snack
		Control Group	Fried Group		
Monday	Chicken and Cabbage Buns (250g) Egg (65g) Purple Sweet Potato (100g) Rice Gruel (400g)	Boiled Tilapias (150g) Szechuan Style Shredded Potato (100g) Steamed Eggplant with Minced Garlic (150g) Sweet Potato (100g) Rice (150g)	Fried Tilapias (150g)	Poached Chicken Leg Meat (150g) Steamed Lettuce (150g) Dried Tofu Strips Salad (100g) Pumpkin (100g) Rice (150g)	Pear (400g) Apricot Kernel (20g)
Tuesday	Turnip and Carrot Buns (250g) Egg (65g) Sweet Potato (100g) Corn Flour Gruel (400g)	Steamed Spanish Mackerel (150g) Corn Salad (150g) Poached Young Chinese Cabbage (150g) Mashed Potato (100g) Rice (150g)		Sliced Cold Chicken (150g) Coleslaw (150g) Braised Frozen Tofu (100g) Sweet Potato (100g) Rice (150g)	Apple (400g) Filbert (20g)
Wednesday	Chicken and Cabbage Buns (250g) Egg (65g) Purple Sweet Potato (100g) Millet Gruel (400g)	Boiled Salmon (150g) Cold Eggplant Side Dish (150g) Stew Potatoes with Beans (150g) Mashed Potato (100g) Rice (150g)	Fried Salmon (150g)	Poached Chicken Breast (150g) Boiled Chinese Baby Cabbage (150g) Dried Tofu Strips Salad (100g) Pumpkin (100g) Rice (150g)	Banana (400g) Cashew (20g)
Thursday	Bean Bundle (250g) Egg (65g) Sweet Potato (100g) Corn Flour Gruel (400g)	Shredded Chicken with Soy Sauce (150g) Boiled Leaf Lettuce (150g) Stewed Tofu with Cabbage (150g) Yam Mud (100g) Rice (150g)		Steamed Cod (150g) Poached Young Chinese Cabbage (150g) Shredded Potatoes with Salad (100g) Pumpkin (100g) Rice (150g)	Watermelon (400g) Peanut (20g)

Friday	Turnip and Carrot Buns (250g) Egg (65g) Sweet Potato (100g) Seaweed and Egg Soup (400g)	Poached Chicken Leg (150g)	Fried Chicken Leg (150g)	Steamed Hairtail (150g) Cold Eggplant Side Dish (150g) Stir-fried Spinach (150g) Yam Mud (100g) Rice (150g)	Dragon Fruit (400g) Kernel of Walnut (20g)
		Okra steamed egg (150g) Poached Chinese Cabbage (150g) Pumpkin (100g) Rice (150g)			
Saturday	Zucchini and Egg Buns (250g) Egg (65g) Boiled Corn (100g) Millet Gruel (400g)	Steamed Cod (150g) Steamed Eggplant with Minced Garlic (150g) Boiled Colza (150g) Purple Sweet Potato (100g) Rice (150g)		Soy Sauce Chicken (150g) Stewed Tofu with Vermicelli (100g) Steamed Lettuce (150g) Mashed Potato (100g) Rice (150g)	Honey-dew Melon (400g) Pistachio (20g)
Sunday	Tomato and Egg Soup (400g) Egg (65g) Sweet Potato (100g) Rice Gruel (400g)	Steamed Chicken with Chili Sauce (150g)	Fried Chicken with Chili Sauce (150g)	Sliced Cold Chicken (150g) Braised Green Beans with Potatoes (100g) Boiled Chinese Baby Cabbage (150g) Sweet Potato (100g) Rice (150g)	Plum (400g) Chinese Chestnut (20g)
		Poached Cabbage (150g) Mixed Dried Bean Curd Sticks (150g) Yam Mud (100g) Rice (150g)			

Supplementary Table 2. Intakes of food components and nutrients during the intervention between the two groups

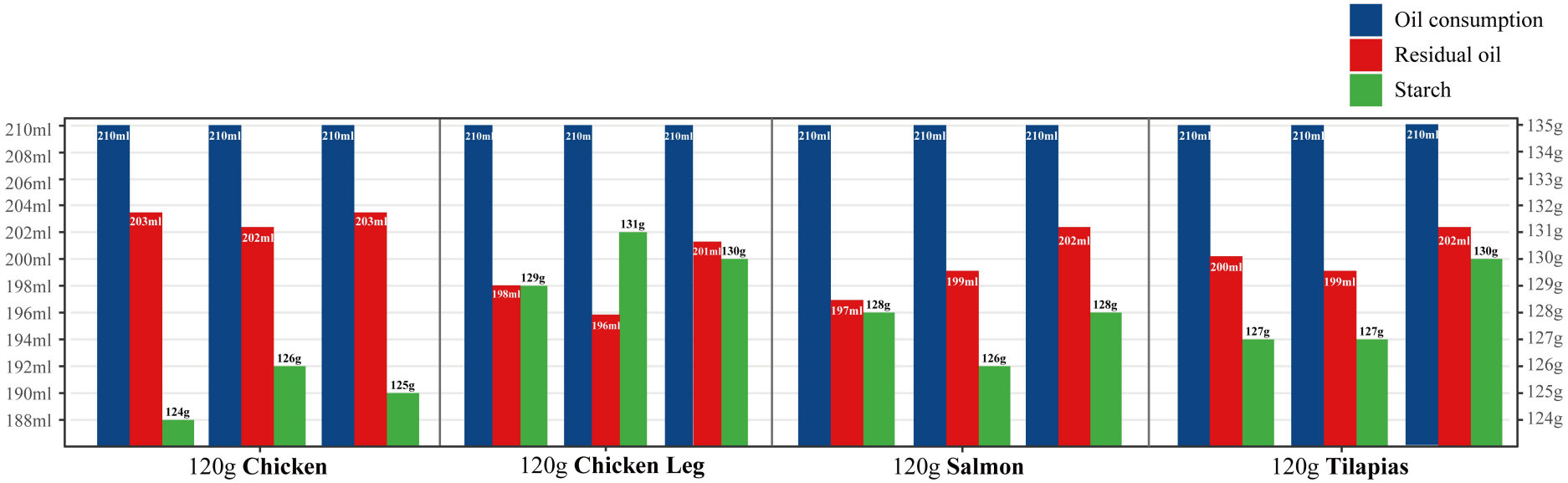
Dietary intakes	Control Group (N=58)	Intervention Group (N=59)	*P* value
Energy, kcal/day	2151.60 (163.62)	2162.23 (165.15)	0.715
AHEI-2010 score	86.02 (1.56)	86.69 (1.44)	0.193
***Food Components**, g*			
Total vegetable	481.62 (5.46)	485.79 (4.96)	0.231
Dark-green vegetable	259.59 (3.48)	257.82 (3.05)	0.982
Red and orange vegetables	52.16 (1.84)	54.96 (2.05)	0.269
Starchy	86.45 (2.84)	83.92 (3.15)	0.153
Beans and peas	51.64 (0.96)	49.06 (1.38)	0.351
Total fruit	346.72 (6.96)	348.28 (5.16)	0.288
Poultry	142.63 (4.68)	146.20 (7.62)	0.870
Fish	145.02 (6.18)	149.52 (4.82)	0.489
Eggs	62.80 (1.48)	61.67 (1.08)	0.203
Soy product	47.61 (4.62)	45.82 (5.82)	0.942
Nuts and seeds	24.59 (0.36)	25.49 (0.72)	0.108
Total grain	237.08 (10.84)	240.61 (11.46)	0.403
Whole grain	104.43 (6.49)	107.09 (5.41)	0.769
Oil	22.36 (0.75)	23.82 (1.07)	0.282
Salt	5.26 (0.24)	5.47 (0.46)	0.149
Red meats and organ	0	0	-
Added sugars	0	0	-
Alcoholic	0	0	-
Nutrients			
Total fat, %TE	21.57 (0.02)	21.96 (0.02)	0.167
Carbohydrate, %TE	64.72 (0.16)	64.16 (0.11)	0.543
Protein, %TE	14.21 (0.12)	13.88 (0.09)	0.152
Dietary fiber, g	19.79 (3.16)	19.53 (3.01)	0.822
SFA, g	10.44 (1.07)	10.39 (1.31)	0.493
MUFA, g	17.23 (2.24)	17.86 (2.19)	0.732
PUFA, g	23.99 (1.97)	24.50 (2.34)	0.523
Vitamin A (RAE), μg	1174.94 (29.01)	1085.10 (27.94)	0.206
Vitamin B1, mg	1.03 (0.29)	1.01 (0.33)	0.882
Vitamin B2, mg	1.06 (0.19)	1.04 (0.24)	0.395
Vitamin C, mg	138.16 (13.50)	139.17 (11.35)	0.932
Vitamin D, μg	4.41 (0.63)	4.29 (0.59)	0.226
Vitamin E, mg	22.99 (3.56)	24.26 (3.39)	0.329
Calcium, mg	746.00 (13.22)	758.96 (11.02)	0.727
Iron, mg	21.19 (4.09)	22.06 (4.48)	0.249
Zinc, mg	10.29 (0.98)	11.11 (1.32)	0.615
Sodium, mg	1906.23 (45.12)	2035.70 (32.41)	0.192

Potassium, mg	3111.27 (29.13)	3029.04 (30.67)	0.246
Selenium, μg	67.59 (2.24)	64.23 (3.29)	0.190

Intakes are presented as mean (standard derivation).

AHEI, Alternative health eating index; %TE, percentage of energy provided by macronutrients; SFA, Saturated fatty acids; MUFA, Monounsaturated fatty acids; PUFA, Polyunsaturated fatty acids; RAE, Retinol activity equivalents.

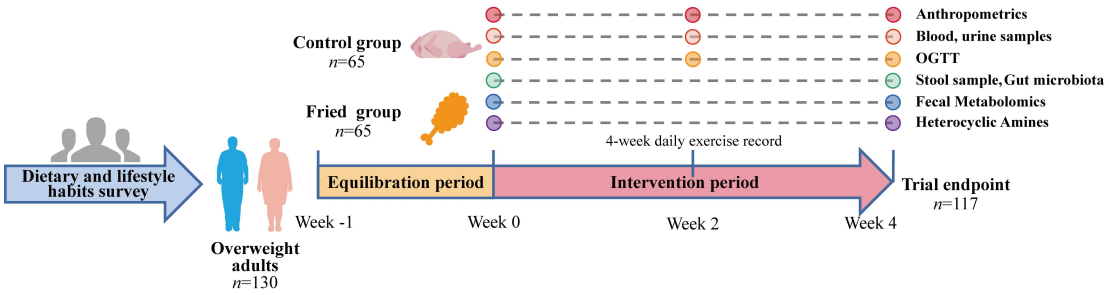
Supplementary Figure 1. The amount of oil and starch per gram of meat required for frying



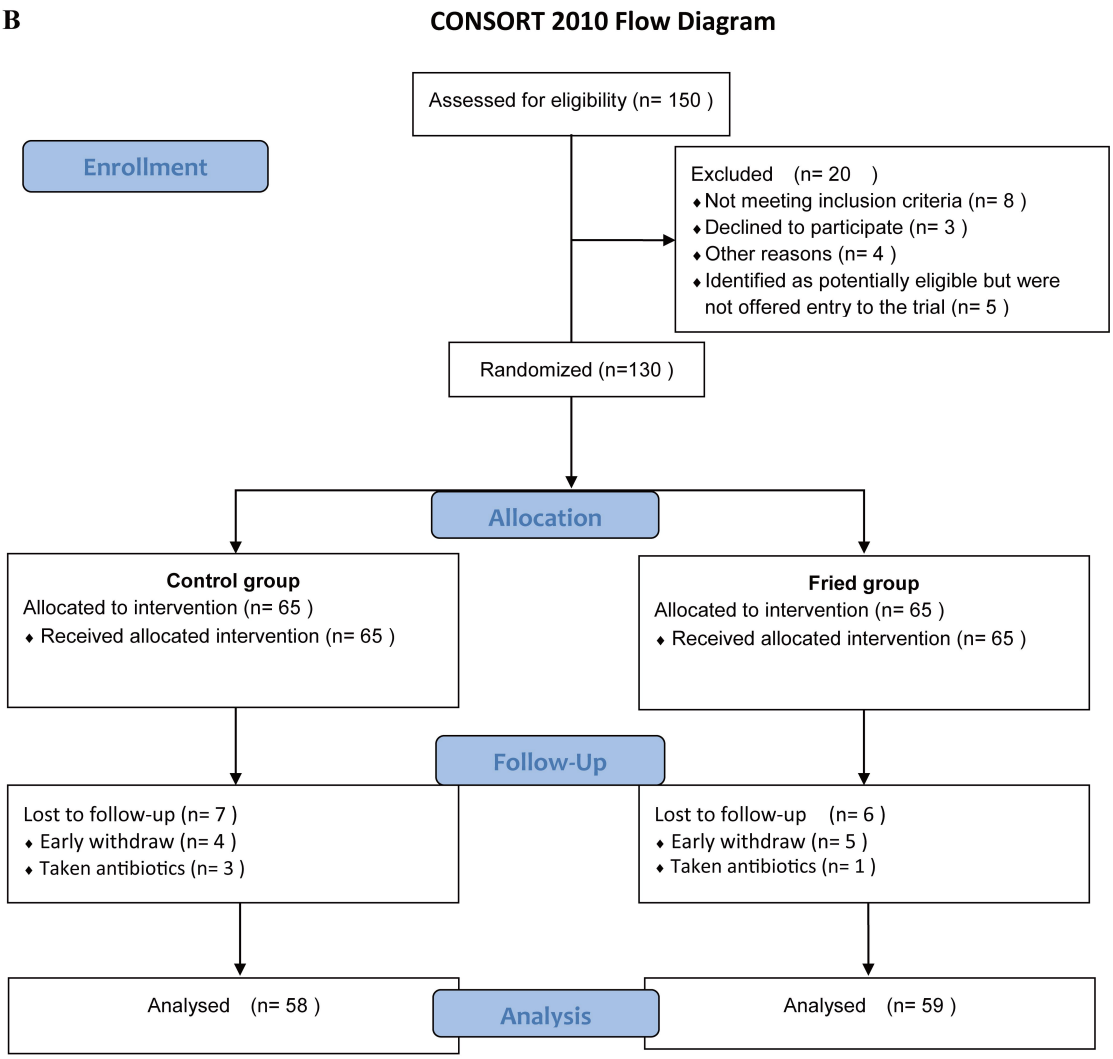
In this pre-experiment, we recorded three times in terms of the amount of oil used for frying and the residual amount of oil after frying, then we calculated the amount of oil that the meat absorbed during frying. We also weighted the meat before and after wrapping the starchy. We therefore calculated the amount of oil and starch per gram of meat required for frying to ensure isocaloric feeding between two groups.

Supplementary Figure 2. The Strategies for samples collection (A) and flow diagram of this study (B)

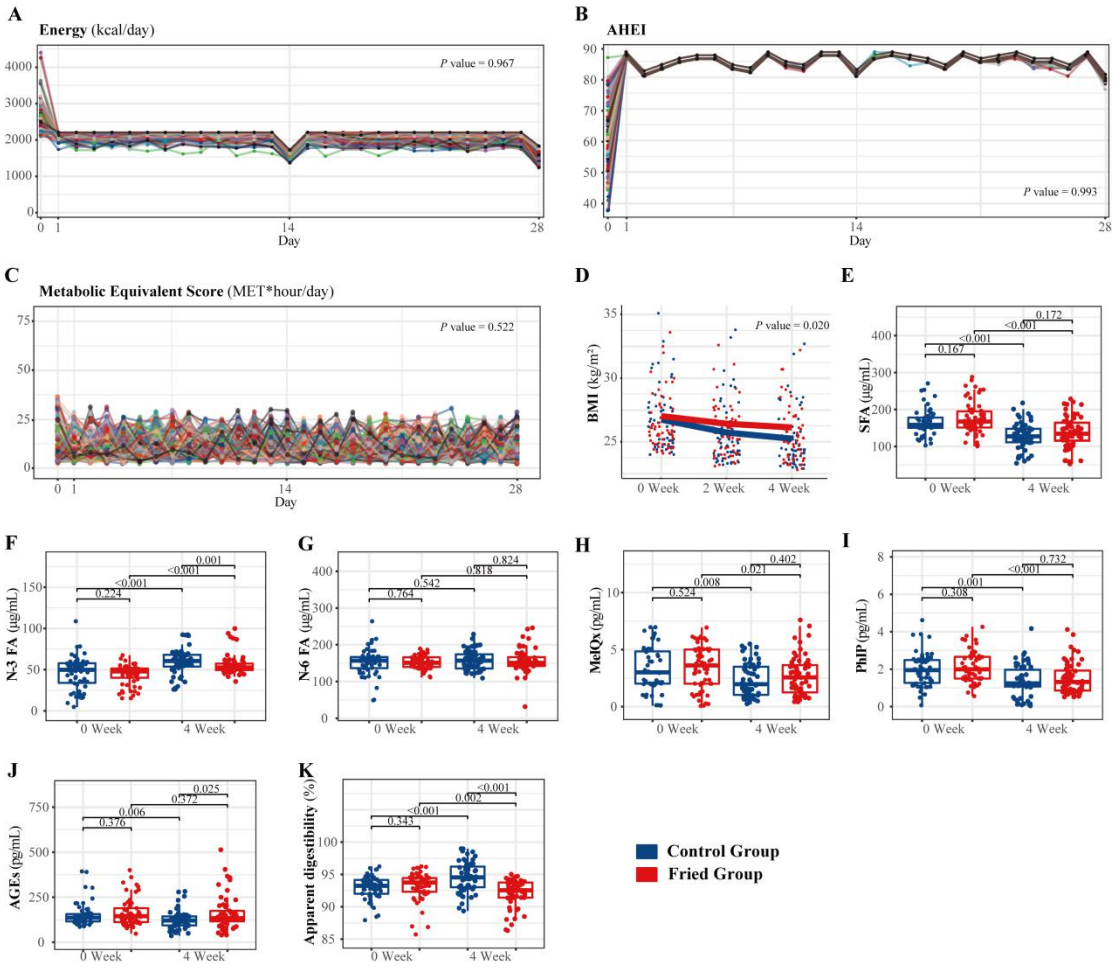
A



B



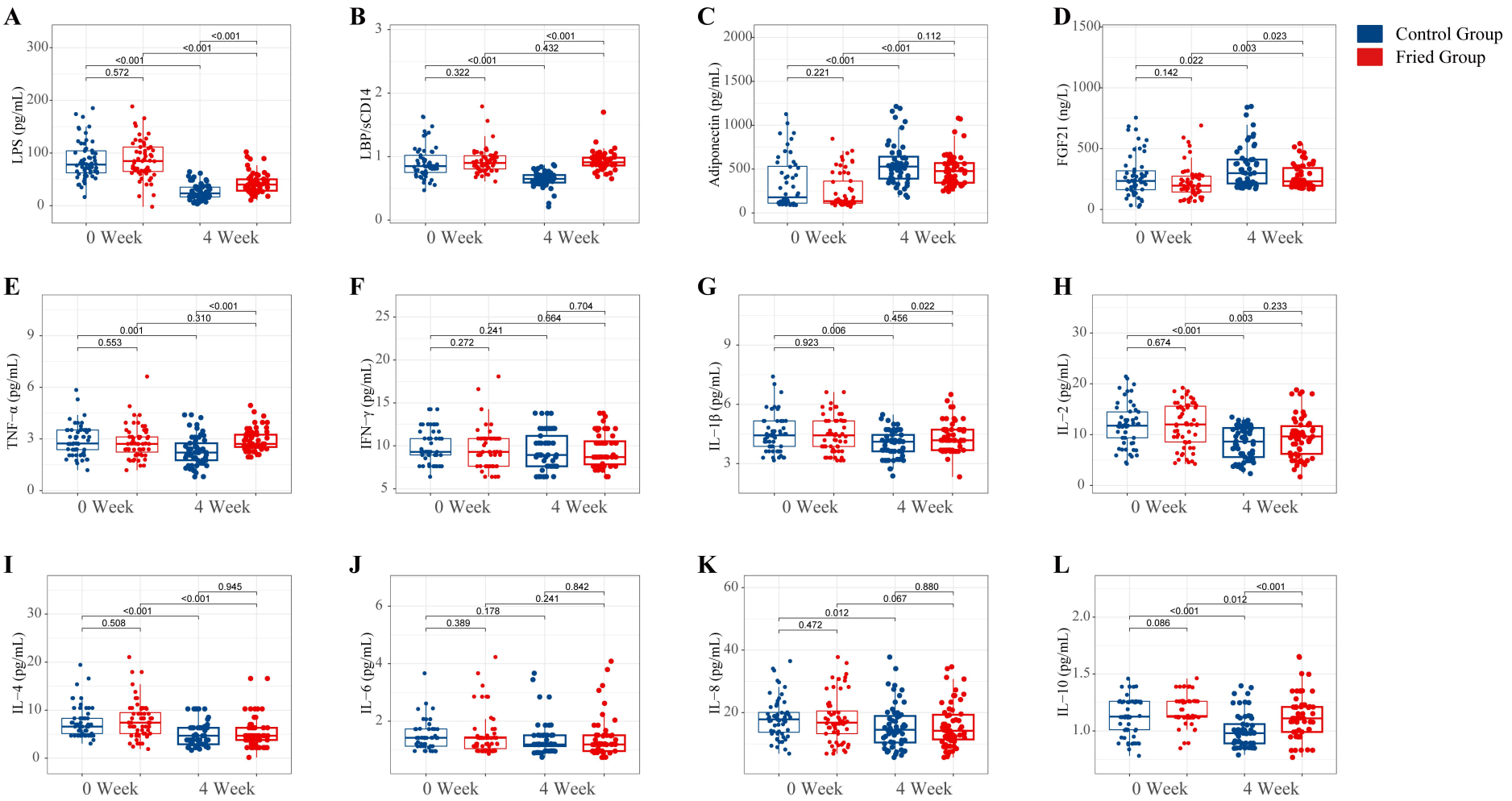
Supplementary Figure 3. Compliance assessment of this randomized controlled-feeding trial.



AHEI, Alternate Healthy Eating Index 2010; MET, metabolic equivalent score; BMI, body mass index; SFA, saturated fatty acids; n-3 FA, n-3 fatty acids; n-6 FA, n-6 fatty acids; MeIQx, 2-Amino-3,8-dimethylimidazo[4,5-f] quinoxaline; PhIP, 2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine; AGEs, advanced glycation end products.

The *P* values in (A), (B), and (C) referred to the differences evaluated by repeated measured general linear models.The *P* values in (D) referred to the differences evaluated by generalized linear mixed models. The *P* values in (E)-(K) referred to the differences evaluated by general linear models.

Supplementary Figure 4. The differences for the secondary outcomes in terms of biomarkers of gut health and systemic inflammations.



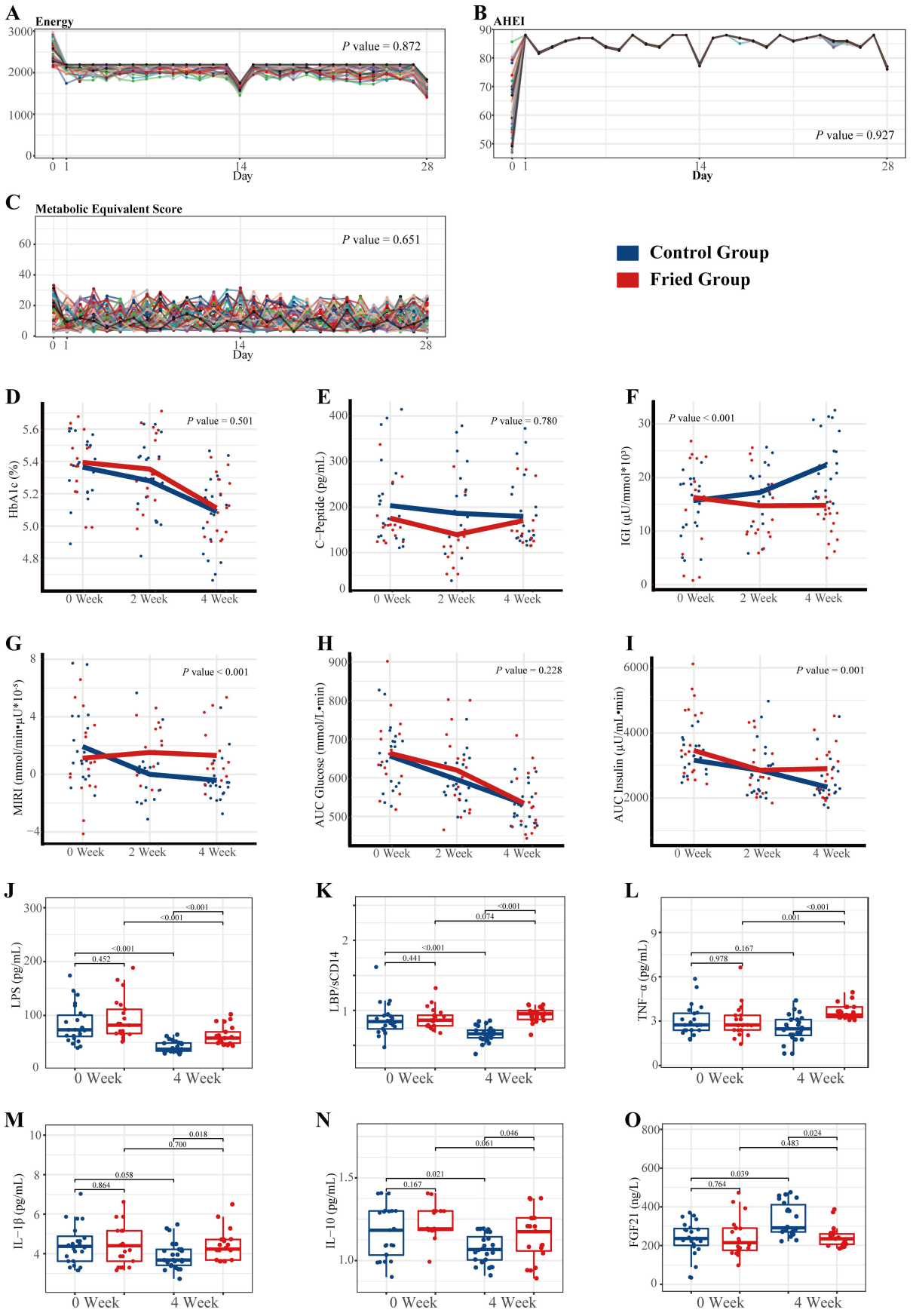
LPS, lipopolysaccharide; LBP, lipopolysaccharide binding protein; SCD14, soluble lipopolysaccharide receptor CD14; FGF21, fibroblast growth factor 21. The *P* values referred to the differences evaluated by general linear models.

Supplementary Table 3 Baseline characteristics in the subgroup analysis for the participants who habitually consumed fried food 1 time per week before participating in the trial (*n*=45)

Dietary intakes	Control Group	Fired Group	*P* value
N	24	21	
Age, year	22.04 (3.07)	21.65 (2.82)	0.243
Female, [N (%)]	15 (62.5%)	14 (66.67%)	0.384
Body mass index, kg/m^2	25.64 (2.97)	25.48 (2.05)	0.786
Body fat rate, %	25.40 (6.76)	26.08 (5.52)	0.274
Waist-hip ratio, %	0.84 (0.09)	0.85 (0.12)	0.905
Laboratory measurements			
HbA_{1c}			
%	5.36 (0.25)	5.39 (0.23)	0.775
mmol/mol	35.19 (2.63)	35.41 (2.42)	0.697
Fasting Glucose, mmol/L	4.46 (0.38)	4.52 (0.39)	0.475
Fasting insulin, uU/mL	4.31 (1.59)	4.48 (1.74)	0.834
IGI, $uU/mmol*10^3$	15.85 (12.68)	16.23 (14.61)	0.483
MIRI, $mmol/min•uU*10^{-5}$	1.96 (1.76)	1.23 (1.94)	0.546
AUC of glucose, mmol*min/L	625.60 (86.73)	630.98 (93.14)	0.797
AUC of insulin, uU*min/mL	3148.88 (1466.39)	3523.34 (1766.08)	0.268
Dietary intake			
Energy, kcal/day	2511.27 (463.62)	2562.23 (465.15)	0.274
Total fat, %TE	25.14 (5.78)	25.96 (6.89)	0.310
Carbohydrate, %TE	64.38 (13.10)	63.37 (11.64)	0.681
Protein, %TE	10.48 (2.59)	10.67 (2.52)	0.584
AHEI-2010 score	56.91 (15.89)	56.37 (15.16)	0.612
Physical exercise habitus, [N (%)]	18 (75.00%)	16 (76.19%)	0.902

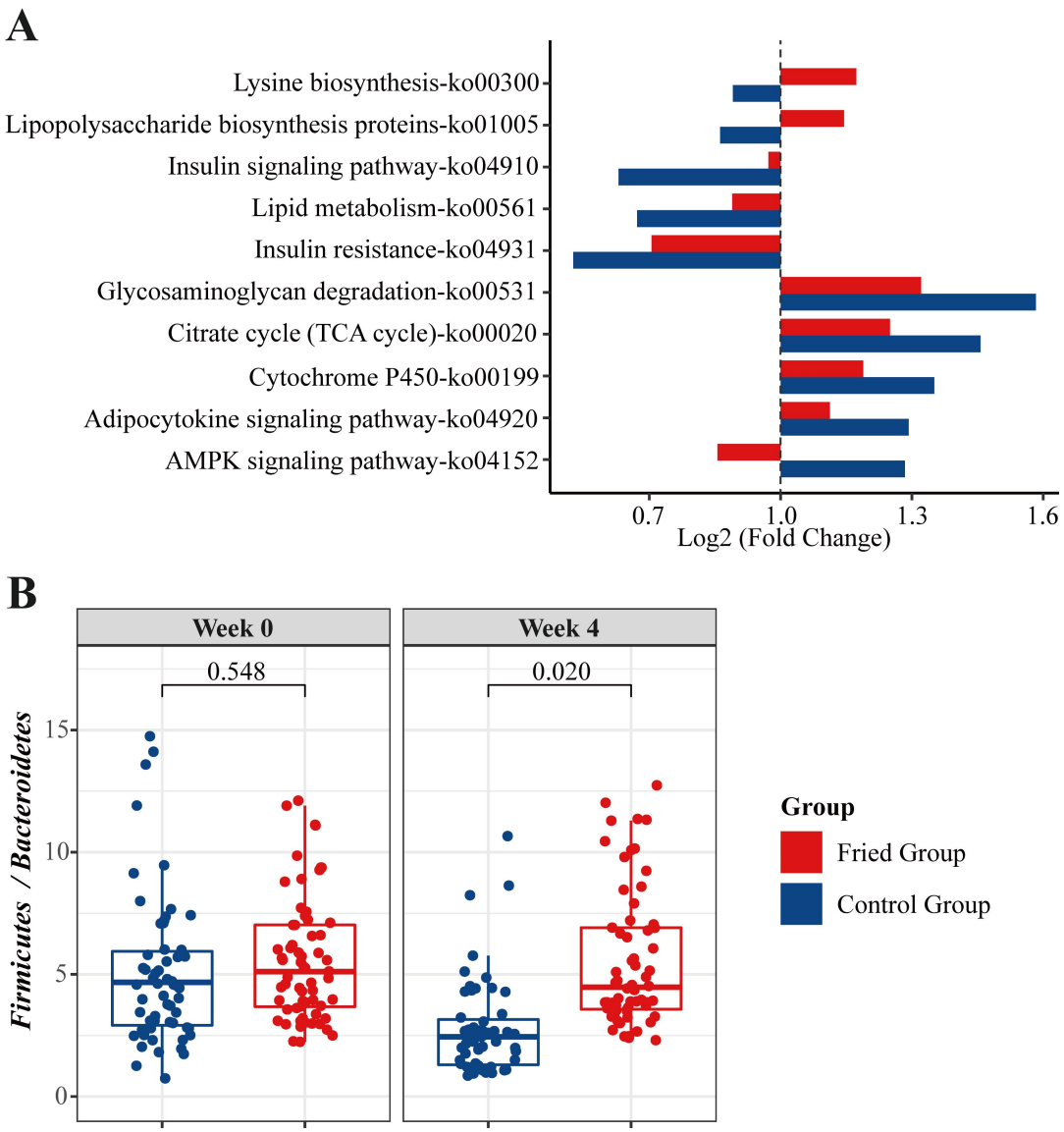
Continuous variables are presented as mean (SD). Categorical variables are presented as n (%). IGI, insulinogenic index; MIRI, muscle insulin resistance index; AHEI-2010, Alternate Healthy Eating Index 2010.

Supplementary Figure 5 Subgroup analysis of participants who habitually consumed fried food 1 time per week before participating in the trial.



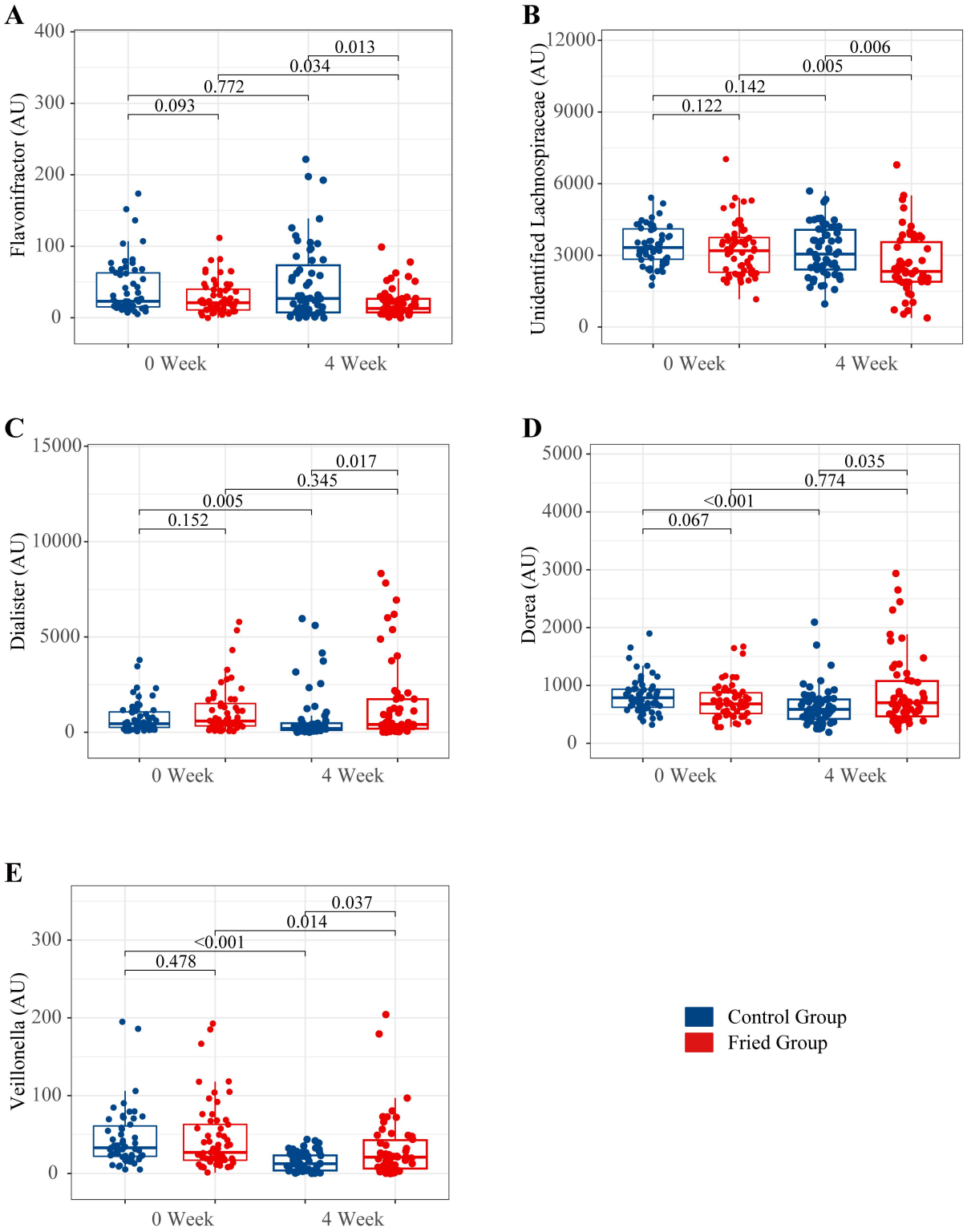
The *P* values in (A), (B), and (C) referred to the differences evaluated by repeated measured general linear models. The *P* values in (D)-(I) referred to the differences evaluated by generalized linear mixed models. The *P* values in (J)-(O) referred to the differences evaluated by general linear models.

Supplementary Figure 6 The microbiota-predicted pathways and the ratio of *Firmicutes* and *Bacteroidetes* in both groups.



(A) the top 10 differential microbiota-predicted pathways between the two groups, *P*-FDR < 0.05; (B) the differences for the ratio of *Firmicutes* and *Bacteroidetes* between the two groups, the *P* values referred to the differences evaluated by general linear models.

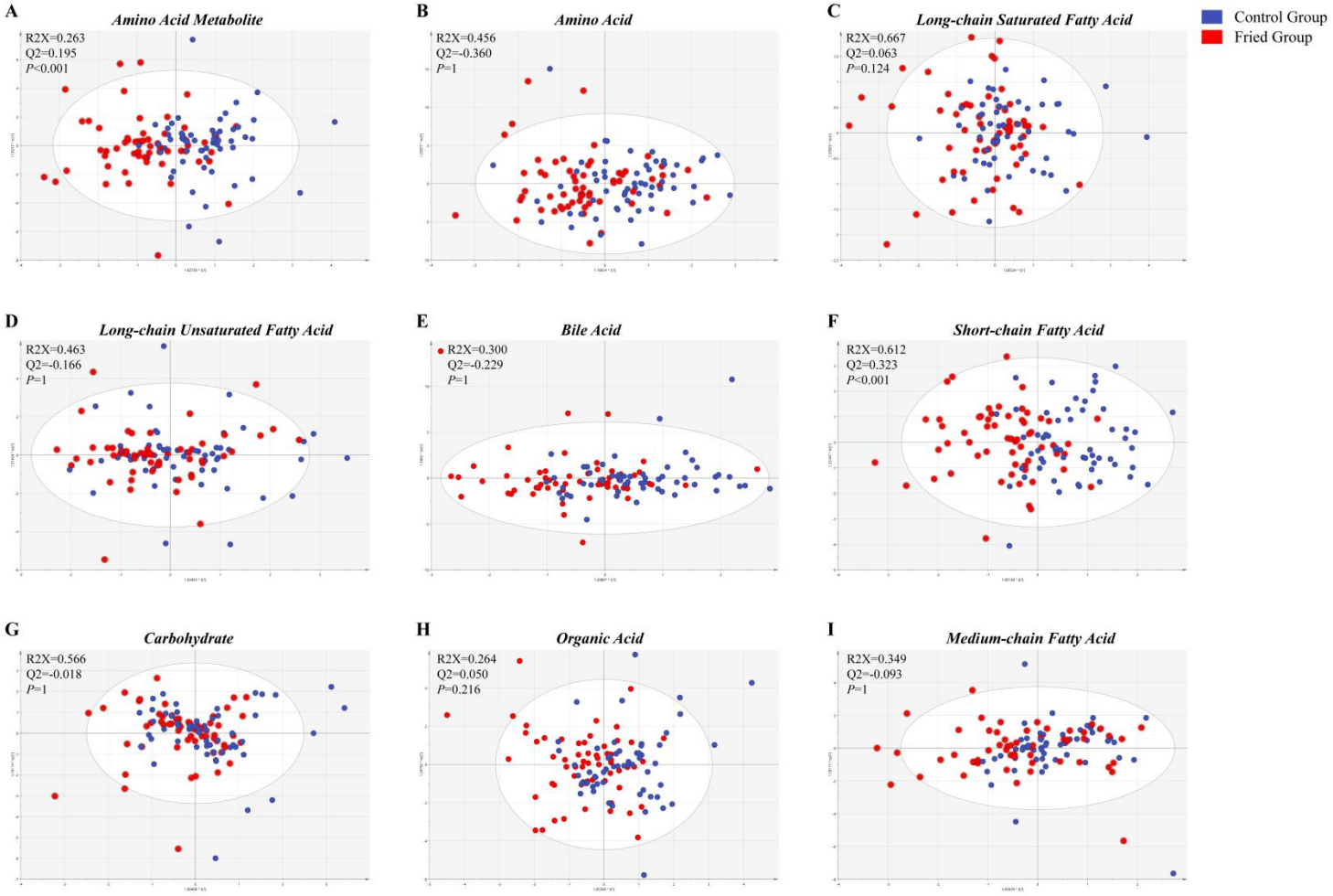
Supplementary Figure 7. Individual analyses for the differences of the 5 identified genera between the two groups



AU, arbitrary units;

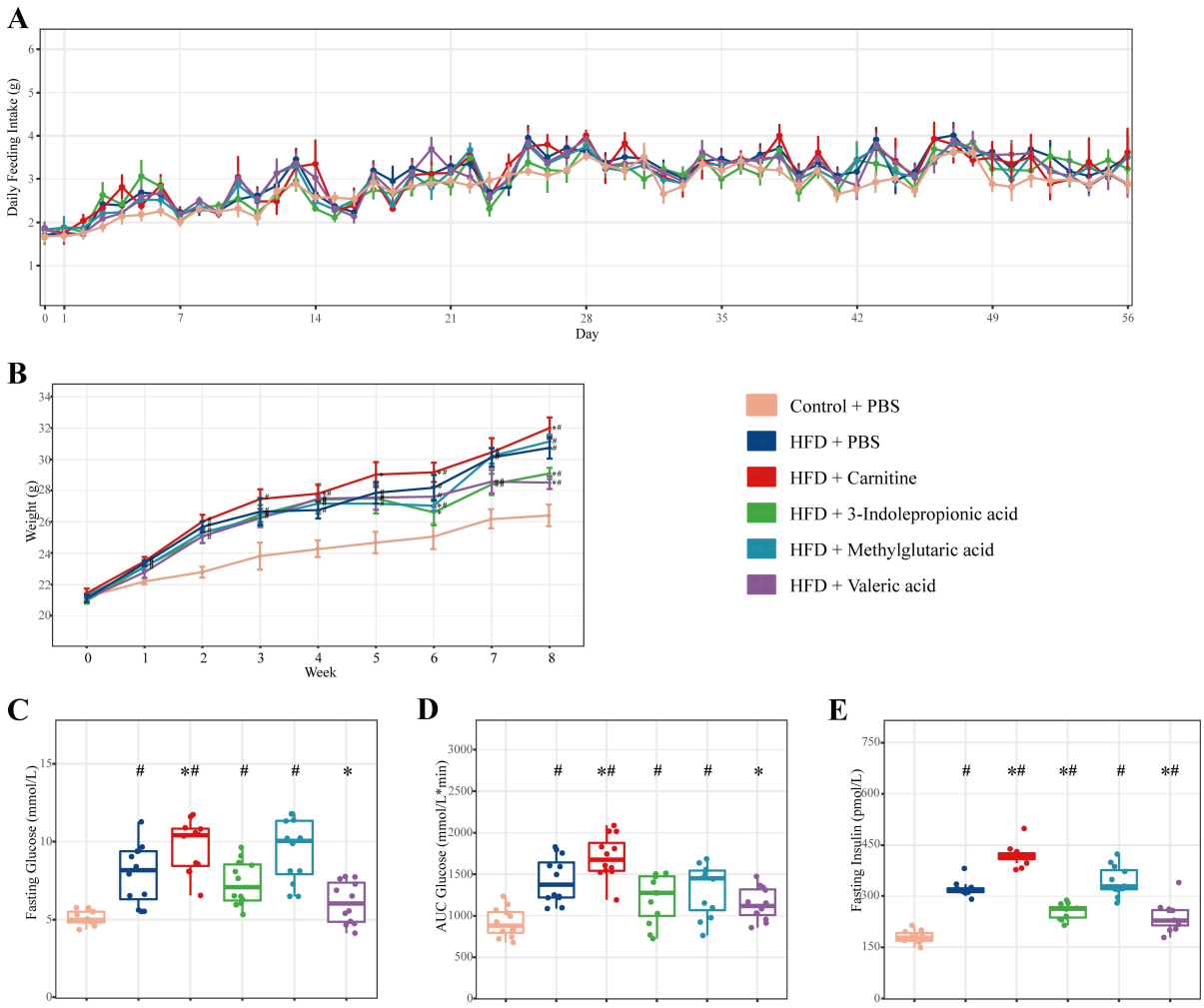
P-values were adjusted using the Benjamini-Hochberg false discovery rate.

Supplementary Figure 8. The stratification by metabolite categories.



R2X, the percentage of matrix information that the model can explain; Q2, the predictive power of the model calculated by cross-validation; *P*, validation of the OPLS-DA from an ANOVA of the cross-validated predictive residuals (CV-ANOVA).

Supplementary Figure 9 The effect of the identified metabolites on daily intake, body weight, and glucose homoeostasis in mice



HFD, high fat diet; AUC, the area under the curve.

P value < 0.05, significance of the differences between Control+PBS and other groups;

* *P* value < 0.05, significance of the differences between HFD+PBS and other groups.