Effects of Diet-Modulated Autologous Fecal Microbiota Transplantation on Weight Regain

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Table of contents

1. Inclusion and exclusion criteria
2. Lifestyle intervention description and outline,aFMT and placebo capsules processing, blood sample analysis
3. Metagenomics analysis BoosterShot pipeline, 16s rRNA sequencing pipeline
4. Multiple imputations for missing follow-up data, sample size calculation, randomization protocol and allocation sequence.
5. Table S1: Chronic Pharmacotherapy of the participants at baseline
6. Table S2: Characteristics of the study population at time 14-month
7. Table S3: Adherence to diet and physical activity at time 6-month
8. Table S4: Adherence to diet and physical activity, across lifestyle intervention arms and treatment groups, at the end of the trial
9. Figure S1: aFMT effect on 6-14 months changes in HOMA-IR, across lifestyle intervention
10. Figure S2: 0 to 6-months species changes across lifestyle intervention arms
11. Figure S3: 0 to 6-months KEGG modules changes across lifestyle intervention arms
12. Figure S4: Shotgun metagenomics processing validation against MetaPhlan2
13. Figure S5: Microbiome taxonomic and functional changes – centered-log-transformed data
14. Figure S6: Microbiome taxonomic and functional changes – excluding subjects with prescribed metformin

Supplementary 1. Inclusion and exclusion criteria

Inclusion criteria:

- Age above 30 years
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    - Waist circumference above 102 cm in men and 88 cm in women.

    Or

    - dyslipidemia (TG>150 and HDL-c≤40 for men; ≤50 for women)

Exclusion criteria:

- Inability to perform physical activity
- Serum creatinine≥2 mg/dL
- Serum ALT or AST greater than 3 times above upper-limit-norm
- Major illness that might require hospitalization
- Pregnancy or lactation
- Active cancer, or chemotherapy treatment in the last 3 years
- Warfarin treatment
- Pacemaker
- Participation in a different trial

Supplementary 2. Sample size calculation

Minimal required sample size was estimated based on the weight loss pattern in our previous CENTRAL trial.[1] Aiming to detect a complete attenuation of the expected 3±4.3kg weight regain, for an alpha of 5% and power of 90%, the calculated sample size was 88 subjects in total.

Supplementary 3. Randomization protocol and allocation sequence

Recruitment to the DIRECT PLUS and aFMT trial was performed by ER, AYM, HZ, AK, and GT. All eligible participants who signed consent to participate in the trial and completed the baseline measurements were randomized into one of the three intervention groups (Healthy dietary guidance, Mediterranean, green-Mediterranean) at a 1:1:1 ratio and within strata of gender and work status (to ensure equal workplace-related lifestyle features between groups).
Participants reducing >3.5% body-weight, without prescribed antibiotics 2 months prior to feces sampling who agreed to participate were randomized in 1:1 ratio to two treatment groups (aFMT and placebo) within strata of gender and lifestyle intervention group.
Randomization was conducted in a single phase using an ad-hoc R-based procedure, the randomization was done by Nirit Keren from the Center for Microbiome Research at Shamir Medical Center, Israel.

Supplementary 4. Lifestyle intervention

All trial participants received free gym memberships and educational sessions, and were encouraged to engage in moderate intensity physical activity, ~80% of which included an aerobic component. The aerobic effort increased gradually, starting with 20 minutes of aerobic training at 65% maximum heart rate, and increased to 45-60 minutes of aerobic training at 80% of maximum heart rate. The full workout program included 45-60 minutes of aerobic training 3-4 times/week, while resistance training started with one set of weights corresponding to 60% of the maximum weight, eventually reaching the use of two sets of weights corresponding to 80% of the maximum weight. The resistance training included leg extensions, leg curls, squats, lateral pull-downs, push-ups, shoulder presses, elbow flexions, triceps extensions, and bent leg sit-ups. PA group participants received basic health-promoting guidelines for achieving a healthy diet.

Outline of dietary and PA interventions

	Healthy dietary guidance	**Mediterranean diet**	**Green Mediterranean diet**
Physical activity	18-months free gym membership, 18-months PA education sessions 45-60 minutes of aerobic training + resistance training, 3-4 times/week.		
Lifestyle group sessions	18-months group sessions in the workplace, weekly for the first month, and monthly thereafter		
General dietary guidance	Limit dietary cholesterol, trans-fat, saturated-fat, sugars, and salt and increase intake of vegetables		
Energy, kcal/day	Guidelines for a healthy Mediterranean diet with no specific recipes or calorie restriction	1500-1800kcal/day for men, 1200-1400kcal/day for women	
Total fat, % of daily consumption		~40% mainly PUFA and MUFA	
Carbohydrates, gr/day		Less than 40gr/day in the first 2 months with increased gradual intake up to 80gr/day	
Specific recommendations		Avoid red and processed meats. Reduced poultry intake.	
Polyphenols, mg/day		+440 mg/day [source: provided walnuts (28g/day]	+1240 mg/day [source: provided walnuts (28g/day), green tea (4 cups/day), *Wolffia globosa* duckweed (Mankai) shake (100g frozen cubes)]
Specific recommendations			

Supplementary 5. aFMT and placebo capsules processing

Capsules processing was carried out under aerobic conditions. A fecal suspension was generated in normal saline without preservatives using a commercial blender. Materials were sequentially sieved to remove particulate material. The final slurry was concentrated by centrifugation and resuspended in saline at 10% of the volume of the initial sample with 20% glycerol added as a bacterial cryoprotectant. Fecal matter suspension was pipetted into size 0 capsules (650 µL), which were closed and secondarily sealed in size 00 acid-resistant hypromellose capsules (DRCaps,Capsugel). Each sample was processed separately, and a dose of 100 capsules containing sieved, concentrated material, was derived from the fecal matter. Placebo capsules consisted of agarose in normal saline/glycerol (the same vehicle as in aFMT capsules).

Supplementary 6. Blood sample analysis
Serum levels of total cholesterol (TC; CV, 1.3%), low-density-lipoprotein (LDL) cholesterol were determined enzymatically with a Cobas 6000 automatic analyzer (Roche). Plasma insulin levels were measured with the use of an enzymatic immunometric assay [Immulite automated analyzer, Diagnostic Products, coefficient of variation (CV)=2.5%].
Plasma levels of high-sensitivity C-reactive protein were measured by ELISA (DiaMed; CV, 1.9%). Plasma leptin levels were assessed by ELISA (Mediagnost), with a coefficient of variation of 2.4%.

Supplementary 7. Metagenomics analysis BoosterShot pipeline
DNA Extraction
Samples were extracted using MO Bio PowerFecal (Qiagen) automated for high throughput on QiaCube (Qiagen), with bead beating in 0.1mm glass bead plates.
DNA Quantification
Samples were quantified with Qiant-iT Picogreen dsDNA Assay (Invitrogen).
Library Preparation & Sequencing
Libraries were prepared with a procedure adapted from the Nextera Library Prep kit (Illumina). Libraries were sequenced on an Illumina NextSeq using single-end 1 x 145 reads with a NextSeq 500/550 High Output v2 kit (Illumina).
Sequence Quality Control
DNA sequences were filtered to remove low quality (Q-Score < 20) reads, and for length (< 50), and adapter sequences were trimmed using cutadapt. Fastq files were converted a single fasta using shi7.
OTU Picking
DNA sequences were aligned to a curated database containing all representative genomes in RefSeq for bacteria with additional manually curated strains. Alignments were made at 98% identity against all reference genomes. Each input sequence was compared to each reference sequence in CoreBiome's Venti database using full gapped alignment with Burst. Ties were broken by minimizing the overall number of unique gene hits. For taxonomy assignment, each input sequence was assigned the lowest common ancestor that was consistent across at least 80% of all reference sequences tied for best hit. The number of counts for each taxon was then normalized to the average genome length. Species accounting for less $1X10^{-6}$ of all species-level markers were discarded. Samples with fewer than 1,000 sequences were also discarded. The normalized and filtered tables were used for all downstream analyses.
Functional Genome Content
Functional groups were observed directly using Kyoto Encyclopedia of Genes and Genome Orthology groups (KEGG KOs) by alignment against a gene database derived from the strain database used above.

Supplementary 8. 16s rRNA sequencing pipeline
DNA Extraction
Fecal microbiota DNA was extracted using QIAamp PowerFecal DNA Kit (Qiagen) and a FastPrep-24™bead beater (MP Biomedicals). DNA quantity and quality was assessed spectrophotometrically by NanoDrop™ (ThermoFisher Scientific).
Library Preparation & Sequencing
For 16S rRNA sequencing amplification of total genomic faecal DNA was carried out using the specific bacterial primer set 341F (5' CCTACGGGNGGCWGCAG 3') and 806R (5' GACTACNVGGGTWTCTAATCC 3') with overhang Illumina adapters, targeting a ~460-bp fragment of the 16S rRNA variable region V3-V4.[2,3]
PCR amplification of each sample was carried out using 25 µl reactions with 0.2 µM of each primer and 12.5 ng template DNA, and employing KAPA HiFi HotStart ReadyMix. PCR amplification was carried out using a GeneAmp PCR System 9700 (Thermo Fisher Scientific) with

the following steps: one cycle at 94°C for 5 minutes, 30 cycles at 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds and one final elongation step at 72°C for 5 minutes. The PCR products were checked on 1.5% agarose gel and cleaned from free primers and primer dimer using the Agencourt AMPure XP system (Beckman Coulter, Brea, CA, USA) following the manufacturer's instructions. Subsequently dual indices and Illumina sequencing adapters Nextera XT Index Primer (Illumina) were attached by 7 cycles PCR (16S Metagenomic Sequencing Library Preparation, Illumina).
The final libraries were quantified using the Quant-IT PicoGreen dsDNA assay kit (Thermo Fisher Scientific) by the Synergy2 microplate reader (Biotek), then libraries were pooled in an equimolar way and analysed on a Typestation 2200 platform (Agilent Technologies, Santa Clara, CA, USA). Barcoded library were sequenced on Illumina® MiSeq (PE300) platform (MiSeq Control Software 2.0.5 and Real-Time Analysis software 1.16.18). Sequences with expected error rate >1.5% were removed from analysis.

Mice study extraction, amplification and sequencing of fecal samples

DNA was extracted from all fecal samples by PureLink Microbiome DNA Purification Kit (Invitrogen, Carlsbad, CA). The V4 region of the bacterial 16S rRNA gene was amplified by polymerase chain reaction (PCR) using the 515F (AATGATACGGCGACCACCGAGATCTACACGCT) barcoded and 806R (TATGGTAATTGTGTGYCAGCMGCCGCGGTAA) primers. A reaction containing a final concentration of 0.04% of each primer and 0.5% of PrimeSTAR® Max DNA Polymerase (Takara, Mountain View, CA) 50µl total volume. PCR reactions were carried out by 35 cycles of denaturation (95°C), annealing (55°C) and extension (72°C), with final elongation at 72°C. PCR products were purified using AMPure magnetic beads (Beckman Coulter, Indianapolis, IN) and quantified using dsDNA fluorescence quantification assay kit (DeNovix Inc, Wilmington, Delaware). Samples were then pooled at equal amounts of 50ng, loaded on 2% agarose E-Gel (Thermo Fisher, Waltham, MA), purified and sent for sequencing using the Illumina MiSeq platform (Genomic center, Azrieli Faculty of Medicine, BIU, Israel).

Supplementary 9. Multiple imputations for missing follow-up data

We performed intention-to-treat analyses, including all 90 participants, by imputing missing follow-up data of a single participant on the 14-month timepoint of both primary outcomes by the multiple imputation technique.[4]
The imputation was done by the R package "mice"[5], wherein the following predictors were used in the imputation model: age, sex, baseline weight and initial 6-months weight-loss.

Table S1: Chronic Pharmacotherapy of the participants at baseline

	All subjects			Healthy dietary guidance group			Mediterranean diet			Green Mediterranean diet		
Treatment	aFMT	Placebo	Pv	aFMT	Placebo	Pv	aFMT	Placebo	Pv	aFMT	Placebo	Pv
Subjects - no.	44	46		8	8		17	18		19	20	
Oral anti-hyperglycemic- no. (%)	3 (6.8)	3 (6.5)	1	1 (12.5)	0 (0.0)	1	0 (0.0)	1 (5.6)	1	2 (10.5)	2 (10.0)	1
Exogenous insulin- no. (%)	1 (2.3)	1 (2.2)	1	0 (0)	0 (0)	1	0 (0)	0 (0)	1	1 (5.3)	1 (5.0)	1
Anti-hypertensive- no.(%)	10 (22.7)	9 (19.6)	.91	3 (37.5)	0 (0.0)	.2	2 (11.8)	4 (22.2)	.71	5 (26.3)	5 (25.0)	1
Cholesterol lowering- no. (%)	6 (13.6)	8 (17.4)	.84	2 (25.0)	1 (12.5)	1	1 (5.9)	1 (5.6)	1	3 (15.8)	6 (30.0)	.5
Anti-platelet- no. (%)	6 (13.6)	2 (4.3)	.24	2 (25.0)	0 (0.0)	.45	1 (5.9)	0 (0.0)	.98	3 (15.8)	2 (10.0)	.95

Table S2: Characteristics of the study population at time 14-month

	All subjects			Healthy dietary guidance group			Mediterranean diet			Green Mediterranean diet		
Treatment	aFMT	Placebo	Pv	aFMT	Placebo	Pv	aFMT	Placebo	Pv	aFMT	Placebo	Pv
Subjects - no.	44	46		8	8		17	18		19	20	
Body mass index	28.92 (3.12)	29.53 (4.21)	.82	29.11 (3.51)	28.31 (1.72)	.73	29.23 (3.69)	29.75 (4.96)	.77	28.55 (2.46)	29.79 (4.16)	.42
Waist circumference- cm	101.45 (7.85)	102.25 (10.84)	.94	100.00 (9.62)	97.00 (4.62)	.86	102.47 (8.66)	103.94 (11.80)	.84	101.16 (6.52)	102.58 (11.33)	.75
Weight- Kg	87.69 (12.58)	86.92 (14.25)	.80	86.15 (14.90)	79.27 (8.57)	.25	87.99 (13.48)	89.63 (15.78)	.70	88.07 (11.35)	87.18 (13.98)	.91
Fasting plasma glucose- mg/dl	103.57 (14.69)	99.78 (9.18)	.29	104.18 (17.13)	98.48 (6.74)	.73	99.28 (7.41)	98.89 (10.65)	.27	107.17 (18.05)	101.17 (8.80)	.52
Fasting plasma insulin- microU/ml	10.16 (4.61)	11.54 (7.05)	.56	10.95 (4.40)	11.34 (7.38)	.83	10.06 (3.84)	10.89 (5.91)	.97	9.92 (5.46)	12.21 (8.08)	.42
HOMA-IR	2.55 (1.29)	2.88 (1.86)	.69	2.53 (1.06)	2.83 (1.98)	.73	2.46 (0.93)	2.67 (1.55)	1	2.65 (1.65)	3.11 (2.14)	.63

Values are presented as means (standard deviation) for continues variables and total number (percent) for categorical variables. No significant differences were observed between placebo or aFMT group in the measured baseline characteristics, overall and across lifestyle interventions. HOMA-IR denotes homeostasis model assessment of insulin resistance

Table S3: Adherence to diet at time 6-month; adherence to physical activity at baseline and time 6-month

	Healthy dietary guidance	Mediterranean diet	Green Mediterranean diet	Pv
Adherence to dietary intervention at 6-months				
% Carbohydrates intake	45.99 (8.13)	32.53 (6.88)	37.19 (8.53)	<.001
% Protein intake	18.97 (3.58)	27.02 (4.23)	23.21 (4.65)	<.001
% Fat intake	36.41 (5.13)	41.15 (6.27)	40.76 (7.25)	.1
Adherence to physical activity intervention				
Baseline METs/week	38.97 (48.68)	42.08 (18.62)	35.08 (22.98)	0.723
6-month METs/week	56.72 (47.17)	72.67 (41.39)	51.36 (33.36)	.212

Values are presented as means (standard deviation). Proportional macronutrient intake (6-months timepoint) and metabolic equivalents (METs; baseline and 6-months timepoint) across lifestyle intervention group. METs unit are defined as the ratio of work metabolic rate to the standard resting metabolic rate.

Table S4: Adherence to diet and physical activity, across lifestyle intervention arms and treatment groups at the end of the trial

	Healthy dietary guidance			Mediterranean diet			Green Mediterranean diet		
Treatment	aFMT	Placebo	Pv	aFMT	Placebo	Pv	aFMT	Placebo	Pv
% Carbohydrates intake	43.90 (6.01)	44.62 (2.05)	.855	38.01 (6.34)	36.94 (8.47)	.727	41.85 (6.55)	39.13 (8.26)	.447
% Protein intake	19.99 (1.76)	20.59 (1.14)	.584	23.91 (3.14)	22.98 (4.79)	.6	20.86 (2.23)	20.82 (3.45)	.861
% Fat intake	37.22 (5.09)	35.44 (2.17)	.715	38.98 (4.59)	41.63 (5.46)	.116	38.74 (5.58)	40.48 (5.67)	.482
METs/week	67.58 (49.77)	55.91 (54.07)	.624	46.27 (26.98)	48.69 (34.80)	.93	36.14 (31.75)	33.86 (27.19)	.804

Values are presented as means (standard deviation). Proportional macronutrient intake and metabolic equivalents (METs) across lifestyle intervention group and aFMT treatment at the end of the trial. METs unit are defined as the ratio of work metabolic rate to the standard resting metabolic rate.

Figure S1: **aFMT effect on 6-14 months changes in HOMA-IR, across lifestyle intervention**

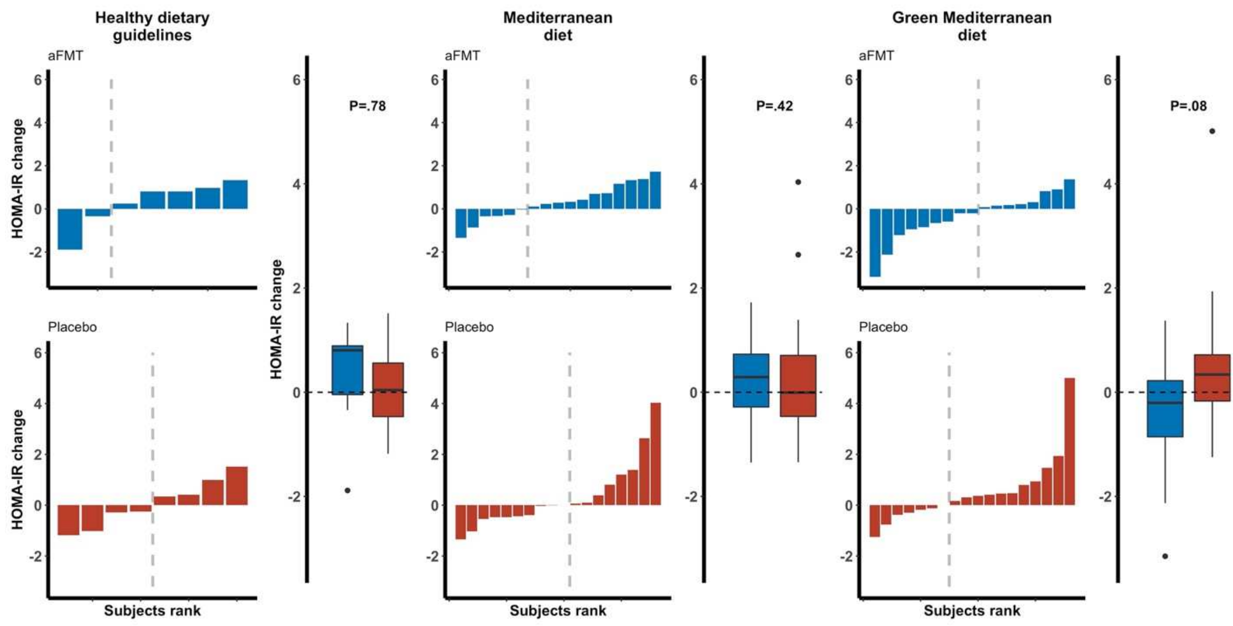

Figure S2: 0 to 6-months taxa changes across lifestyle intervention arms

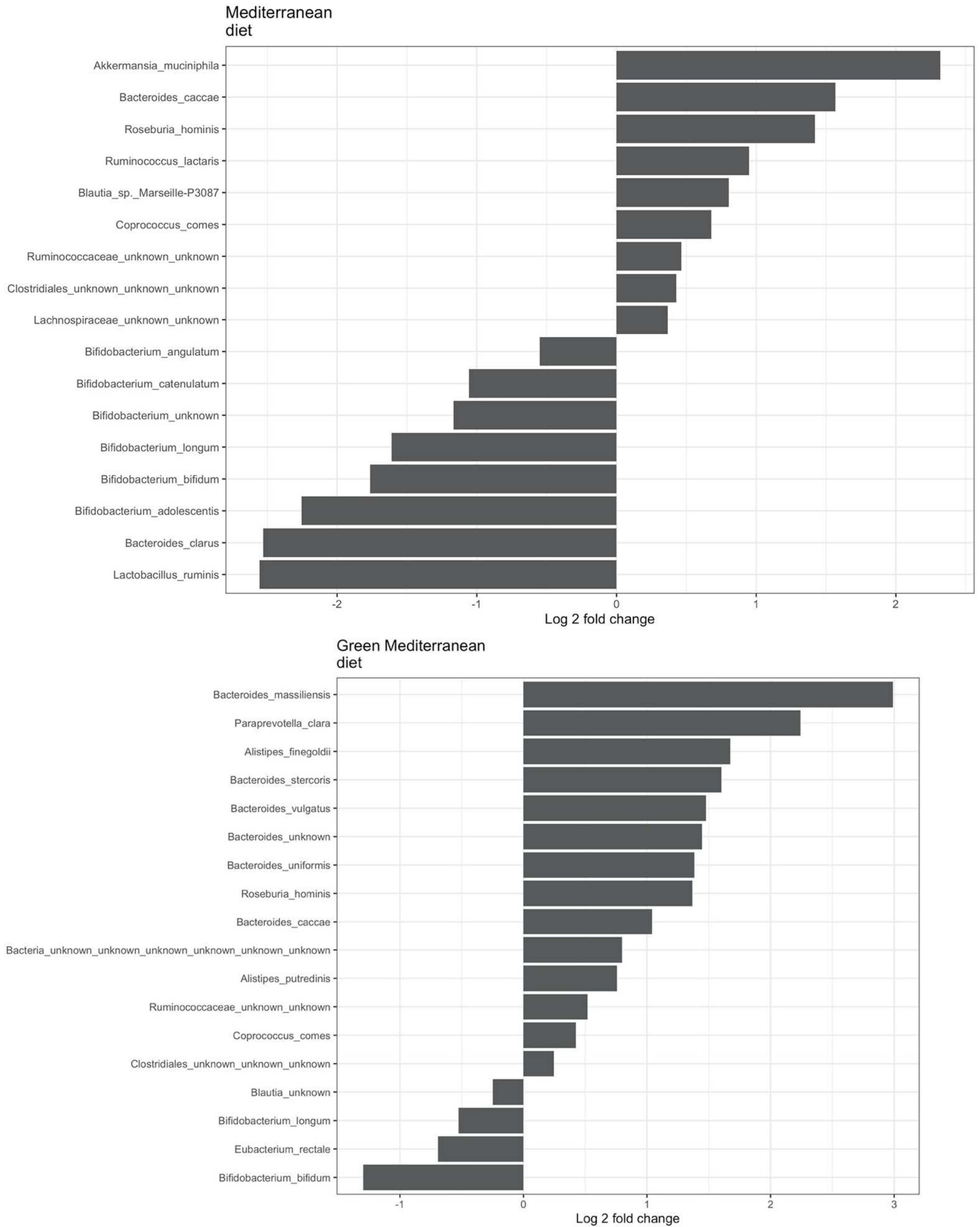

Figure S2: 0 to 6-months taxa changes across lifestyle intervention arms
Species that were significantly changed by weight loss, across lifestyle intervention groups. Values represents log 2 fold change from baseline, by bacteria. No significant changes were observed among the Healthy dietary guidance group

Figure S3: 0 to 6-months KEGG modules changes across lifestyle intervention arms

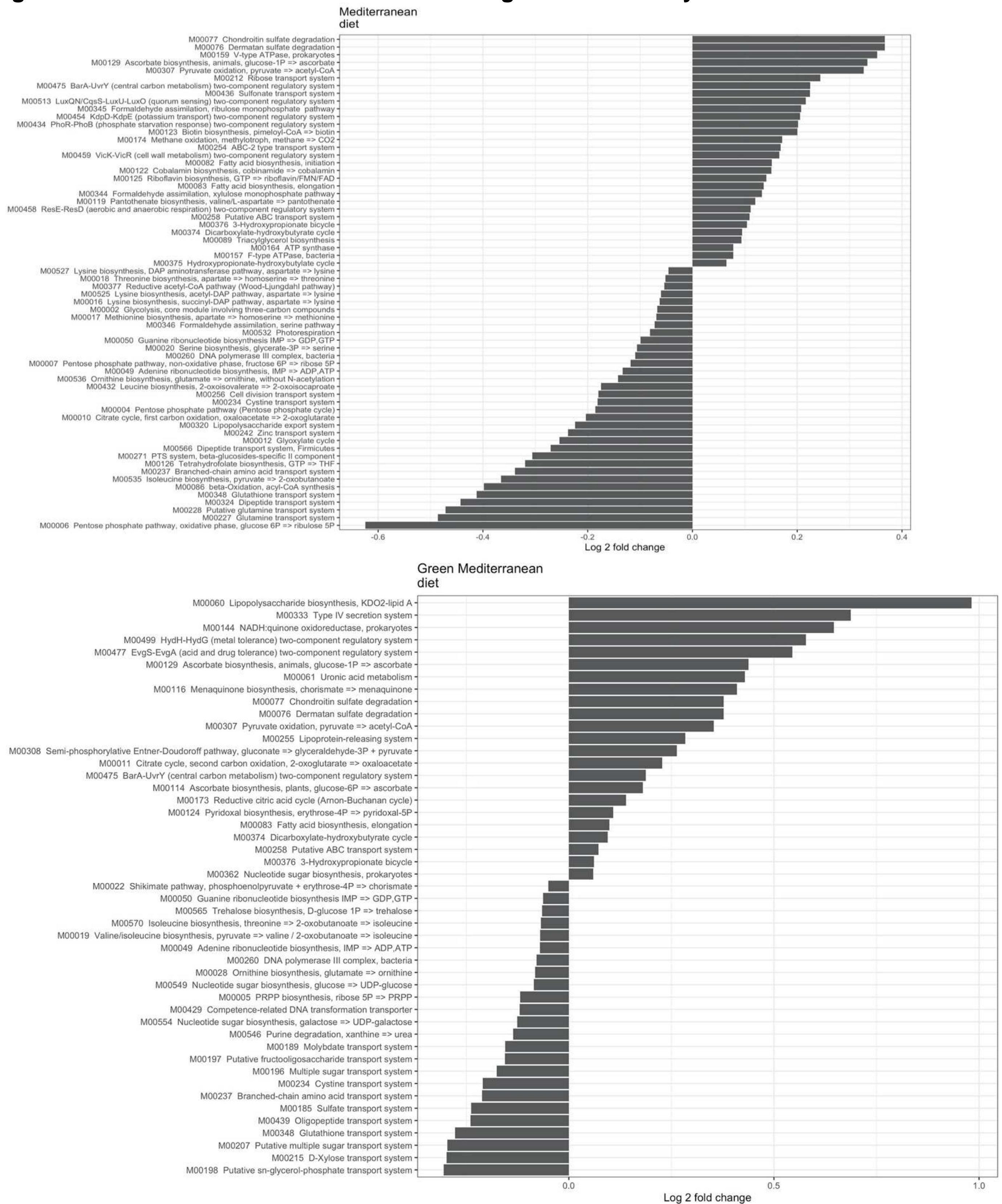

Figure S3: 0 to 6-months KEGG modules changes across lifestyle intervention arms
KEGG modules that were significantly changed by weight loss, across lifestyle intervention groups. Values represents log 2 fold change from baseline, by module. No significant changes were observed among the Healthy dietary guidance group.

Figure S4: Shotgun metagenomics processing validation against MetaPhlan2

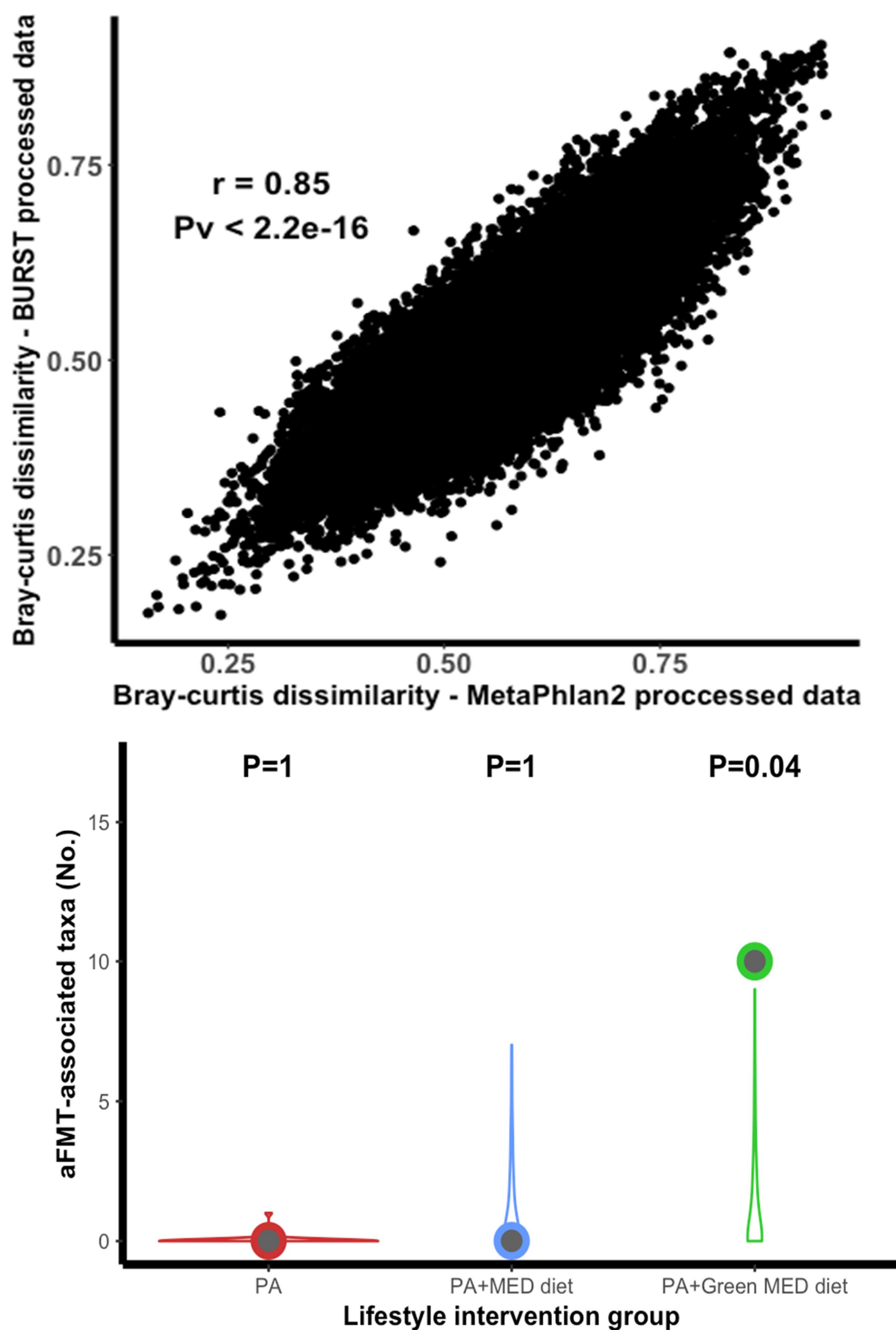

Figure S4: Shotgun metagenomics processing validation against MetaPhlan2
(a) Correlation between pairwise samples dissimilarity, processed by BURST vs. MetaPhlan2, for all possible pairs of sequenced samples. (b) Comparison between the number of observed aFMT-associated taxa, generated by MetaPhlan2, to the number expected by a permuted null model by an iterative randomization (n=1000) of sample labeling across group, treatment and time. The gray dot denotes the observed number of taxa in each group, while the violins enclose 95% of the permuted results. P value was calculated as observed/expected.

Figure S5: Microbiome taxonomic and functional changes – centered-log-transformed data

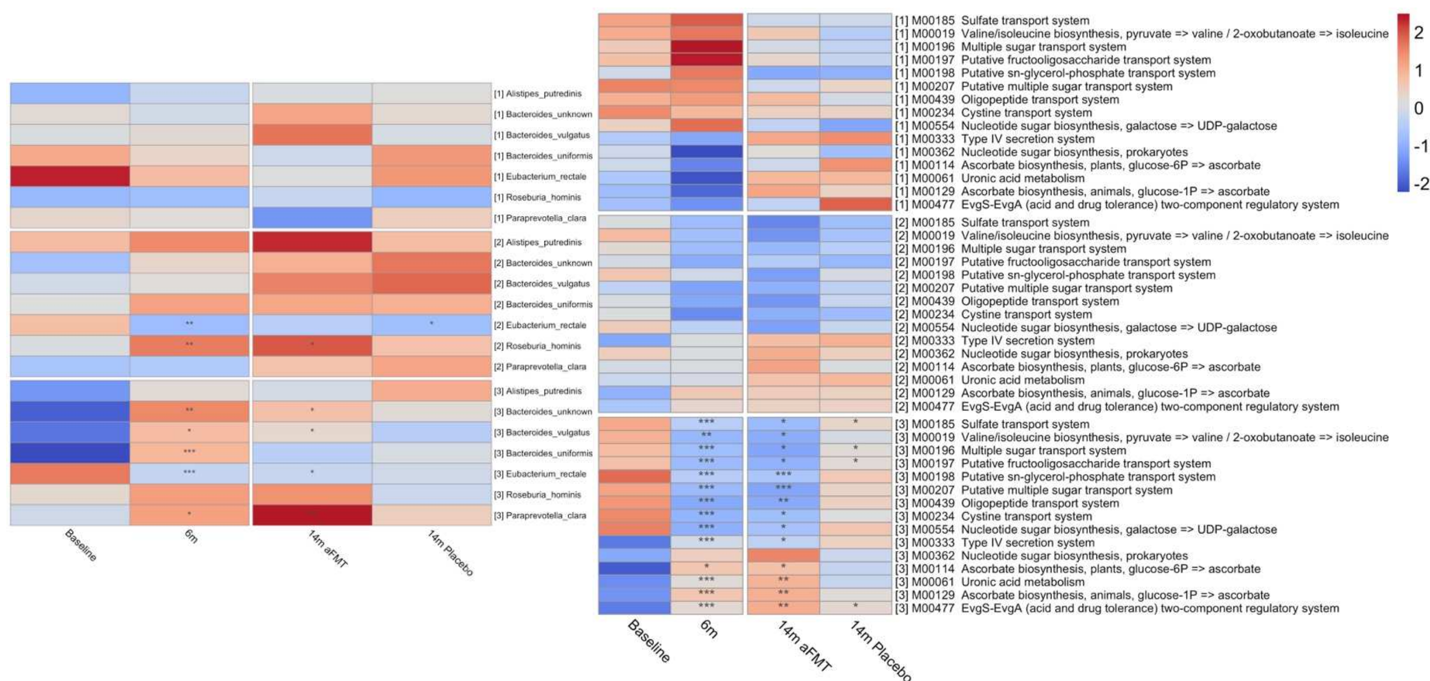

Figure S5: Microbiome taxonomic and functional changes – centered-log-transformed data
Shotgun metagenomics assessed bacteria and metabolic pathways (KEGG modules) after centered log transformation that were significantly changed by weight loss and maintained by the aFMT treatment, across lifestyle intervention groups.

Figure S6: Microbiome taxonomic and functional changes – excluding subjects with prescribed metformin

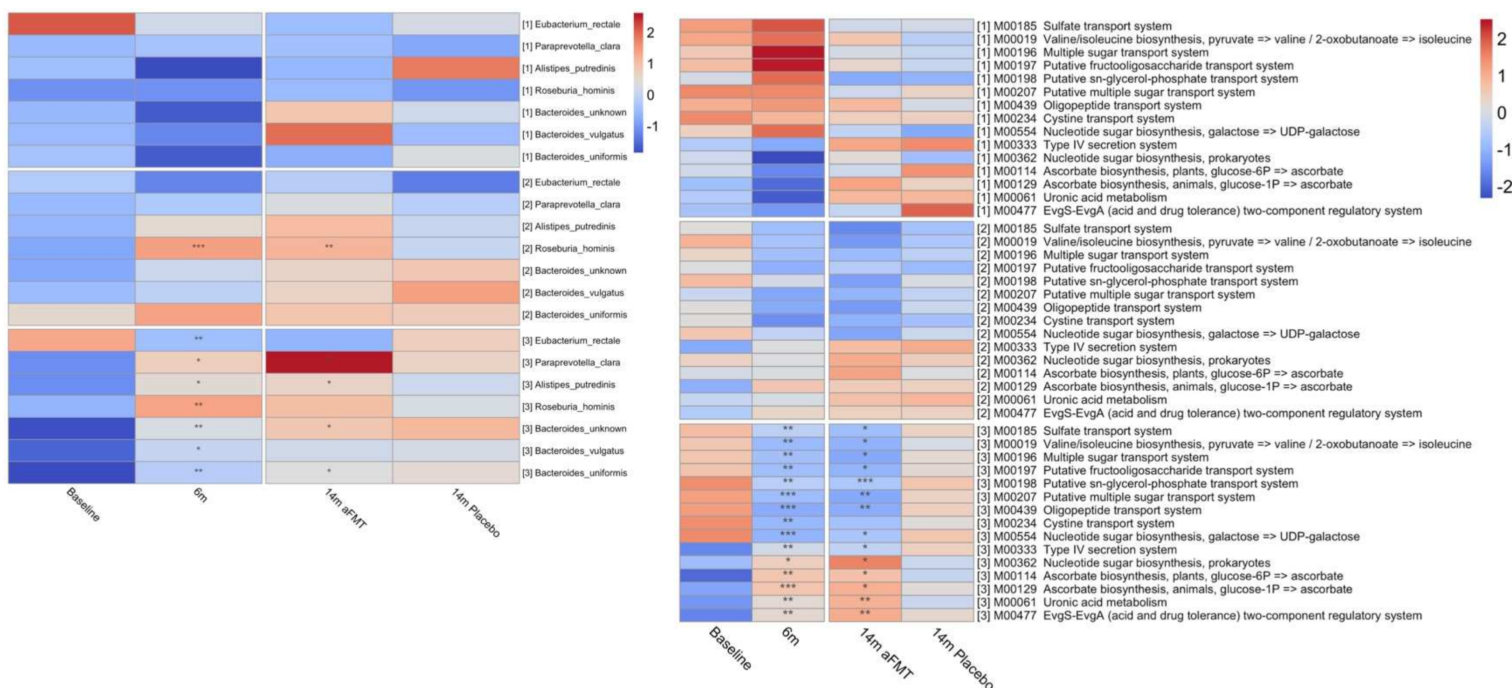

Figure S6: Microbiome taxonomic and functional changes – excluding subjects with prescribed metformin
Shotgun metagenomics assessed bacteria and metabolic pathways (KEGG modules), excluding subject with prescribed metformin, that were significantly changed by weight loss and maintained by the aFMT treatment, across lifestyle intervention groups.