High adherence to the Mediterranean diet is associated with a diverse faecal microbiome and reduced systemic inflammation in a cohort of pregnant women

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Methods

Quantification of short-chain fatty acids (SCFA) in serum by UPLC-MS

Chemicals

All solvents, namely, water, methanol, acetonitrile, and isopropanol, were LC-MS grade and were purchased from ThermoFisher. Standards of acetic acid, butyric acid, hexanoic acid, isobutyric acid, isovaleric acid, propionic acid, valeric acid, 2-methyl-butyric acid and formic acid were obtained from Sigma-Aldrich (St Louis, MO, USA). Reagents O-benzylhydroxylamine hypochlorite and N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride were purchased from Sigma-Aldrich (St Louis, MO, USA). Last, isotopically labelled standards of acetic acid-d4 were

obtained from Sigma- Aldrich (St Louis, MO, USA); propionic acid-13c3, Isobutyric acid-d7, butyric acid-d7, iso-valeric acid-d9, hexanoic acid-d11 were purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA).

Calibration and internal standards

Calibration curves were built in the linearity range of 0.2 - 80 µM for all the analytes. An internal standards (IS) mix solution containing 8 µM of acetic acid-d4, propionic acid-13c3, Isobutyric acid-d7, butyric acid-d7, iso-valeric acid-d9, and hexanoic acid-d11 in methanol was prepared and stored at -20 °C until further use.

Sample preparation

Frozen 50 µL samples aliquoted in glass vials (Screw Neck Max Recovery vials, Waters Corporation, MA, USA), calibration standard solutions, and internal standards solutions were thawed in a cool room at 4 °C. A volume of 150 µL of IS mix solution was added to all sample vials. Then, the vials were capped and mixed by vortexing at 600 rpm for 5 min at 10 °C (ThermoMixer C, Eppendorf, Germany) and later centrifuged at 3900 rpm for 20 min at 10 °C. After centrifugation, 40 µL of the upper layer supernatant were transferred to 1.5 mL maximum recovery vials (Water Corporation, MA, USA). Then, 40 µL of LC-MS grade water, 10 µL of O-benzylhydroxylamine hydrochloride (0.1 M) in methanol, and 10 µL of N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (0.25 M) in methanol were added into the maximum recovery vials. The vials were capped and placed onto a thermal mixer (ThermoMixer C, Eppendorf, Germany) to be incubated at 25 °C and 600 rpm for 1 h. After incubation, 400 µL of 50 % methanol in water were added into the vials and mixed by vortexing, followed by the addition of 600 µL of dichloromethane and vortexing again. The glass vials were then centrifuged at 3900 rpm for 20 min at 10 °C. A volume of 500 µL was transferred from the lower organic layer into clean 1.5 mL recovery vials. The extraction step using dichloromethane was repeated once again. Finally, the glass vials were left to dry inside a fume hood overnight. The dried pellet was reconstituted by adding 200 µL of 50% methanol in water and vortexing for 20 s. Extracts

from the same sample were pooled into 4 mL glass vials, mixed by vortexing, and stored at -20 °C until analysis.

LC-MS analysis

Chromatographic separation was performed using an Acquity I-Class UPLC (Waters Corporation, USA), and mass spectrometry analysis was performed on a Xevo TQ-XS MS system (Waters Corporation, USA). A Kinetex 2.6 µm, C18, 100 Å, 100 x 2.1 mm, LC column (Phenomenex, USA) thermostated at 40 °C was used. The mobile phase consisted of 0.1 % formic acid in water (A) and 0.1 % formic acid in methanol:isopropanol (9:1, v:v) (B). The 6.0 min gradient was as follows: 32 % B (0-0.5 min) at a flow rate of 0.4 mL/min. Then, 60 % B (4 - 4.8 min), 65 % B (4.8 - 4.9 min), 98 % B (4.9-5.2 min), 98 % B (5.2-5.3 min), and 32 % B (5.3-6.0 min) at a flow rate of 0.6 mL/min. An injection volume of 5 µL was used. In between sample injections, the injection loop was washed 3 times with 900 µL of a solution of water:acetonitrile (95:5, v:v) and 300 µL of isopropanol. In addition, the following conditions were used in the mass spectrometer: Capillary Voltage: 3.0 kV; Cone Voltage: 30 V; Desolvation temperature: 650 °C; Gas-flow desolvation: 1000 L/h; Cone gas flow: 150 L/h; Nebulizer: 7.0 Bar, and Collision gas flow: 0.16 mL/min. Mass spectrometry analysis was performed in positive ion mode in Multiple Reaction Monitoring (MRM). The MS characteristics of the analysed compounds are reported in Table S1.

LC-MS data pre-processing

Mass spectrometry data were collected from the Acquity Xevo TQ-XS system with MassLynx 4.2 (Waters Corporation, Milford, MA, USA). Data was processed using the TargetLynx package to generate calculated concentrations. Linear fitting of the calibration curve was performed by applying a weighting factor of 1/x.

Table S1. Mass spectrometry characteristics of the analysed SCFA in serum samples

Analyte	Precursor (m/z)	Product (m/z)	Collision energy (eV)	Retention time (min)
Acetic acid (C2:0)	166.1	91.1	16	1.24
Propionic acid (C3:0)	180.1	91.1	16	1.60
Iso-butyric acid (Iso-C4:0)	194.1	91.1	16	1.99
Butyric acid (C4:0)	194.1	91.1	16	2.13
Iso-valeric acid (Iso-C5:0)	208.1	124.1	10	2.67
Valeric acid (C5:0)	208.1	124.1	10	2.84
Hexanoic acid (C6:0)	222.1	124.1	10	3.54
2-Methylbutyric acid (2-Me-C4:0)	208.1	124.1	10	2.50
3-Methylvaleric acid (3-Me-C5:0)	222.1	124.1	10	3.32
Internal isotopically-labelled standards				
C2:0-d4	170.1	91.1	1.24	16
C3:0-13C3	183.1	91.1	1.60	16
Iso-C4:0-d7	194.1	91.1	1.97	16
C4:0-d7	201.1	91.1	2.10	16
Iso-C5:0-d9	217.1	91.1	2.64	16
C6:0-d11	233.1	91.1	3.49	20

Table S2. PERMANOVA results based on Hellinger distances comparing weeks 20 and 36.

Variable	Df	SumOfSq_Residuals	R^2	F	P(>F)
MDI_category	1	0.948	0.0259	2.0456	0.0005
Time	1	0.283	0.0077	0.6101	0.9902
Age	1	0.864	0.0236	1.8645	0.0009
BMIprepreg	1	0.783	0.0214	1.6891	0.0062
MDI_category:Time	1	0.271	0.0074	0.5841	0.9957
Residual	72	33.360	0.9137		
Total	77	36.508	1		

Table S3. PERMANOVA results based on Hellinger distances comparing weeks 28 and 36

Variable	Df	SumOfSq_Residuals	R2	F	P(>F)
MDI_category	1	1.0614	0.0336	2.2823	0.0001
Time	1	0.3423	0.0108	0.7361	0.9125
Age	1	1.1025	0.0.034	2.3707	0.0001
BMIprepreg	1	0.7596	0.024	1.6334	0.0096
MDI_category:Time	1	0.3724	0.0118	0.8008	0.8261
Residual	60	27.9024	0.88465		
Total	65	31.5405	1		

Table S4. Results of the differential abundance analysis for the samples at week 20/28 from Maaslin2*.

Feature	**Metadata**	**Value**	**Coef**	**Stderr**	**N**	**N.not.0**	**pval**	**qval**
Order_Oscillospirales	MDIcategory	HMDI	2.64	0.79	48	37	0.0018	0.1855
Turicibacter	Age_imp	Age_imp	-0.91	0.28	48	48	0.0026	0.1855
Alistipes	MDIcategory	HMDI	1.74	0.60	48	45	0.0063	0.2406
Bacteroides	BMIprepreg_imp	BMIprepreg_imp	-0.84	0.31	48	48	0.0084	0.2406
Bifidobacterium	MDIcategory	HMDI	-2.30	0.81	48	48	0.0068	0.2406
Romboutsia	Age_imp	Age_imp	-0.68	0.26	48	48	0.0126	0.3022
Turicibacter	BMIprepreg_imp	BMIprepreg_imp	0.73	0.29	48	48	0.0149	0.3065
Class_Clostridia	MDIcategory	HMDI	1.53	0.69	48	48	0.0312	0.3098
Clostridioides	Age_imp	Age_imp	-0.77	0.33	48	46	0.0237	0.3098

Clostridium	Age_imp	Age_imp	-0.81	0.33	48	48	0.0197	0.3098
Collinsella	Age_imp	Age_imp	-1.14	0.51	48	42	0.0291	0.3098
Family_Eggerthellaceae	Age_imp	Age_imp	-0.68	0.30	48	41	0.0284	0.3098
Family_Oscillospiraceae	MDIcategory	HMDI	2.01	0.90	48	36	0.0294	0.3098
Lachnospira	MDIcategory	HMDI	1.86	0.79	48	31	0.0238	0.3098
Ruminococcus_A	Age_imp	Age_imp	-1.18	0.53	48	44	0.0323	0.3098
Coprococcus	MDIcategory	HMDI	2.30	1.07	48	31	0.0374	0.3167
Phylum_Firmicutes_A	MDIcategory	HMDI	1.97	0.91	48	29	0.0368	0.3167

*This table is shown as it was obtained from Maaslin2. Key: coefficient (coef), standard error (stderr), sample size (N), number of samples where the feature is not zero (N.not.0), p-value (pval), adjusted p-value (qval).

Table S5. Results of the differential abundance analysis for the samples at week 36 from Maaslin2*.

Feature	Metadata	Value	Coef	Stderr	N	N.not.0	pval	qval
Family_Ruminococcaceae	BMIprepreg_imp	BMIprepreg_imp	-0.69	0.24	48	48	0.0069	0.2757
Family_Ruminococcaceae	Age_imp	Age_imp	-0.67	0.24	48	48	0.0069	0.2757
Order_Oscillospirales	BMIprepreg_imp	BMIprepreg_imp	-1.14	0.40	48	41	0.0074	0.2757
Turicibacter	Age_imp	Age_imp	-0.87	0.29	48	48	0.0046	0.2757
Coprococcus	MDIcategory	HMDI	2.21	0.86	48	33	0.0138	0.2956
Family_Bacteroidaceae	BMIprepreg_imp	BMIprepreg_imp	-1.13	0.43	48	40	0.0113	0.2956
Lachnospira	MDIcategory	HMDI	1.89	0.72	48	29	0.0123	0.2956

Family_Anaerovoracaceae	BMIprepreg_imp	BMIprepreg_imp	-0.67	0.28	48	45	0.0197	0.2969
Family_Oscillospiraceae	BMIprepreg_imp	BMIprepreg_imp	-0.96	0.39	48	38	0.0193	0.2969
Ruminococcus_B	MDIcategory	HMDI	-2.42	1.00	48	31	0.0198	0.2969
Order_Oscillospirales	MDIcategory	HMDI	1.92	0.81	48	41	0.0221	0.3013
Blautia_A	BMIprepreg_imp	BMIprepreg_imp	0.27	0.12	48	48	0.0265	0.3249
Class_Clostridia	BMIprepreg_imp	BMIprepreg_imp	-0.83	0.37	48	47	0.0282	0.3249
Evtepia	BMIprepreg_imp	BMIprepreg_imp	-0.84	0.40	48	34	0.0400	0.4283

Table S6. Maternal DASS-21 score at weeks 20 and 36 of pregnancy

Time point	Depression DASS scores					
	Normal		Mild		Moderate	
	LMDI (n)	HMDI (n)	LMDI (n)	HMDI (n)	LMDI (n)	HMDI (n)
Week 20	14	11	1	2	1	1
Week 36	21	20	1	1	0	1

LMDI: Low Mediterranean Diet Index, HMDI: High Mediterranean Diet Index, DASS: Depression Anxiety Stress Scale.

Time point	Anxiety DASS scores							
	Normal		Mild		Moderate		Severe	
	LMDI (n)	HMDI (n)	LMDI (n)	HMDI (n)	LMDI (n)	HMDI (n)	LMDI (n)	HMDI (n)
Week 20	10	11	2	0	4	2	0	1
Week 36	17	21	1	0	4	0	0	1

LMDI: Low Mediterranean Diet Index, HMDI: High Mediterranean Diet Index, DASS: Depression Anxiety Stress Scale.

Time point	Depression DASS scores					
	Normal		Mild		Moderate	
	LMDI (n)	HMDI (n)	LMDI (n)	HMDI (n)	LMDI (n)	HMDI (n)
Week 20	16	13	0	1	0	0
Week 36	19	20	3	1	0	1

LMDI: Low Mediterranean Diet Index, HMDI: High Mediterranean Diet Index, DASS: Depression Anxiety Stress Scale.

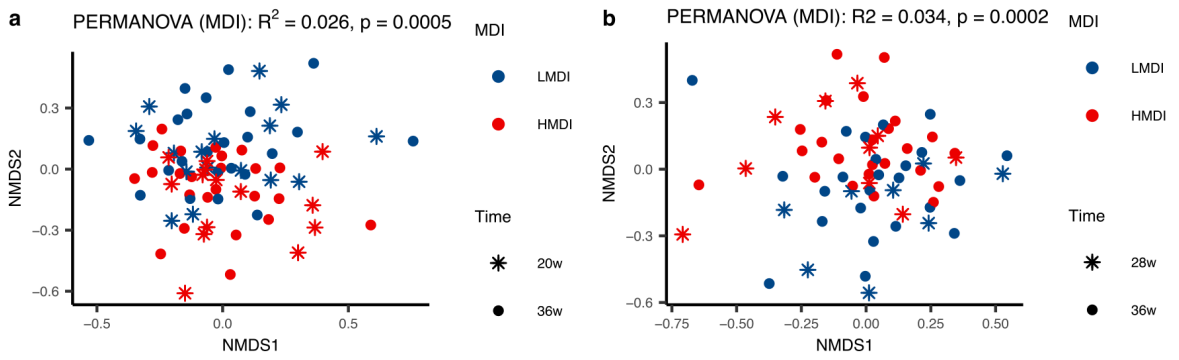

Figure S1. Non-metric multidimensional scaling (NMDS) plots of the microbial composition between samples. A) Comparison between samples collected at weeks 20 and 36. B) Comparison between samples collected at weeks 28 and 36. MDI: Mediterranean Diet Index groups, LMDI: Low Mediterranean Diet Index, HMDI: High Mediterranean Diet Index.

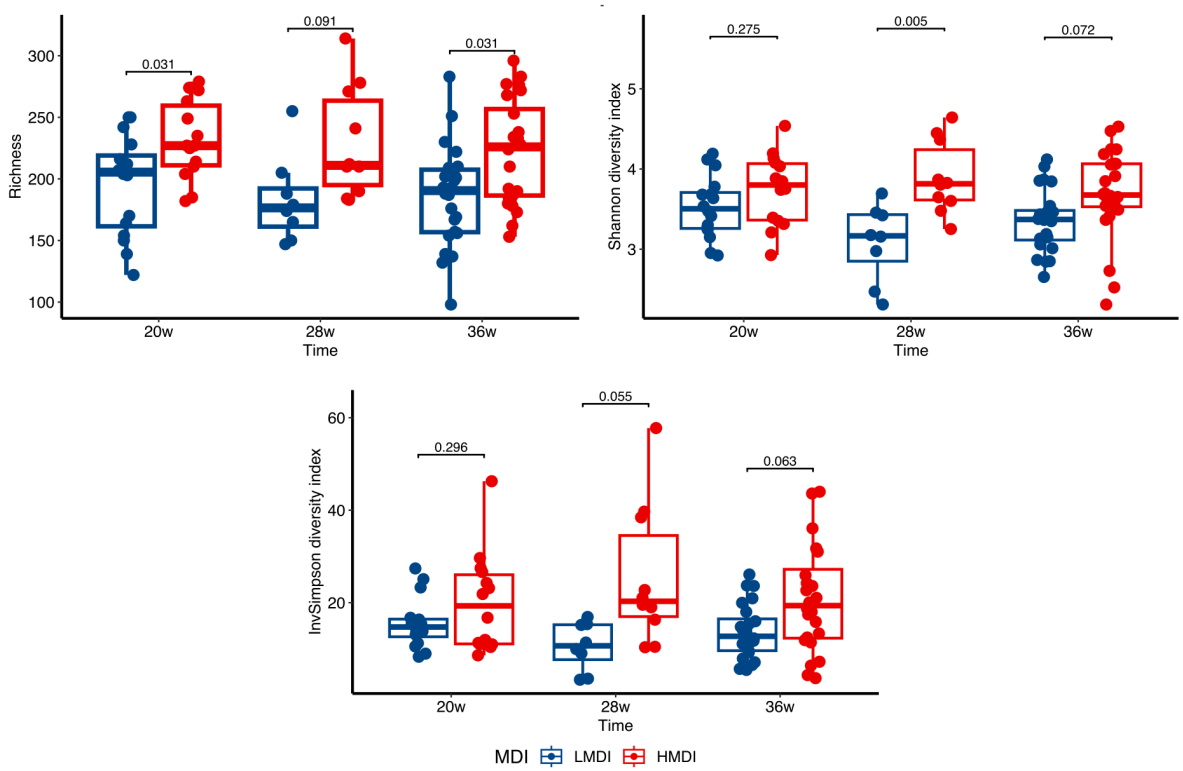

Figure S2. Alpha diversity metrics. A) Richness (total number of ASVs in the sample); B) Shannon diversity index; C) Inverse Simpson diversity index. MDI: Mediterranean Diet Index groups, LMDI: Low Mediterranean Diet Index, HMDI: High Mediterranean Diet Index. The horizontal bars over the box plots indicate the adjusted p-value of the effect of MDI in the linear models.

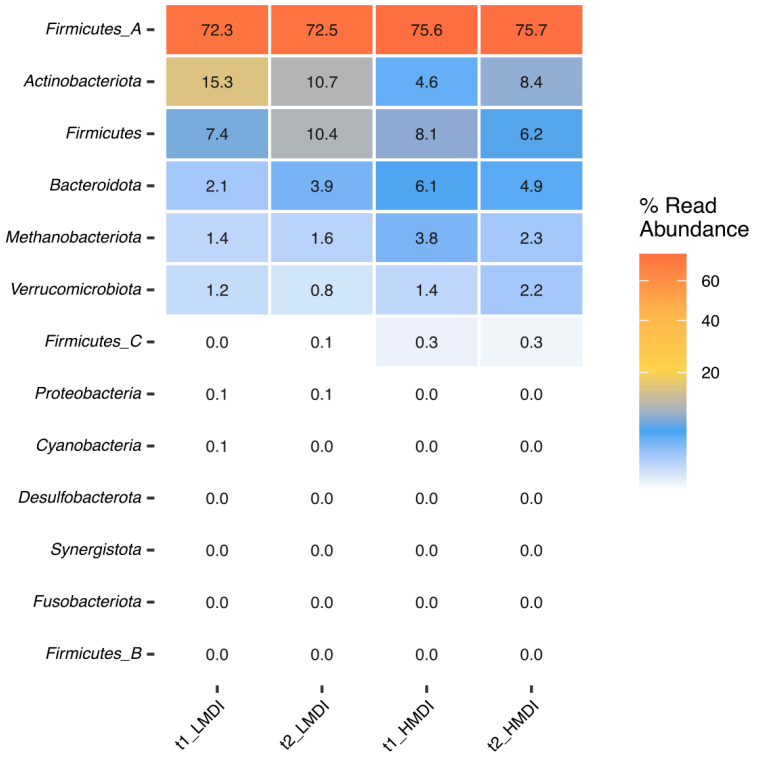

Figure S3. Heatmap of the microbial community composition at the Phylum level. This heatmap shows the average composition of all the samples within a group. The taxonomy was collapsed to the Phylum taxonomic level. MDI: Mediterranean Diet Index, LMDI: Low Mediterranean Diet Index, HMDI: High Mediterranean Diet Index. t1 represents the pooled samples from participants at weeks 20 or 28; t2 refers to samples collected from participants at week 36.

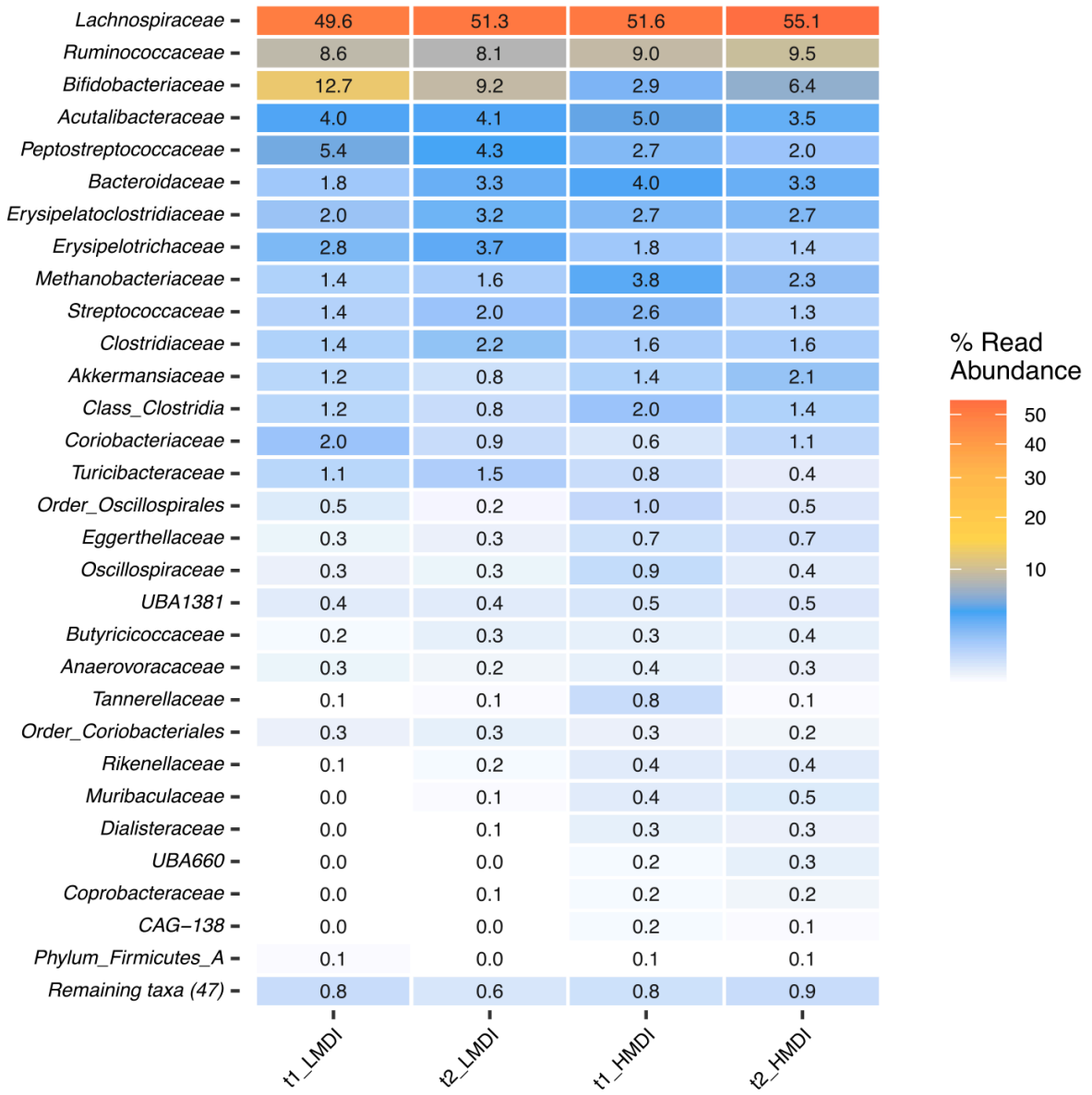

Figure S4. Heatmap of the microbial community composition at the Family level. This heatmap shows the average composition of all the samples within a group. The taxonomy was collapsed to the Family taxonomic level. Category "NA" refers to the proportion of reads that could not be classified to the Family level. MDI: Mediterranean Diet Index, LMDI: Low Mediterranean Diet Index, HMDI: High Mediterranean Diet Index. t1 represents the pooled samples from participants at weeks 20 or 28; t2 refers to samples collected from participants at week 36.