Effect on gut microbiota of a 1-y lifestyle intervention with Mediterranean diet compared with energy-reduced Mediterranean diet and physical activity promotion: PREDIMED-Plus Study

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ABSTRACT

Background: The Mediterranean diet is a well-recognized healthy diet that has shown to induce positive changes in gut microbiota. Lifestyle changes such as diet along with physical activity could aid in weight loss and improve cardiovascular risk factors.

Objectives: To investigate the effect of an intensive lifestyle weight loss intervention on gut microbiota.

Methods: This is a substudy of the PREDIMED-Plus (Prevención con Dieta Mediterránea-Plus), a randomized controlled trial conducted in overweight/obese men and women (aged 55–75 y) with metabolic syndrome. The intervention group (IG) underwent an intensive weight loss lifestyle intervention based on an energy-restricted Mediterranean diet (MedDiet) and physical activity promotion, and the control group (CG) underwent a non-energy-restricted MedDiet for 1 y. Anthropometric, biochemical, and gut microbial 16S rRNA sequencing data were analyzed at baseline ($n = 362$) and 1-y follow-up ($n = 343$).

Results: IG participants had a weight loss of 4.2 (IQR, –6.8, –2.5) kg compared with 0.2 (IQR, –2.1, 1.4) kg in the CG ($P < 0.001$). Reductions in BMI, fasting glucose, glycated hemoglobin, and triglycerides and an increase in HDL cholesterol were greater in IG than in CG participants ($P < 0.05$). We observed a decrease in *Butyricicoccus*, *Haemophilus*, *Ruminiclostridium 5*, and *Eubacterium hallii* in the IG compared with the CG. Many genera shifted in the same direction within both intervention groups, indicating an overall effect of the MedDiet. Decreases in *Haemophilus*, *Coprococcus 3*, and few other genera were associated with a decrease in adiposity parameters in both intervention groups. Changes in *Lachnospiraceae NK4A136* were positively associated with changes in MedDiet adherence.

Conclusions: Weight loss induced by an energy-restricted MedDiet and physical activity induce changes in gut microbiota. The role of MedDiet-induced changes on the host might be via short-chain fatty acid producing bacteria, whereas with energy restriction, these changes might be modulated with other mechanisms, which need to be explored in future studies. This trial was registered at http://www.isrctn.com/ISRCTN89898870 as ISRCT 89898870. *Am J Clin Nutr* 2021;114:1148–1158.

Keywords: weight loss, gut microbiota, Mediterranean diet, energy restriction, obesity

Introduction

Microbiota colonizes the human gut during or shortly after birth and continues to grow and develop until it establishes a stable environment in adults. During adulthood, the variability and complexity of the human gut microbiome are influenced by several lifestyle choices, including dietary and nondietary factors such as physical activity, stress, or smoking habits (1). Also, environmental factors, aging, medications, and diseases shift the composition and functionality of our microbes. Individuals with conditions such as diabetes, metabolic syndrome (MetS), and cardiovascular risks have shown to have a dysbiotic gut with opportunistic pathogens (2). Obesity has been associated with lower diversity and richness of the microbiota, as well as a decreased Bacteroidetes-to-Firmicutes

ratio (B/F) (3), but this remains inconclusive as some studies have failed to show this association (4, 5). Different studies support gut microbiota as an environmental factor related to the progress of obesity and metabolic disturbances (2, 6), even though the causal nature of this has not been completely understood.

Weight loss is an effective strategy for obese and overweight individuals to reduce the risk of developing metabolic disorders and cardiovascular diseases (CVDs). Lifestyle changes using different dietary strategies and increasing physical activity promotion have been recommended to lose weight (7). Diet is an important factor in modulating not only weight but also gut microbiota composition and function. Several studies have shown a change in the gut microbiota associated with specific dietary factors or patterns (8–10). A recent study conducted in the NU-AGE (New dietary strategies addressing the specific needs of elderly population for an healthy ageing in Europe) trial demonstrated that higher adherence to a Mediterranean diet (MedDiet) pattern for 1 y was associated with specific gut microbiome changes that were associated with improved health status and reduced frailty (11). Another recent study, conducted among overweight and obese participants adhering to the MedDiet or an isocaloric control diet for 8 wk, showed significant improvements in a decrease in circulating total cholesterol, insulin resistance, and fecal bile acids related to changes in gut microbiota (12). Combining the beneficial effects of an energy-restricted MedDiet and physical activity in a weight loss perspective could aid in the betterment of cardiometabolic risk factors through changing gut microbiota profile. In this substudy conducted in the framework of the PREDIMED-Plus (Prevención con Dieta Mediterránea-Plus) randomized trial, as the primary objective, we evaluated the 1-y effect of an energy-reduced MedDiet weight loss lifestyle intervention program compared with non-energy-restricted MedDiet intake on gut microbiota composition in overweight/obese adults with MetS. As a secondary objective, we explored the associations of the gut microbiota composition with respect to the components of the intervention.

Methods

Study design and participants

The present study was conducted in the frame of the PREDIMED-Plus study, with further details in **Supplemental Method 1**. The primary outcome of the parent study, PREDIMED-Plus, is weight loss and a composite of CVD incidence. Evaluation of gut microbiota composition is an intermediate outcome of the PREDIMED-Plus study. Eligible participants were community-dwelling men and women aged 55–75 y and 60–75 y, respectively, without a documented history of CVD at baseline of overweight/obesity [BMI (in kg/m^2) $\geq$27 and $\leq$40] and with at least 3 components of MetS according to the American Heart Association and National Heart, Lung, and Blood Institute. Details of the trial have been described elsewhere (13). Further details on the study can be found at https://www.predimedplus.com/. This trial was registered at http://www.isrctn.com/ISRCTN89898870 as ISRCT 89898870. Participants were not involved in the design, conduct, or reporting of the study; further information can be found in Supplemental Method 1.

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The data sets generated and analyzed during the current study are not expected to be made available outside the core research group, as neither participants' consent forms nor ethics approval included permission for open access. However, the researchers will follow a controlled data-sharing collaboration model, as in the informed consent participants agreed with a controlled collaboration with other investigators for research related to the project's aims. Therefore, investigators who are interested in this study can contact the PREDIMED Steering Committee by sending a request letter to predimed_scommittee@googlegroups.com. A data-sharing agreement indicating the characteristics of the collaboration and data management will be completed for the proposals that are approved by the Steering Committee.

Supplemental Tables 1–5, Supplemental Figures 1–6, and Supplemental Methods 1–3 are available from the "Supplementary data" link in the online posting of the article at https://academic.oup.com/ajcn/.

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Abbreviations used: BCAA, branched-chain amino acid; B/F, Bacteroidetes-to-Firmicutes ratio; CG, control group; CVD, cardiovascular disease; FDR, false discovery rate; IG, intervention group; MedDiet, Mediterranean diet; MetS, metabolic syndrome; MedScore, Mediterranean diet adherence score; P/B, Prevotella-to-Bacteroides ratio; PCoA, principal coordinate analysis; SCFA, short-chain fatty acid; sPLS-DA, sparse partial least squares discriminant analysis; T2D, type 2 diabetes; VIP, variable importance in projection.

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In this substudy, a total of 400 participants matched for age, sex, and BMI were randomly selected from the intervention group (IG, $n = 200$) and control group (CG, $n = 200$) from 2 PREDIMED-Plus study centers (Reus and Malaga). Briefly, participants randomly allocated to the IG were instructed to adhere to an energy-reduced MedDiet, accompanied by physical activity promotion, to accomplish specific weight loss objectives. Trained dietitians conducted an individual motivational interview, a group session, and a phone call each month during the intervention follow-up (1 y). The IG received an intensive intervention consisting of individualized behavioral support, and participants in the CG received information on maintaining ad libitum unrestricted caloric MedDiet with no advice on weight loss strategies such as to increase physical activity. In the case of the CG, participants received only 1 individual session and 1 group session every 6 mo to motivate and adhere to the intervention. Trained dietitians and nurses conducted the intervention and collected baseline and 1-y measurements and biological samples.

Evaluation of food consumption and anthropometric and biochemical measurements

At baseline and 12-mo follow-up visits, nurses measured waist circumference (midway between the lowest rib and the iliac crest, using an anthropometric tape), weight (using electronic calibrated scales), and height (using a wall-mounted stadiometer) twice. Dietary consumption was estimated by the dietitians using a validated FFQ, and energy and nutrient consumption were calculated using the Spanish food composition tables. Mediterranean diet adherence score (MedScore) was calculated from a modified version of a previously validated questionnaire (14) (17-point validated tool), and information on physical activity was collected using a validated questionnaire (15). Serum and plasma samples were collected at baseline and 1 y following the intervention after an overnight fast and then aliquoted and stored at –80°C. Standard enzymatic methods were conducted to evaluate serum total cholesterol, HDL cholesterol, and triglyceride concentrations. LDL cholesterol was calculated by the Friedewald formula whenever triglycerides were <300 mg/dL.

Fecal sample collection and processing

Supplemental Method 2 describes fecal sample collection. Fecal DNA extraction was conducted using the QIAamp PowerFecal DNA Kit (Qiagen) according to the manufacturer's protocol, and an additional bead-beating step of 5 min using the FastPrep-24 5G Homogenizer (MP Biomedicals) was added to the first lysing step. The quantity of DNA was evaluated using Qubit 2.0 Fluorometer-dsDNA (High Sensitivity Kit; Invitrogen). After extraction, the DNA was stored at –20°C until further processing.

16S rRNA sequencing and processing

Supplemental Method 3 provides detail on 16S rRNA gene sequencing. Briefly, we used the Ion Ribosomal 16S Kit (Thermo Fisher Scientific) that includes 2 primer sets selectively amplifying the corresponding hypervariable regions of the 16S region in bacteria: primer set V2–4–8 and primer set V3–6, 7–9. After sequencing, the individual sequence reads were filtered using Ion Reporter Software V4.0 (Thermo Fisher Scientific)to remove low-quality and polyclonal sequences. Data were processed and separated into 6 hypervariable regions using an adapted script available from Mas-Lloret et al (16). Only variable region V4 was used for further analyses. These files were imported to QIIME2, and the DADA2 pipeline was followed (see Supplemental Method 3). Taxonomy was assigned to the clustered sequences with SILVA 132 as the 16S classifier database. Mitochondrial features and features unidentified at the phylum level were removed in the preprocessing step in R (v 3.6) (17). The MetagenomeSeq package was used to normalize the samples using the cumulative sum scaling and log transformation method.

Bioinformatics and statistical analysis

Baseline characteristics of study participants were described as mean and SD or median with 25% and 75% IQR (based on distribution) for quantitative variables and as percentages for categorical variables. Differences in baseline characteristics were evaluated with χ^2 tests for categorical variables, t tests (for normally distributed variables), and Wilcoxon tests (for nonnormally distributed variables). Effects of intervention on changes in different variables were evaluated using Wilcoxon tests and are shown appropriately according to their distribution. Abovementioned Wilcoxon and t tests were evaluated using package MatrixTests in R (v 3.6.2) (18), and significance was determined at $P < 0.05$.

For the microbiome analysis, normalized data from the MetagenomeSeq package were used (19). The α diversity (chao1, Shannon index), B/F, log of *Prevotella*-to-*Bacteroides* ratio (P/B) [adapted from Roager et al. (20)], and phylogenetic distance were evaluated using packages microbiome and picante (21, 22). Effect of intervention (Time × Treatment) adjusted by baseline weight, sex, and study center was used to estimate the changes in α diversity, B/F, P/B, and phylogenetic distance by a linear mixed model. In addition, for the B/F and P/B, we also adjusted by baseline ratio values. Principal coordinate analysis (PCoA)–based β diversity (weighted UniFrac distance, unweighted UniFrac, Bray–Curtis dissimilarity) was evaluated with the vegan package in R (v 3.6.2), and PERMANOVA (permutational multivariate analysis of variance) was conducted with the *adonis* function (999 permutations) using participants as *strata* and also adjusting for baseline weight, sex, and study center (23). The condition for homogeneity was verified using the *betadisper* function.

To investigate the changes in microbial genera between the intervention groups, the *fitZig* function from the MetagenomeSeq package that implements a zero-inflated Gaussian model was used. We accounted for the repeated measures with a mixed model, and the analysis was carried out at the genus level. According to the authors' recommendation (24), we calculated effective sample sizes and retained only the genera that had an effective sample size more than the median of all samples. To reduce the type I error rate in multiple testing, we used the false discovery rate (FDR) approach to correct P values. An FDR of 10% was set for the between-group analysis. For the within-group analysis, we used the *fitfeature* function that uses a zero-inflated

TABLE 1 Baseline characteristics of study participants[1]

Characteristic	IG	CG
No.	183	179
Age, y	64.3 (5.1)	65.1 (4.9)
Sex, M/F, *n*	97/86	77/102
Weight, kg	89.7 (13.6)	86.7 (11.56)[2]
BMI, kg/m^2	33.4 (30.8, 36.0)	32.9 (30.5, 35.6)
Waist circumference, cm	110.7 (9.8)	108.9 (9.55)
Diabetes (yes), % (*n*)	26.2 (48/135)	20.6 (37/142)
Hypercholesteremia, (no/yes), % (*n*)	94.5 (10/173)	93.8 (11/168)
Total cholesterol, mg/dL	203.0 (177.0, 224.5)	197.0 (172.5, 226.5)
LDL cholesterol, mg/dL	116.0 (94.5, 140.8)	115.0 (97.0, 139.5)
HDL cholesterol, mg/dL	46.0 (40.0, 57.0)	47.0 (42.0, 54.0)
Triglycerides, mg/dL	151.0 (55.3, 246.8)	152.0 (68.5, 235.5)
Glucose, mg/dL	104.0 (92.5, 118.0)	103.0 (94.0, 116.0)
Glycated hemoglobin, %	5.8 (5.6, 6.3)	5.8 (5.5, 6.3)
Physical activity, METs-min/wk	1627 (682, 3650)	1767 (839, 3308)
Energy intake, kcal/d	2546.5 (543.7)	2416.6 (514.7)
17-point Mediterranean adherence score	7.7 (2.1)	8 (2.4)
Smoking, *n*		
Current smoker	32	24
Former smoker	65	68
Never	85	87
No data	1	
Study center (Malaga/Reus), *n*	66/117	73/106

[1] Values expressed as Mean (SD) for normally distributed variables and Median (25%, 75% IQR) for non-normal distributions unless otherwise indicated. Chi-square, Wilcoxon, and *t* tests were conducted for categorical, nonnormal, and normally distributed variables, respectively. CG, control group; IG, intervention group; METs, Metabolic equivalent of task.

[2] Significant difference < 0.05.

lognormal model. Log-fold changes in *fitfeature* were calculated from the coefficients of the zero-inflated lognormal model. In addition, we calculated effective sample sizes and report the only genus that passes the threshold. For this analysis, an FDR of 5% was set.

We also used a second approach using sparse partial least squares discriminant analysis (sPLS-DA) to compare the results from those obtained in MetagenomeSeq. This supervised method from the mixOmics package selects features that can best discriminate the 2 intervention groups at the end of the intervention (25). The samples were center log-ratio transformed (using package Hotelling) and indexed with respect to their baseline samples, which accounts for within-participant variations [adapted from Lee et al. (26)]. The number of components and features per component were calculated using the *tune.splsda* function, based on minimum balanced error rate. Each feature selected has an associated loading representing the relative importance of that feature on the component for discriminating the groups. This is represented as variable importance in projection (VIP), and a feature with a VIP of >1 is regarded as important for discrimination. Features having VIP >1 were chosen to be compared with the results of MetagenomeSeq.

The associations between changes in measured biochemical variables and changes in microbial genera that significantly changed in the IG or the CG (and VIP >1) were analyzed using a NBZIMM package in R, which uses a negative binomial mixed model and allows to adjust for covariates (27). Coefficients obtained from this along with adjusted *P* values were visualized in R software using ggplots2 (28). To detect the associations in the overall population, we adjusted for group of intervention, study center, sex, and baseline weight. *P* values were corrected by FDR for multiple testing.

Phylogenetic Investigation of Communities by Reconstruction of Unobserved States analysis

Predicted metagenome functions were performed using Phylogenetic Investigation of Communities by Reconstruction of Unobserved States plugin (29) within QIIME2 with the q2-picrust2 plugin. MetaCyc pathways (30) were normalized within QIIME2 and analyzed using the open-source software STAMP with Welch's *t* test option (31). Those pathways with a $P < 0.05$ were posteriorly analyzed in QIIME2 with the longitudinal plugin for paired sampled comparisons. For this analysis, an FDR of 10% was set.

Results

Characteristics of the study population

A flowchart of selected participants is represented in **Supplemental Figure 1**. A total of 400 participants matched by age, sex, and BMI were randomly allocated to this study (200 per intervention group). After preprocessing steps (as mentioned in Supplemental Figure 1 and Supplemental Method 3), data at baseline were available for 183 participants in the IG and 179 participants in the CG, corresponding to 171 participants in the IG and 172 participants in the CG after 1 y. There were no significant differences in the measured baseline variables between groups (**Table 1**), except for higher body weight in the IG ($P = 0.03$).

TABLE 2 Effects of intervention on anthropometric and biochemical variables measured[1]

Characteristic	Changes in IG ($n = 171$)	Changes in CG ($n = 172$)	P value
Weight, kg	–4.2 (–6.8, –2.5)	–0.2 (–2.1, 1.4)	<0.001
BMI, kg/m^2	–1.6 (–2.5, –0.9)	–0.05 (–0.8, 0.6)	<0.001
Waist circumference, cm	–5 (–9.0, –1.8)	0.0 (–2.5, 2.0)	<0.001
Total cholesterol, mg/dL	–1.0 (–17.5, 14.0)	–2.0 (–22.0, 14.0)	0.767
LDL cholesterol, mg/dL	1.0 (–14.5, 14.5)	–2.0 (–19.0, 13.0)	0.577
HDL cholesterol, mg/dL	3.0 (–0.5, 6.0)	2.0 (–2.3, 6.0)	0.012
Triglycerides, mg/dL	–19.0 (–52.5, 9.5)	–3.5 (–41.5, 28.0)	0.028
Glucose, mg/dL	–5.0 (–14.0, 2.0)	0.5 (–7.3, 8.0)	<0.001
Glycated hemoglobin, %	–0.1 (–0.3, 0.1)	0.0 (–0.1, 0.2)	0.002
Physical activity, METs-min/wk	1154 (0, 2633)	0 (–787, 743)	<0.001
17-point Mediterranean adherence score	6.0 (4.0, 8.5)	2.0 (1.0, 5.0)	<0.001
Energy intake, kcal/d	–318.2 (–655.6, 3.2)	44.3 (–329.3, 391.7)	<0.001

[1] Values expressed as Median (25%, 75% IQR). Wilcoxon test was conducted for evaluating the differences between 2 groups of intervention. CG, control group; IG, intervention group; METs, Metabolic equivalent of task.

Diet, food groups (**Supplemental Table 1**), and physical activity changes (**Table 2**) were in the expected direction, with significant improvements in the IG compared with the CG.

After 1 y (Table 2), IG participants lost an average of 4.2 (IQR, –6.8, –2.5) kg compared with 0.2 (IQR, –2.1, 1.4) kg in the CG ($P < 0.001$). Reductions in BMI, waist circumference, and concentrations of triglycerides, glucose, and glycated hemoglobin were greater in IG than in CG participants (all, $P < 0.05$), whereas a significantly higher increase in HDL cholesterol was observed in the IG compared with the CG ($P < 0.05$) (Table 2). Even though participants belonged to a Mediterranean region, the baseline MedDiet score was equal to or below the median MedDiet adherence score (low, ≤7; medium, 8–10; and high, 11–17) in both arms of intervention (32). This adherence increased with 1 y of intervention in both groups.

Changes in α and β diversity

No significant differences in α diversity indices (Chao1, Shannon) adjusted for body weight at baseline between the 2 intervention groups or within groups were observed (**Table 3**). Time and treatment interaction did not vary significantly for weighted UniFrac, unweighted UniFrac, or Bray–Curtis dissimilarity (Table 3, **Figure 1**). Likewise, no differences were noted at baseline and the 1-y time point (**Supplemental Table 2**). B/F increased significantly in the IG compared with the CG ($P < 0.05$), but no changes in P/B were observed (**Supplemental Table 3**, **Supplemental Figure 2**A,B). No differences in baseline α and β diversity, B/F, and P/B were observed between the groups.

TABLE 3 Effects intervention on changes in α and β diversity metrics[1]

Diversity measures ($n = 343$)	Treatment * Time (P value)
Chao1[2]	0.16
Shannon diversity[2]	0.15
Phylogenetic distance[2]	0.21
Weighted UniFrac[3]	0.72
Unweighted UniFrac[3]	0.23
Bray Curtis dissimilarity[3]	0.33

[1] Effect of intervention (Treatment * Time) evaluated by linear mixed model adjusted for sex, study center, and baseline weight for chao1, Shannon diversity, and phylogenetic distance. Weighted UniFrac, unweighted UniFrac, and Bray–Curtis dissimilarity were evaluated by PERMANOVA (permutational multivariate analysis of variance) adjusted for sex, study center and baseline weight, and participants as strata.

[2] α diversity indexes.

[3] β diversity indexes.

Effect of intervention on changes in gut microbiota

Differential abundance analysis between the 2 groups of intervention conducted at the genus level showed *Haemophillus*, *Butyricicoccus*, *Eubacterium hallii*, and *Ruminiclostridium 5* were reduced and *Coprobacter* and uncultured bacterium (from Rhodospirillales order) increased in the IG compared with the CG (all FDR $P < 0.1$) (**Table 4**, **Supplemental Figure 3**A–H) while adjusting for sex, study center, and baseline weight. LogFC represents the coefficient of change in the MetagenomeSeq model evaluated comparing the IG with the CG. Some of the genera (*Haemophillus*, *E. halii*, *Ruminococcus NK4A214*) that were found to vary significantly between the groups in the MetagenomeSeq model also contributed to characterizing the IG and the CG in the sPLS-DA model (**Supplemental Figure 4**).

Figure 2 shows the Venn diagram of genera that shifted within both groups. Fifteen genera in the IG (Figure 2) and 16 genera in the CG (Figure 2) were significantly different from baseline to 1 y within each intervention group and had a VIP >1 from the sPLS-DA model. Within IG analysis, 7 among 15 genera reducing in relative abundance belonged to the family Lachnospiraceae, whereas some of these such as *Roseburia* and *Dorea* increased in the CG (**Supplemental Tables 4–5**). An increase in some short-chain fatty acid (SCFA) producers such as *Lachnospira* and *Lachnospiraceae NK4A136* group was observed in both intervention groups (Supplemental Tables 4–5). Overall predominant changes in both groups belonged to genera from Lachnospiraceae and Ruminococcaceae families.

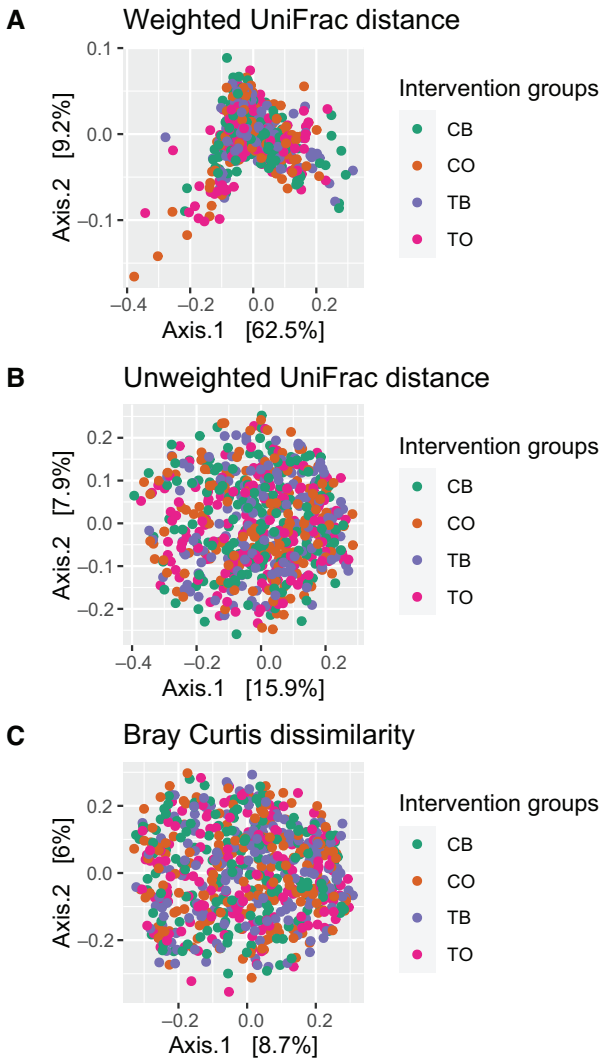

FIGURE 1 (A) Principal coordinate analysis (PCoA) of weighted UniFrac distance showing 2 groups of intervention at 2 time points. (B) PCoA of unweighted UniFrac distance. (C) PCoA of Bray–Curtis distance ($n = 343$). CB, control group at baseline; CO, control group at year 1; TB, intervention group at baseline; TO, intervention group at year 1.

Associations between changes in gut microbiota and measured variables

In the overall population, as well individually within groups, changes in *Eubacterium eligens* were negatively associated with changes in weight (FDR $P < 0.05$), waist circumference (insignificant FDR), glucose (insignificant FDR), and HbA1c (insignificant FDR) (**Figure 3**, **Supplemental Figures 5**A, **6**A). *Haemophilus*, which varied significantly between the groups of intervention, was positively associated with weight changes in the overall population (Figure 3). *Parabacteroides* was positively associated with triglyceride concentrations in the overall population, as well as in the IG and the CG (Figure 3, Supplemental Figures 5A, 6A). Interestingly, *Phascolarbacterium*, which was positively associated with energy intake, also followed the same direction for weight, BMI, waist circumference, and glucose but was negatively associated with physical activity. Changes in fiber intake were negatively associated with changes in *Haemophilus* but positively associated with changes in *E. hallii* and *Ruminococcaceae UCG-003* (**Figure 4**). *Lachnospiraceae NK4A136* group was also positively associated with MedScore (Figure 4). Few other associations within the IG and the CG were observed (Supplemental Figures 5A,B, 6A,B).

Changes in bacterial predicted metagenomics functions during intervention

Metabolic pathways belonging to the biosynthesis of nucleotides, nucleosides, and amino acids and carbohydrates changed significantly between the 2 intervention groups (**Figure 5**). Compared with the CG, fermentation pathways leading to the generation of energy were reduced in the IG.

Discussion

We report for the first time, to our knowledge, the effect of a large long-term lifestyle-based weight loss intervention with energy-reduced MedDiet and increased physical activity on gut microbiota. Several changes in the relative abundance of genera have been observed within and between the intervention groups that can be attributed to weight loss, diet, and physical activity. Changes observed in the gut microbiota profile were also associated with changes in some CVD risk factors.

We observed a significant change in the relative abundance of members belonging to the Firmicutes phylum (decreasing: *Butyricoccus*, *Ruminiclostridium 5*, and *E. hallii*; increasing: *Ruminococcaceae NK4A214*, *Coprobacter*) and a significant increase in the B/F in the IG compared with the CG, which could partly be explained by higher weight loss in the IG compared

TABLE 4 Differentially abundant genus between groups of intervention[1]

Genus	logFC (ΔIG–ΔCG)	*P* value	Adjusted *P* value
Haemophilus	–7.6	<0.001	<0.001
Butyricicoccus	–4.2	<0.001	<0.001
Ruminiclostridium 5	–2.2	0.003	0.09
Eubacterium hallii	–2.2	0.006	0.08
O_Rhodospirillales_F_uncultured_uncultured bacterium	4.3	0.006	0.05
Ruminococcaceae NK4A214	2.6	0.007	0.08
Coprobacter	2.3	0.030	0.08

[1]Model adjusted for baseline weight, sex, and study center. logFC is the β estimate of the adjusted model. *P* value adjusted by false discovery rate for multiple testing. CG, control group; IG, intervention group.

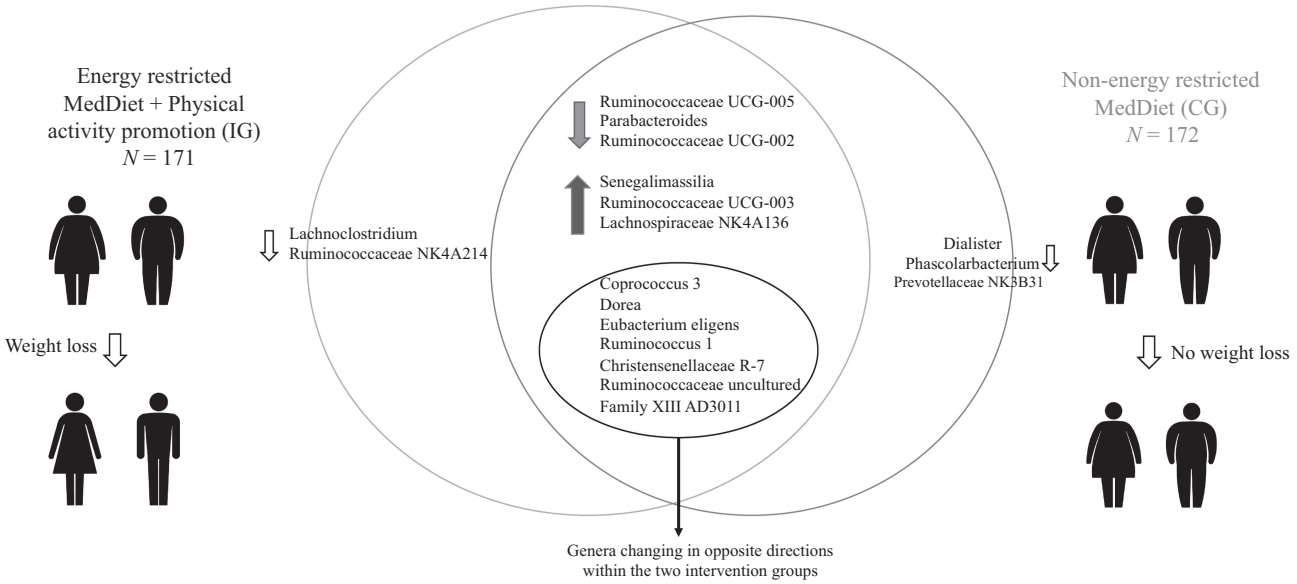

FIGURE 2 Venn diagram representing IG and CG by genera varying within groups evaluated with MetagenomeSeq and having a variable importance in projection >1 from the sparse partial least squares discriminant analysis model. Genera shifting in the same direction as well in opposite directions within each intervention groups are shown. CG, control group; IG, intervention group.

with the CG. Even though widely debated, it has been reported that during weight loss, the B/F increases, suggesting that it may respond to energy restriction (3, 33, 34). An increase in B/F has also been reported with higher adherence to MedDiet as well as low animal protein intake (35).

Other results from the above MedDiet adherence study (35), indicating an increase in the relative abundance of *Dorea*, *Roseburia*, and *Coprococcus* (all reported as SCFA producers of the Lachnospiraceae family), also were in line with our results only in the non-energy-restricted MedDiet group (CG). However, in the IG, we observed these taxa to reduce in 1 y of intervention. Correspondingly, we also observed a decrease in the predicted fermentation pathways in the IG compared with the CG. Although the reduction in these carbohydrate/fiber-using SCFA producers could indicate contradictory findings, some studies have observed an increase in SCFA gut production in obese compared with normal-weight individuals (36, 37). Whether this increase in SCFA producers may be the cause or the consequence

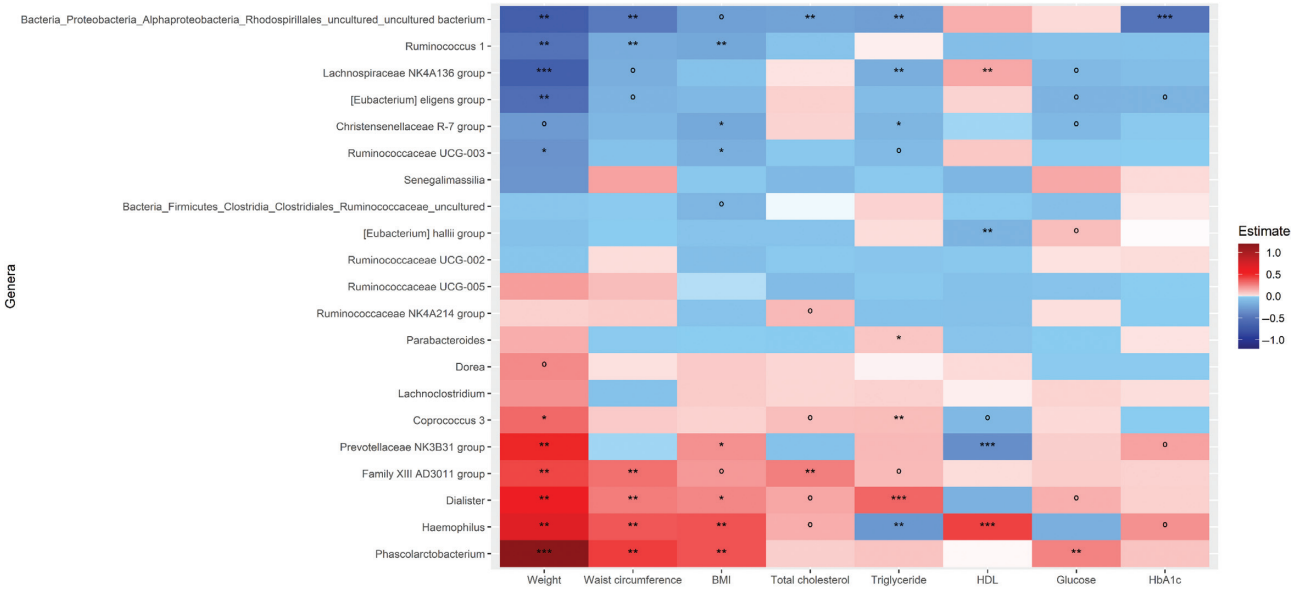

FIGURE 3 Heat plot showing associations in overall study population ($n = 343$) between changes in microbial genera and clinical variables. Model evaluated by negative binomial mixed model, adjusting for covariates sex, study center, and baseline body weight; adjusted P value denoted by $^{***}P < 0.001$, $^{**}P < 0.05$, $^{*}P < 0.1$, and $^{\circ}P < 0.2$.

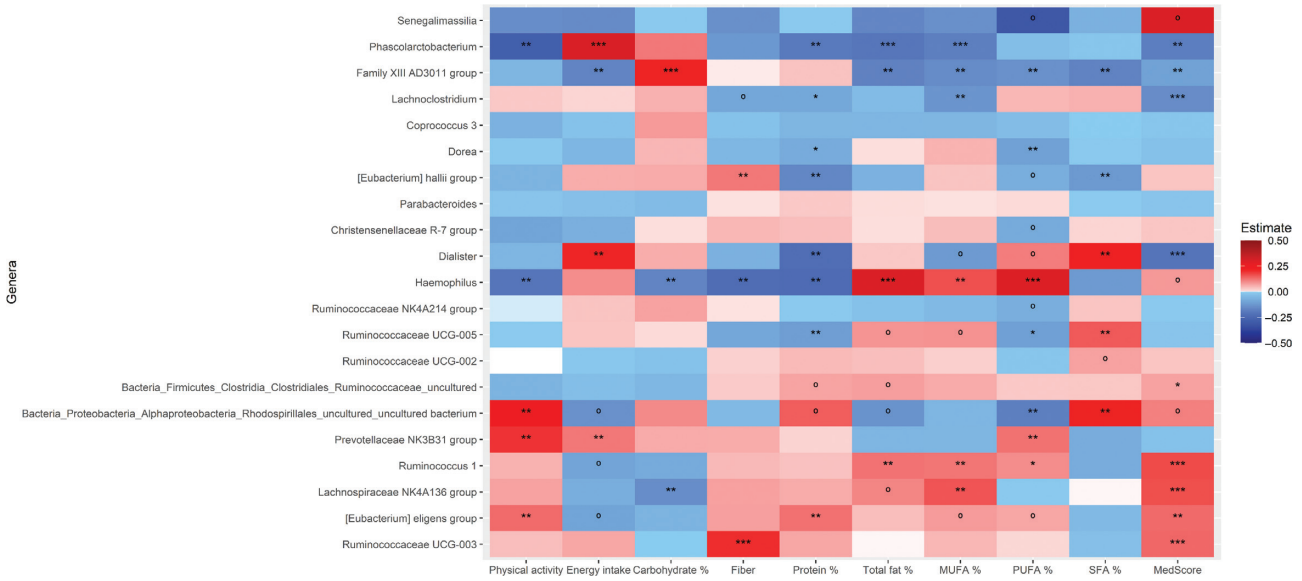

FIGURE 4 Heat plot showing associations in overall study population ($n = 343$) between changes in microbial genera and energy intake variables. Model evaluated by negative binomial mixed model, adjusting for covariates sex, study center, and baseline body weight; adjusted P value denoted by $^{***}P < 0.001$, $^{**}P < 0.05$, $^{*}P < 0.1$, and $^{o}P < 0.2$.

of obesity remains to be elucidated. The high-energy deriving capacities of carbohydrate/polysaccharide-using bacteria could create a net energy excess for the host, contributing to obesity. However, SCFAs, especially butyrate and their producers, have been well associated with several beneficial health effects (38); hence, a careful evaluation of their composition as well quantity is required to infer further.

Even though there were reductions in certain SCFA producers in the IG, we observed within the same group a selective increase in other SCFA producers (39) such as the *Lachnospiraceae*

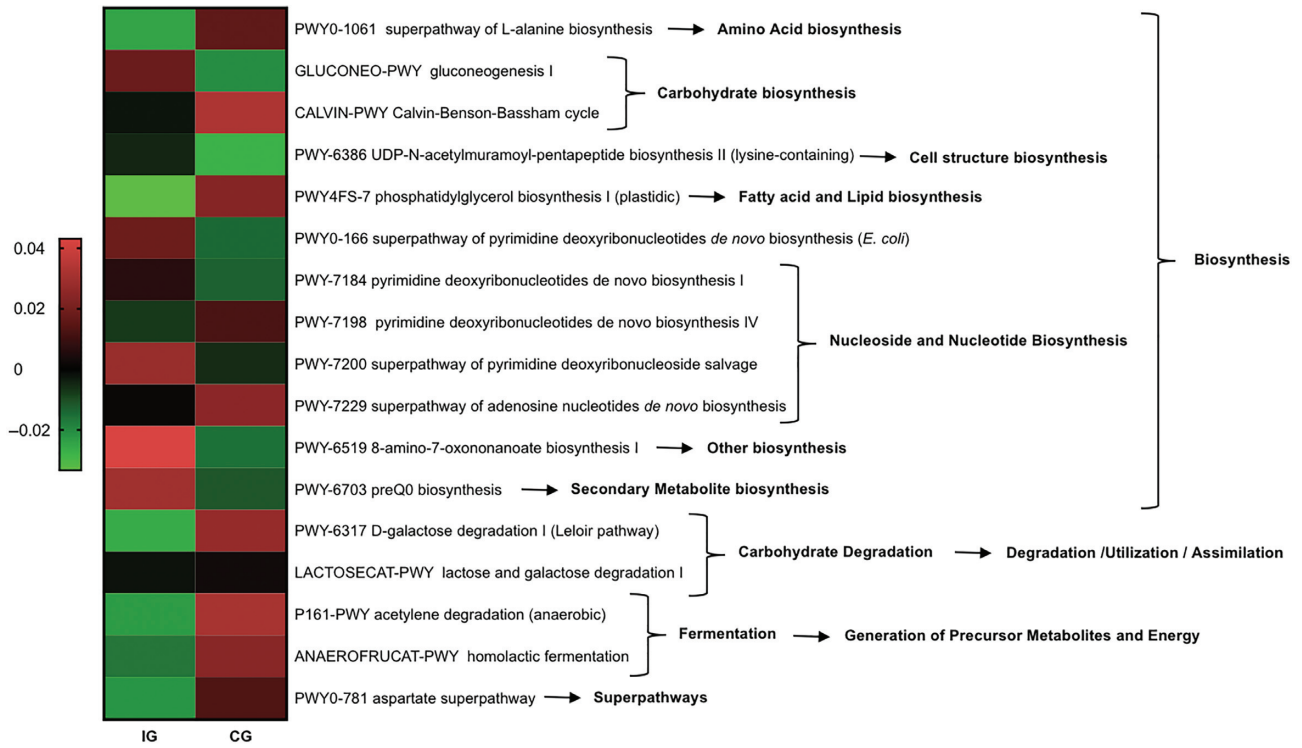

FIGURE 5 Heat map representing median values of significantly increasing or decreasing predicted metagenome pathways in the IG ($n = 171$) and CG ($n = 172$). CG, control group; IG, intervention group.

NK4A136 group and *Ruminococcaceae* (*UCG-003*, *UCG-002*), which also were associated positively with MedScore. We also observed that some SCFA-producing genera (*Lachnospira*, *Lachnospiraceae NK4A136* group, and *Alistipes*) shifted in the same direction within both intervention groups, reflecting overall the effect of MedDiet on gut microbiota. Increases of proteins, polyphenols, and unsaturated fats have shown inhibitory activities to certain bacterial genera (40–42). In parallel, in the IG, participants consumed higher protein, polyphenols, and unsaturated fats compared with those in the CG, possibly leading to selective enrichment in certain SCFA producers compared with others that might be inhibited by a synergy of the abovementioned components. It has been demonstrated in a mice study that calorie restriction could limit butyrogenic enzymes and promote propiogenic enzymes, which could lead to competition and selective growth of SCFA producers (43, 44).

Changes in *Coprococcus 3* were positively associated with changes in weight, total cholesterol, and triglycerides and negatively with HDL cholesterol in the overall population. In line with our results, enrichment of the *Coprococcus* genus has been associated with a high lifetime CVD risk profile in Bogalusa Heart study participants, as well as with the obese phenotype (45).

Not only *Coprococcus* but also other genera majorly belonging to the Lachnospiraceae family (*Blautia*, *Dorea*, *Roseburia*, *Coprococcus 3*) and *Ruminococcus 1* were observed to be changing in opposite directions in the IG and the CG. We observed a positive association for changes in the relative abundance of *Coprococcus 3* and *Dorea* with changes in weight significantly in the overall population and nonsignificantly in both intervention groups, consistent with a Swedish study (46). This study also reported a positive association of these genera with plasma branched-chain amino acids (BCAAs), usually increased in type 2 diabetes (T2D) and MetS (47, 48). Similar observations were made in the METSIM (METabolic Syndrome In Men) cohort, in which *Blautia* was associated with higher BMI and also higher circulating BCAAs, whereas *Chistensenellaceae* R-7 group abundance was negatively associated with BCAAs (49). Consistently, we found a negative association in the *Chistensenellaceae* R-7 group with changes in weight, BMI, triglycerides, and plasma glucose. It has been demonstrated that following the MedDiet enriched with extra virgin olive oil reduced circulating concentrations of BCAAs and was associated with a lower risk of T2D (50). Taking these findings into consideration, we suspect BCAAs as one of the pathways for glucose regulation in the IG via MedDiet-associated weight loss and corresponding changes in gut microbiota (51, 52). These results could also indicate that even with following the same dietary pattern, factors such as energy restriction and physical activity could play an additional beneficial role in overweight/obese individuals by altering glucose regulation via BCAAs (53).

In the IG, we also observed changes in some previously bile acid–associated bacteria, such as *Lachnoclostridium* (containing members of 7α-dehydroxylating capacity) and *Bilophila* (deconjugator of taurine–bile acid), that have shown to control lipid and glucose metabolism in mice studies (54, 55). Consistently, we observed a positive (nonsignificant) association between *Lachnoclostridium* and glucose. The observations we make above are specific to the IG, indicating that calorie restriction along with an increase in physical activity could modulate bile-related bacteria (56). Compared with dietary interventions, very few studies have been conducted studying the effect of physical activity on gut microbiota, with contradictory results. *Haemophilus* and *Phascolarctobacterium*, which overall had shown a positive association in this study with risk factors assessed, were also negatively associated with changes in physical activity. We suspect the associations we observe here are not solely dependent on physical activity but rather a synergy between energy homeostasis and nutrient intake.

Predicted metagenomics functions have been shown to differ between adults with different body weight and health status. In our study, we observed that predicted functions of the bacterial community in the gut of the IG were trying to adapt to energy restriction by increasing biosynthesis pathways, especially carbohydrate and nucleotide biosynthesis. However, as protein and fat intake increased in the IG, we also observed a decrease in amino acids and lipid biosynthesis, indicating an adaptation to diet. Many of the observations made in this study should also be interpreted in terms of calorie restriction as it has been reported that calorie restriction could alter gut microbiota and their functionality independent of a dietary regimen (57, 58).

With the exception of a few landmark studies (11, 59), our study explores the effect of a healthy lifestyle intervention on gut microbiota in a comparatively large sample population and follow-up (35, 44, 60). The randomized controlled trial design of our study allows us to establish causality when assessing the effect of the interventions, being one of the most important strengths, but this does not apply when we assess associations as secondary analyses. Another strength of the present study is that we have observed significant differences between groups in all components of the intervention (weight loss, adherence to MedDiet, and physical activity) in the expected direction, allowing us to test for potential effects of the intervention on gut microbiota. The nature of the intervention comprising dietary intervention, behavioral therapy, and physical activity promotion indicates the multilevel intervention strategy that promotes participants to follow the intervention and obtain clinical benefits.

As much as this multifaceted intervention strategy is beneficial, it implies a limitation on the inference of results that cannot be attributed solely to a single component of the intervention. Along with this, some limitations of this study also deserve to be mentioned. First, our findings are limited to adults with high BMI who also met the criteria for MetS and were living in a Mediterranean country. Therefore, they cannot be generalized to other populations or all individuals with MetS. Second, the lack of data on fecal metabolites and species-level taxonomy does not allow us to infer further the pathways associated with the associations we have observed. Third, the dietary records were collected from a self-reported questionnaire, which might over/underestimate the intake of certain food groups. Future studies with a comprehensive set of metabolomics, metagenomics, and intermediate time points would allow us to better understand the transition of gut microbiota during the weight loss period.

Overall, in this 1-y lifestyle-based intervention, we observed that an energy-restricted Mediterranean diet with physical activity and behavioral support induced weight loss and improved CVD-associated risk factors. A decrease in several members of Firmicutes, especially belonging to the Lachnospiraceae, and a

selective increase in some SCFA producers were observed in the IG. This work identifies that even with similar healthy dietary patterns, the addition of an intervention program enhancing calorie restriction and physical activity could have a significant benefit on the CVD risk factors potentially modulated via the gut microbiota.

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The authors' contributions were as follows—JS-S, MB, MAM-G, FJT, DC, JVL, JV, and MF: designed the study; JM, SG, AA, IM-I, JCF-G, LT-C, RF-C, RO, AMG-P, OC and MRB-L: provided sample collection and processing; JM, IMI, SG, and AA: conducted the statistical analysis; MB, FT, and JS-S: provided supervision; JM, IMI, JS-S, MB, and FJT: wrote the manuscript; and all authors: read and approved the final manuscript.

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