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# Effect of 1-year lifestyle intervention with energy-reduced Mediterranean diet and physical activity promotion on the gut metabolome and microbiota: a randomized clinical trial

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## Abstract

**Background**: The health benefits of the Mediterranean diet (MedDiet) have been linked to the presence of beneficial gut microbes and related metabolites. However, its impact on the fecal metabolome remains poorly understood.

**Objective:** Our goal was to investigate the weight loss effects of a 1-year lifestyle intervention based on an energy-reduced MedDiet coupled with physical activity (intervention group), compared to an *ad libitum* MedDiet (control group), on fecal metabolites, fecal microbiota, and their potential association with cardiovascular risk factors

**Methods:** A total of 400 participants (200 from each study group), aged 55-75 years, and at high cardiovascular risk, were included. Dietary and lifestyle information, anthropometric measurements, blood biochemical parameters, and stool samples were collected at baseline and after 1 year of follow-up. Liquid chromatography-tandem mass spectrometry was used to profile endogenous fecal metabolites, and 16S amplicon sequencing was employed to profile the fecal microbiota.

**Results:** Compared to the control group, the intervention group exhibited greater weight loss and improvement in various cardiovascular risk factors. We identified intervention effects on four stool metabolites and subnetworks primarily composed of bile acids, ceramides, and sphingosines, fatty acids, carnitines, nucleotides, and metabolites of purine and the Krebs cycle. Some of these were associated with changes in several cardiovascular risk factors. Additionally, we observed a reduction in the abundance of the genera *Eubacterium Hallii* group and *Dorea*, and an increase in alpha diversity in the intervention group after 1 year of follow-up. Changes in the intervention-related microbiota profiles were also associated with alterations in different fecal metabolite subnetworks and some cardiovascular risk factors.

**Conclusions:** An intervention based on an energy-reduced MedDiet and physical activity promotion, compared with an *ad libitum* MedDiet, was associated with improvements in cardiometabolic risk factors, potentially through modulation of the fecal microbiota and metabolome.

**Abbreviations:**

3-MAA, 3-methyl-adipic acid
BMI, body mass index; FDR, false discovery rate
CG, control group
CVD, cardiovascular disease
DPA, docosapentaenoic acid
FDR, false discovery rate
HOMA-IR, homeostasis model assessment of insulin resistance
IG, intervention group
LDL, low-density lipoprotein
LC-MS, liquid chromatography-tandem mass spectrometry
MET, metabolic equivalent of tasks
MEDAS, Mediterranean Diet Adherence Screener
MedDiet, Mediterranean diet
PCoA, principal coordinate analysis
PERMANOVA, permutational multivariate analysis of variance
PREDIMED, PREvención con Dieta MEDiterránea
PUFA, polyunsaturated fatty acids
WGCNA, Weighted gene co-expression network analysis

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**Keywords**: Lifestyle intervention; Mediterranean diet; cardiocascular risk factor; metabolic syndrome; fecal microbiota; fecal metabolome

## Introduction

The traditional Mediterranean diet (MedDiet) is characterized by a high intake of vegetables, fruits, legumes, whole cereals, and nuts; moderate consumption of fish and seafood; moderate-low consumption of dairy products; low consumption of meat and meat products; moderate alcohol

intake (in the form of red wine during meals); and the use of olive oil as the main source of fat (1). It has been widely demonstrated that the MedDiet pattern represents a nutritional strategy with significant beneficial effects for the prevention of cardiovascular diseases (CVD) (2), obesity (3), and related metabolic consequences (4), and reducing all-cause mortality (5).

Greater adherence to the MedDiet has also been positively associated with beneficial gut bacteria and derived microbiota-related metabolites (6). These effects have been partially explained by the increase of fiber-degrading species and anti-inflammatory responses in the human body (7). However, the effect of the MedDiet on gut microbiota and plasma metabolome is heterogenous across the studies and the potential effects on cardiovascular risk factors remain unsettled (8,9).

Blood metabolome is commonly used in human studies to explore the associations of gut microbiota-derived metabolites with cardiometabolic diseases. Combining the results of plasma metabolomics and 16S sequencing, it is possible to identify specific networks that suggest an interplay between diet, circulating metabolites, and gut microbiota (10). For instance, higher adherence to the MedDiet improved postprandial glucose metabolism and insulin sensitivity in subjects with obesity/overweight possibly mediated by gut microbiota metabolites, such as butyric acid derived from the fermentation of dietary fiber in the colon (11). Although the effect of diet on gut microbiota and plasma or urine metabolites and its relationship with cardiovascular risk factors has been reported by different studies, few of them have been focused on the fecal metabolome.

In participants with obesity and overweight exposed to the MedDiet intervention, a decrease in plasma and urine levels of carnitine, and significant reductions in plasma cholesterol and fecal bile acids were reported (12). Additionally, the metagenomic analysis showed increased levels of the fiber-degrading bacteria and genes for microbial carbohydrate degradation linked to butyrate metabolism (12).

After 2 months on a MedDiet intervention, participants with metabolic syndrome showed enrichment in gut bacterial genera related to bile acid metabolism and increased levels of fecal cadaverine and these changes were associated with an improvement in insulin sensitivity (13).

Even if the effects of the MedDiet in reducing the risk of numerous non-communicable diseases have been described, more studies are needed to help understand the potential effects using a more detailed examination of microbes and metabolites (9), especially considering recent findings highlighting potential difficulties when inferring microbiome-cardiometabolic disease associations from either blood or fecal metabolome data (8).

Hence, within the framework of the PREDIMED (PREvención con DIeta MEDiterránea)-Plus randomized trial, we explored the effects of a 1-year intensive lifestyle intervention based on an energy-reduced MedDiet, physical activity, and behavioral support (Intervention Group; IG) versus an *ad libitum* MedDiet (Control Group; CG), on fecal metabolites and fecal microbiota of 400 individuals with overweight/obesity and metabolic syndrome.

## Methods

### Participants and study design

The present study was conducted within the frame of the PREDIMED-Plus trial, with further details provided in the Supplementary material.

This study encompasses the analysis of a subsample of 400 participants (CG, n = 200; IG, n = 200) from the PREDIMED-Plus recruiting centers of Alicante, Barcelona, Reus, and Valencia, with available fecal microbiota 16S data and fecal metabolomics data at both time points (baseline and 1-year of intervention) to evaluate the effect of 1-year lifestyle intervention on fecal metabolome and microbiota.

**Intervention**

The PREDIMED-Plus intervention was designed to last six years plus two years of follow-up. Participants randomized in the IG were trained by a dietitian to modify their lifestyle through an er-MedDiet (energy reduction of 30% of individual estimated energy requirements) and increase their physical activity to reduce their body weight (14). IG participants received lifestyle recommendations (related to diet and physical activity) and behavioral support through a face-to-face educational program. In addition, during the first year of the intervention, IG participants attended two monthly visits (one group session and one individual session) and received one monthly phone call.

A 17-item questionnaire was used to assess adherence to the er-MedDiet (15). This 17-item questionnaire is a modified version of the previously validated 14-item Mediterranean Diet Adherence Screener (MEDAS) questionnaire (16). Specifically, this modified questionnaire limits the consumption of the less recommended food groups for weight loss (i.e., red and processed meats, butter and margarine, sugary drinks, and refined cereals).

All participants were also encouraged to increase their usual physical activity by recommending brisk walking for at least 45 min/day or equivalent activity and performing specific exercises to increase strength, balance, and flexibility (aim to increase moderate-to-vigorous physical activity $\geq$ 150 min/week). These activities and sedentary behavior were evaluated with questionnaires validated for the Spanish population and administered periodically (17). Total physical activity was calculated in metabolic equivalent of tasks (MET) min/week and sedentary behavior in h/day as previously described (18). Participants in the CG received recommendations to improve their adherence to the MedDiet in twice-a-year group sessions and did not receive recommendations to

increase physical activity. A schematic representation of the PREDIMED-Plus intervention is reported in Figure 1.

**Clinical variables, anthropometric measurements, and blood biochemistry**

Detailed information regarding dietary assessments, collection of non-dietary variables, anthropometric measurements, and blood biochemical parameters is provided in the supplementary material.

**Stool samples collection**

Stool samples at the baseline visit and 1 year were collected by the participants in a sterilized airtight flask. They were instructed to bring the sample to the study center within 12 hours of excretion under refrigerated conditions (i.e., to be kept frozen at –20ºC at home until delivery to the laboratory). Participants who were using antibiotics or pre/probiotics 15 days before sample collection (n = 4) were identified and excluded from the final sample size. The stool samples were then divided into 250 mg aliquots and stored at –80 °C until analysis.

**Fecal metabolomics analyses**

Metabolomics analyses of stool samples collected at baseline and after 1 year of follow-up were conducted using a liquid chromatography-tandem mass spectrometry (LC-MS) metabolomics platform. A detailed description is provided in the supplementary material.

**Fecal bacterial DNA extraction and 16S amplicon sequencing**

A detailed description of the fecal microbial DNA extraction, amplicon libraries preparation, 16S sequencing procedure, and pipeline utilized to obtain the final data is provided in the supplementary material.

**Statistical analyses**

The baseline clinical characteristics and changes during the follow-up were described according to the study groups. Numerical variables were summarized using means and standard deviations, whereas categorical variables were described as numbers and percentages. Group differences in anthropometric, biochemical, and lifestyle parameters were tested with Student's t-test, and $p < 0.05$ was deemed significant in the exploratory analysis.

A detailed description of the metabolomics statistical analyses is provided in the supplementary material (Supplementary Methods). Briefly, linear regression models were used to assess differences in 1-year changes in stool metabolites (i.e., the change in the metabolite data over time) between study groups. Weighted gene co-expression network analysis (WGCNA) (19) was used to identify metabolomic sub-networks based on correlation patterns using baseline stool metabolomics data. The associations between the 1-year changes in the subnetworks and the study groups were computed using multivariate linear regression models. In the same way, we assessed the associations between the changes in the four subnetworks that were significantly modified by the intervention and 1-year changes in cardiovascular risk factors, using linear regression models.

A detailed description of the microbiota statistical analyses is provided in the supplementary material (Supplementary Methods). Briefly, linear regression models were used to assess the effect of a 1-year PREDIMED-Plus intervention on calculated fecal microbiota alpha diversity indices. The effect of the intervention on fecal microbiota community dissimilarity was assessed with permutational multivariate analysis of variance (PERMANOVA) on the Bray-Curtis distance. Per-feature analysis was performed using the R package MaAsLin2 (20) (version 1.10.0), to detect the intervention effect on taxonomic feature changes over time. The associations between intervention-related fecal microbiota features and fecal metabolites subnetworks were assessed through linear mixed models. In addition, the association between changes in calculated alpha

diversity indexes and changes in fecal metabolites subnetworks was assessed through linear regression models.

## Results

### General characteristics of the study population

A flowchart of selected participants is represented in Supplementary Figure 1. Participants were selected from four PREDIMED-Plus recruiting centers (Alicante, Barcelona, Reus, and Valencia) and available secuencing data from stool samples (n = 782). Participants without available 1-year secuencing data (n = 125), low sequencing quality (n = 26), and reported antibiotic use (n = 4) were excluded. From this subset (n = 627), 400 participants were randomly selected by age, sex, body mass index, and study group (n = 200 for each study group), and fecal metabolomics analysis was conducted.

The general baseline characteristics of the study population according to the PREDIMED-Plus study groups are shown in Table 1. The baseline and changes after a 1-year follow-up in anthropometric, biochemical, and lifestyle parameters according to different PREDIMED-Plus study groups are described in Table 2. Participants included in the IG showed greater weight loss (–4.2 ± 4.8 kg) and lower waist circumference (–4.4 ± 7.4 cm), body mass index (BMI) (–1.5 ± 1.8 kg/m$^2$), as well as a total energy intake (–113.9 ± 714.0 kcal) after 1-year of lifestyle intervention compared to the participants in the CG. In addition, the participants in the IG showed a reduction in glycated hemoglobin (–0.1 ± 0.8 % over total) and increased adherence to MedDiet (3.4 ± 4.5) and physical activity (117.3 ± 501.9 METs/day) compared to those in the CG.

### Fecal metabolomics and network analysis

Of the 532 fecal metabolites, only four showed significant differences in changes (FDR (false discovery rate) < 0.05) between study groups after 1-year of intervention (Figure 2). Compared to the participants in the CG, the 4,7,10,13,16-docosapentaenoic acid (DPA) (IG mean: –0.40 ± 1.44; CG mean: –0.08 ± 1.61) and adrenic acid decreased (IG mean: –0.33 ± 1.20; CG mean: –0.08 ± 1.33), and oleic acid (IG mean: 0.17 ± 0.94; CG mean: -0.10 ± 1.01) and 3-methyl-adipic acid (3-MAA) (IG mean: 0.25 ± 1.11; CG mean: 0.02 ± 1.10) increased in those in the IG. In addition, significant differences were observed in another 56 metabolites that disappeared after FDR correction (FDR > 0.05) (Supplementary Table 1).

WGCNA grouped 532 baseline metabolites into 16 subnetworks of different sizes **(**Supplementary Table 2**).** Grey60 network was the subnetwork with fewer connected metabolites (n = 5), while the brown network was the highest connected subnetwork (n = 265 metabolites). Four subnetworks (Black, Midnight Blue, Pink, and Salmon) showed statistically significant between-group differences in changes after 1-year of intervention (Table 3). Metabolites selected in the Black subnetwork included mainly ceramides and sphingosines, the Midnight blue subnetwork included purines, the Pink included fatty acids and carnitines, and the Salmon subnetwork included bile acids. Compared to the CG, the participants in the IG showed a decrease in the Black, Midnight blue, and Pink subnetworks. The Salmon subnetwork increased in the IG compared to the CG.

The pair-wise partial correlations between metabolites accounting for the metabolites within the same networks were shown in Supplementary Figures 2-5. At baseline, the most connected hub for each subnetwork according to their intramodular connectivity was ceramide 18:1; 02/18:0 (kWithin = 10.72) for the Black subnetwork; fumaric acid or maleic acid (kWithin= 2.02) for the Midnight Blue subnetwork; 4,7,10,13,16-docosapentaenoic acid (kWithin= 6.68) for the Pink subnetwork; and glycochenodeoxycholic acid (kWithin= 4.27) for the Salmon subnetwork. After

1-year of intervention, the main hubs were similar: ceramide 18:1; 02/18:0 (kWithin= 9.60) for the Black subnetwork; 6,8-dihydroxy purine (kWithin= 1.67) for the Midnight blue subnetwork; 4,7,10,13,16-docosapentaenoic acid (kWithin= 6.66) for the Pink subnetwork; and glycochenodeoxycholic acid (kWithin= 4.36) for the Salmon subnetwork.

1-year changes in the Pink subnetwork were positively associated with 1-year changes in body weight (β: 0.11, 95% CI: 0.01, 0.21), homeostasis model assessment of insulin resistance (HOMA-IR) index (β: 0.12, 95% CI: 0.01, 0.24), insulin (β: 0.11, 95% CI: 0.01, 0.22), and fasting plasma glucose (β: 0.11, 95% CI: 0.01, 0.23), and negatively with changes in low-density lipoprotein (LDL) cholesterol (β: –0.11, 95% CI: –0.21, –0.01). 1-year changes in the Black subnetwork were negatively associated with changes in LDL cholesterol (β: –0.11, 95% CI: –0.20, –0.01) (Figure 3).

**Microbiota profiles associated with 1-year PREDIMED-Plus intervention**

We observed an increase in alpha diversity indexes Chao1 (mean and SD: 5.5 ± 17.5) , and Shannon (0.1 ± 0.5) after 1-year of follow-up in participants in the IG compared to those in the CG (β = 6.376, $p$ = 0.0005; β = 0.131, $p$ = 0.013 respectively) (Figure 4). The top 2 axes from principal coordinate analysis (PCoA) calculated over the Bray-Curtis distance explained 36% of the total variance between samples. The cluster based on interventions was not obvious (Supplementary Figure 6). PERMANOVA test based on the Bray-Curtis distance did not show statistically significant differences between study groups after 1-year of lifestyle intervention (Supplementary Table 3).

We observed a decrease in the abundance of the *Eubacterium hallii* group (–0.02 ± 1.1) in the IG compared to the CG after 1-year of follow-up (β = –0.365, FDR = 0.046). We also reported a marginal decrease in the abundance of genus *Dorea* (–0.2 ± 1.2) in the IG compared to the CG (β

= –0.346, FDR = 0.169) after 1-year of follow-up (Figure 5). The effect of the intervention on total fecal microbiota genera abundances is shown in Supplementary Table 4.

**Associations between intervention-related fecal microbiota profiles, fecal metabolites subnetworks, and cardiovascular risk factors**

We observed a negative association between 1-year change in metabolite Pink subnetwork and 1-year change in alpha diversity indexes Chao1 (β = –0.0005, $p$ = 0.0005) and Shannon (β = –0.012, $p$ = 0.010) (Figure 6). Furthermore, we observed a positive association between *E. halii* group and *Dorea* genera abundance and metabolites subnetworks Black (β = 0.007, $p$ = 0.00007; β = 0.004, $p$ = 0.012 respectively), Midnight blue (β = 0.007, $p$ = 0.00005; β = 0.060, $p$ = 0.0003 respectively), and Pink (β = 0.004, $p$ = 0.037; β = 0.004, $p$ = 0.031 respectively) (Figure 7).

In addition, we observed a positive association between *E. halii* group and *Dorea* genera abundance and changes in body weight (β = 0.158, $p$ = 0.004; β = 0.173, $p$ = 0.001 respectively), waist circumference (β = 0.150, $p$ = 0.003; β = 0.119, $p$ = 0.020 respectively), BMI (β = 0.156, $p$ = 0.005; β = 0.171, $p$ = 0.002 respectively), triglycerides (β = 0.077, $p$ = 0.043; β = 0.121, $p$ = 0.002 respectively), insulin (β = 0.104, $p$ = 0.007; β = 0.111, $p$ = 0.006 respectively), and the HOMA-IR index (β = 0.089, $p$ = 0.024; β = 0.103, $p$ = 0.012 respectively) (Figure 8). No association was observed between calculated alpha diversity indexes and changes in cardiovascular risk factors (Supplementary Figure 7).

## Discussion

Diet can affect the gut microbiome and interacts with the host (21). The fecal metabolome has been proposed as a functional readout of the gut microbiome (22). Thus, studies with both analyses, fecal metabolome, and microbiome, are essential for the understanding of how dietary

interventions influence the metabolism of the host. In the present study, we demonstrated that an intensive intervention based on an er-MedDiet and physical activity promotion, compared to a control *ad libitum* MedDiet, significantly affects both gut microbiota and fecal metabolites with important relationships between them, indicating a possible interplay.

As previously described in the context of the PREDIMED-Plus study (23), in the present analysis, we observed that participants allocated to the IG, after 1 year of intervention, showed a greater reduction in adiposity and improvements in lipid profile and markers of glucose metabolism. Within this context, the fecal metabolome analysis has established that two metabolites (DPA and adrenic acid) and three subnetworks (Black, Midnight blue, and Pink) were decreased in the IG compared to the CG, whereas two metabolites (oleic acid and 3-MAA) and one subnetwork (Salmon) were increased in the IG and over time. These differences in gut metabolite changes may reflect the differential effect of the interventions that could result directly from food or its digestion or be ascribed to endogenous host secretions or changes in gut microbiota metabolism.

The Black subnetwork was mainly constituted by sphingolipids, the Pink subnetwork was mainly constituted by polyunsaturated fatty acids (PUFAs) and cholesterol esters, and the Midnight blue subnetwork was mainly constituted by metabolites from purine metabolism, the Krebs cycle, and nucleotides. These subnetworks were reduced in the IG compared to the CG. Interestingly, two components of the Pink subnetwork, DPA, a ω3-PUFA, and adrenic acid, a ω6-PUFA, also significantly decreased in the IG. DPA acts as an intermediate between eicosapentaenoic and docosapentaenoic acids and can be found in high concentrations in marine foods (typically included in MedDied), red meat, and milk (24). Plasma and erythrocyte levels of DPA have been reported to decrease after weight loss (25), but we cannot discard that changes in this stool metabolite could be produced by intervention-related changes in the gut microbiota. Adrenic acid

is a non-dietary ω6-PUFA derived from arachidonic acid that to the best of our knowledge has not previously related to changes in gut microbiota. The gut microbiota plays an important role in fatty acid metabolism. Several studies have shown that PUFA can be produced and modulated by the intestinal microbiota and, in turn, the concentration of PUFA can modify the functionality of the microbiota after high-fat diets such as the MedDiet (26,27). Similarly, sphingolipids can be produced endogenously, come directly from food, or be the end-products of microbial metabolism (28). Endogenous sphingolipids are restricted by the breakdown of ceramides (29), so their content in the digestive tract hardly comes from the host. Since the dietary origin of sphingolipids vary considerably (30), and their intestinal absorption change depending on their origin (31), the differences observed between our study groups could be explained by higher consumption of certain foods, such as refined wheat or whole milk, in the CG compared to the IG. Additionally, some bacteria from the *Bacteroides* genus can assimilate and produce sphingolipids that can be absorbed by intestinal epithelial cells and modify the sphingolipids plasma pool (32). Although plasma sphingolipids have been related to impaired glucose metabolism and insulin resistance (33), in our study no such association between this sphingolipid subnetwork and the *Bacteroides* genus was shown. Nevertheless, changes in the Pink subnetwork were positively related to 1-year changes in body weight, HOMA-IR, insulin, and glucose, and inversely associated with changes in LDL cholesterol, suggesting that this network may play an important role in the metabolism of the host.

The Salmon subnetwork only included metabolites of bile acids metabolism. Bile acids have a key role in regulating energy metabolism, satiety, and body weight (34), and changes in bile acid metabolism have been reported in the low-grade inflammation status related to obesity and diabetes (35). Animal studies have shown that physical activity induces an increase in biliary bile

283 acid secretion and bile acid concentrations in feces (36), but in adults with obesity, higher
284 adherence to the MedDiet was associated with lower concentrations of bile acids in feces (12) and
285 higher BMI was associated with increased levels of bile acids in plasma (37). Therefore, the effect
286 of interventions on fecal bile acids may reflect higher physical activity and weight loss achieved
287 in the IG. The increase in fecal oleic acid and 3-MAA in the IG could be explained by the higher
288 adherence to the MedDiet achieved by the participants. Oleic acid is a monounsaturated ω9 fatty
289 acid present mainly in olive oil (38), and the 3-MAA, a methyl-branched fatty acid involved in the
290 catabolism of phytanic acid (39), may be derived from food such as fatty fish or cheese (typical in
291 MedDiet), but also meat.
292 Low gut bacteria diversity has been associated with several diseases (40). A systematic review of
293 observational and randomized clinical trials reported a positive relationship between MedDiet
294 adherence and alpha diversity using data from observational studies (9). However, they also
295 reported inconclusive findings from randomized trials regarding the effects of MedDiet on gut
296 microbiota diversity. In a small study involving adult volunteers living in a Mediterranean area,
297 those with higher adherence showed an increase in microbial richness (41). In line with these
298 findings, we observed an increase in alpha diversity measured using different indexes among the
299 participants in the IG compared to those in the CG. In our study, we observed significant positive
300 effects of the intervention on fecal microbiota richness and diversity after 1 year of follow-up, and
301 the increased alpha diversity secondary to the intervention was associated with one of the fecal
302 metabolite subnetworks identified. Moreover, compared to the CG we observed a decrease in the
303 abundance of *E. hallii* and *Dorea* spp. after 1-year of intervention compared to the CG. The
304 abundance of these bacterial taxa was directly associated with four of the fecal metabolite
305 subnetworks identified and different cardiovascular risk factors. These findings are consistent with

306 those from our previous study which assessed the effects of the intervention in a different
307 subsample of individuals and using another genotyping platform (42).
308 The decrease in the abundance of *E. hallii*, the former name of reclassified *Anaerobutyricum*
309 *soehngenii* (43), can be partially explained by the ability of this bacterium to produce its
310 fermentation end products from lactate, increased levels of which have been described in the small
311 intestine of insulin-resistant subjects, in whom an increased abundance of lactate-producing
312 bacteria have also been reported (44). Lactate is an intermediate in the metabolism of glucose that
313 has been implicated in the pathogenesis of insulin resistance in individuals with obesity (45).
314 Chondonikola and colleagues conducted a controlled trial in which participants were randomized
315 to a 6-month weight maintenance or weight loss intervention, showing the interrelationships
316 among weight loss, glucose metabolism, insulin sensitivity, and lactate concentration (46).
317 Accordingly, we showed that *E. hallii* was positively associated with body weight, waist
318 circumference, BMI, insulin, and HOMA-IR index. It has also been reported that oral
319 administration of *E. hallii* improved insulin sensitivity, increased energy expenditure, increased
320 fecal butyrate concentrations, and modified bile acid metabolism in mice with obesity and diabetes
321 (47). Furthermore, we reported a decrease in the abundance of *Dorea* spp. in fecal samples of
322 participants in the erMedDiet + physical activity intervention. These findings are consistent with
323 our previous findings, which showed an increase in the abundance of *Dorea* within the CG (42).
324 Western dietary pattern was previously associated with a higher abundance of *Dorea* spp. (48).
325 *Dorea* spp. has been found consistently elevated in prediabetes and is positively associated with
326 blood glucose concentrations (49). Consistently, we observed a significant difference in glycated
327 hemoglobin changes between study groups, after 1-year of intervention, and a positive association
328 between this genus, insulin levels, and the HOMA-IR index.

These findings have to be interpreted in the context of some limitations. First, given the multifaceted nature of the study intervention, the results cannot be attributed to a single component of the intervention. Second, the participants included in our study are Mediterranean older adults with overweight/obesity and metabolic syndrome. Therefore, the results may not be generalized to other populations outside of this specific context. Third, the nature of 16S sequencing limits taxonomic profiling to genus-level resolution, as the primers used for amplification bind to regions not conserved across all bacteria, not allowing to differentiate between closely related bacteria at species level. This limitation in taxonomic identification also reduces the possibility to infer the functionality of the microbiome.

The present study also has some strengths. Although the analysis was conducted in a sample that is not representative of the overall population, it is important to mention that individuals at high risk of cardiometabolic diseases represent an important proportion of the global population and hence our findings are relevant to similar community-dwelling older adults who may benefit from approaches to support good health. In addition, the randomized controlled study design, and the significant differences between the components of the intervention (weight loss, adherence to the MedDiet, and physical activity) allowed us to establish causality and assess the potential effects of the intervention. In addition, as we adjusted for major potential confounders when conducting our analyses, residual confounding is highly reduced. Finally, despite the limitations of 16S sequencing, it is important to mention that it is a very suitable technique to analyze a large number of samples.

In conclusion, in this lifestyle intervention-based study, we observed that an energy-reduced MedDiet and physical activity promotion, compared with an *ad libitum* MedDiet, produced significant changes in gut metabolomics and microbiota in a Mediterranean population of older

adults with overweight/obesity and metabolic syndrome and these changes were related to changes in several cardiovascular risk factors. These findings highlight that even with similar healthy dietary patterns, the high intensity of the dietary intervention and weight-loss intervention components, such as caloric restriction and physical activity, could have significant benefits on CVD risk factors, potentially through modulation of the fecal microbiota and metabolome.

The impact of our findings extends beyond individual health outcomes. Investigating the effects of Mediterranean diet and physical activity interventions on the gut microbiome provides insights into the underlying mechanisms by which these interventions improve cardiometabolic biomarkers. Understanding the role of the gut microbiome in mediating the health benefits of these interventions can inform more targeted and effective public health strategies. Elucidating the relationship between diet, physical activity, and the gut microbiome can contribute to the development of personalized health recommendations. Public health policies and interventions can be tailored to individual microbiome profiles, allowing for more precise and effective strategies for preventing and managing cardiometabolic diseases.

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**Data Availability:**

The datasets generated and analyzed during the current study are not publicly available due to data regulations and for ethical reasons, considering that this information might compromise research participants' consent because our participants only gave their consent for the use of their data by the original team of investigators. However, collaboration for data analyses can be requested by sending a letter to the PREDIMED-Plus steering Committee (predimed_plus_scommittee@googlegroups.com). The request will then be passed to all the members of the PREDIMED-Plus Steering Committee for deliberation.

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**Table 1.** General baseline characteristics of the study population according to the PREDIMED-Plus study groups

| | **CG (n = 200)** | **IG (n = 200)** |
|---|---|---|
| Sex, women, n (%) | 88 (44.0) | 88 (44.0) |
| Age, years, mean ± SD | 64.7 ± 5.0 | 64.5 ± 4.9 |

| | | |
|---|---|---|
| Recruiting center, n (%) | | |
| Alicante | 49 (24.5) | 42 (21.0) |
| Barcelona | 20 (10.0) | 26 (13.0) |
| Reus | 114 (57.0) | 110 (55.0) |
| Valencia | 17 (8.5) | 22 (11.0) |
| Education, n (%) | | |
| Primary school or less | 107 (53.5) | 111 (55.5) |
| High School | 51 (25.5) | 58 (29.0) |
| College | 42 (21.0) | 31 (15.5) |
| Civil status, n (%) | | |
| Married | 163 (81.5) | 146 (73.0) |
| Single/divorced/separated | 22 (11.0) | 35 (17.5) |
| Widower | 15 (7.5) | 19 (9.5) |
| Smoke status, n (%) | | |
| Never smoker | 93 (46.5) | 99 (49.5) |
| Former smoker | 81 (40.5) | 76 (38.0) |
| Smoker | 26 (13.0) | 25 (12.5) |
| Disease prevalence, n (%) | | |
| Overweight (BMI=25-29.9 kg/m$^2$) | 144 (72.0) | 157 (78.5) |
| Obesity (BMI $\geqslant$ 30 kg/m$^2$) | 56 (28.0) | 43 (21.5) |
| Hypertension prevalence | 162 (81.0) | 163 (81.5) |
| Type 2 diabetes prevalence | 44 (22.0) | 44 (22.0) |

SD, standard deviation; CG, control group; IG, intervention group; BMI, body mass index.

**Table 2.** Baseline and 1-year changes in anthropometric, biochemical, and lifestyle parameters according to the PREDIMED-Plus study groups.

| | **CG** **n = 200** | **IG** **n = 200** | **Between group difference (CG–IG)** | ***p*-value** |
|---|---|---|---|---|
| Body weight, kg | | | | |
| Baseline | 86.7±12.7 | 88.0±13.4 | | |
| 1-yr change | –0.8±2.8 | –4.9±4.1 | –4.2±4.8 | <0.001 |
| Waist circumference, cm | | | | |
| Baseline | 107.4±10.3 | 108.1±10.0 | | |
| 1-yr change | –1.1±4.1 | –5.5±6.2 | –4.4±7.4 | <0.001 |
| BMI, kg/m$^2$ | | | | |
| Baseline | 32.7±3.6 | 33.0±3.5 | | |
| 1-yr change | –0.3±1.1 | –1.8±1.5 | –1.5±1.8 | <0.001 |
| Total cholesterol, mg/dL | | | | |
| Baseline | 201.8±38.8 | 201.6±36.4 | | |
| 1-yr change | –0.4±33.9 | 0.6±30.6 | 1.0±44.9 | 0.758 |
| HDL cholesterol, mg/dL | | | | |
| Baseline | 49.0±10.5 | 49.9±12.3 | | |
| 1-yr change | 2.2±7.7 | 2.8±7.3 | 0.6±11.2 | 0.491 |
| LDL cholesterol, mg/dL | | | | |
| Baseline | 122.2±32.2 | 121.5±31.6 | | |
| 1-yr change | –1.0±29.6 | –0.3±25.1 | 0.7±39.9 | 0.825 |

| | | | | |
|---|---|---|---|---|
| Triglycerides, mg/dL | | | | |
| Baseline | 169.1±107.6 | 160.3±93.4 | | |
| 1-yr change | –3.5±92.9 | –11.8±87.0 | –8.4±123.8 | 0.345 |
| FPG, mg/dL | | | | |
| Baseline | 116.0±25.1 | 114.2±22.5 | | |
| 1-yr change | –4.3±21.0 | –3.7±17.7 | 0.6±27.2 | 0.770 |
| Insulin, mU/mL | | | | |
| Baseline | 19.7±9.7 | 19.2±11.6 | | |
| 1-yr change | –1.9±7.6 | –2.8±7.5 | –0.9±10.8 | 0.273 |
| HOMA-IR | | | | |
| Baseline | 5.8±3.5 | 5.6±4.1 | | |
| 1-yr change | –0.7±2.8 | –1.0±2.8 | –0.2±3.9 | 0.349 |
| HbA1c, % over total | | | | |
| Baseline | 6.0±0.8 | 6.0±0.8 | | |
| 1-yr change | 0.1±0.6 | –0.1±0.4 | –0.1±0.8 | 0.042 |
| Total energy intake, kcal | | | | |
| Baseline | 2543.1±550.4 | 2516.6±592.7 | | |
| 1-yr change | –141.2±537.4 | –255.2±566.4 | –113.9±714.0 | 0.025 |
| MedDiet adherence score | | | | |
| Baseline | 8.3±2.5 | 8.0±2.5 | | |
| 1-yr change | 2.2±2.9 | 5.6±3.1 | 3.4±4.5 | <0.001 |
| Alcohol intake, g/day | | | | |
| Baseline | 11.0±14.5 | 10.3±11.9 | | |

| | | | | |
|---|---|---|---|---|
| 1-yr change | –0.6±10.1 | –1.7 ± 8.4 | –1.1±13.4 | 0.246 |
| Physical activity, METs/day | | | | |
| Baseline | 367.1±314.3 | 366.9±329.8 | | |
| 1-yr change | 51.1±299.5 | 168.4±407.4 | 117.3±501.9 | 0.001 |

CG, control group; IG, intervention group; BMI, body mass index; HDL, high-density lipoprotein; LDL, low-density lipoprotein; FPG, fasting, plasma glucose; HOMA-IR, homeostatic model assessment of insulin resistance; HbA1c, glycated hemoglobin; MedDiet, Mediterranean diet. Data expressed as mean ± standard deviation. Differences between group differences according to the study groups tested with Students' t-test and $p < 0.05$ were deemed significant.

**Table 3.** Effect of 1-year PREDIMED-Plus intervention on metabolomic subnetworks.

| Metabolomic subnetworks | CG (n = 200) Mean±SD | IG (n = 200) Mean±SD | $\beta$ Coefficient | 95%CI |
|---|---|---|---|---|
| Black | 0.006±0.056 | –0.006±0.055 | –0.012 | (–0.020, –0.001) |
| Blue | –0.005±0.062 | 0.004±0.060 | 0.008 | (0.000, 0.020) |
| Brown | –0.001±0.040 | 0.001±0.043 | –0.001 | (–0.010, 0.010) |
| Cyan | –0.002±0.052 | 0.000±0.042 | –0.001 | (–0.010, 0.010) |
| Green | –0.002±0.048 | 0.002±0.052 | 0.006 | (0.000, 0.010) |
| Green Yellow | 0.001±0.047 | –0.002±0.051 | –0.005 | (–0.010, 0.000) |
| Grey60 | 0.004±0.055 | –0.003±0.050 | –0.005 | (–0.010, 0.000) |
| Light Cyan | –0.002±0.052 | 0.003±0.053 | 0.003 | (–0.010, 0.010) |
| Magenta | –0.004±0.059 | 0.003±0.054 | 0.002 | (–0.010, 0.010) |
| Midnight Blue | 0.005±0.052 | –0.005±0.052 | –0.011 | (–0.020, –0.001) |
| Pink | 0.004±0.050 | –0.004±0.048 | –0.010 | (–0.020, –0.001) |
| Purple | 0.005±0.053 | –0.004±0.050 | –0.007 | (–0.020, 0.000) |
| Red | –0.002±0.039 | 0.002±0.038 | 0.005 | (0.000, 0.010) |
| Salmon | –0.005±0.052 | 0.005±0.060 | 0.010 | (0.001, 0.020) |
| Tan | 0.000±0.061 | –0.001±0.053 | –0.005 | (–0.010, 0.000) |
| Yellow | 0.004±0.050 | –0.004±0.046 | –0.008 | (–0.020, 0.000) |

SD, standard deviation; CG, control group; IG, intervention group. Multivariable linear regression models adjusted for recruiting center (Alicante, Barcelona, Reus, Valencia), smoking status (former smoker, never smoker, smoker), type 2 diabetes status, sex, body mass index (BMI) categories (overweight, BMI=25-29.9 kg/m$^2$; obesity, BMI ≥ 30 kg/m$^2$); age categories (below the median, ≤65 years old; above the median, >65 years old), alcohol intake (g/day$^2$), and hypertension status.

**Figure Legend**

**Figure 1:** Schematic representation of the PREDIMED-Plus lifestyle intervention. Participants randomized in the intervention group (n = 200) were exposed to a weight-loss energy-reduced Mediterranean diet (er-MedDiet) and increased physical activity, also attending two additional monthly visits. Participants in the control group (n = 200) received recommendations to improve their adherence to the Mediterranean diet in twice-a-year group sessions and did not receive recommendations to increase physical activity. Diet and physical activity assessments, stool samples for metabolomics and 16S sequencing, cardiovascular risk factors, and anthropometric measurements were collected at baseline and one year of follow-up.

**Figure 2**: Volcano plot showing the 1-year effect of the PREDIMED-Plus intervention on fecal metabolites. Multivariable linear regression models were adjusted for recruiting center (Alicante, Barcelona, Reus, Valencia), smoking status (former smoker, never smoker, smoker), type 2 diabetes status, sex, body mass index (BMI) categories (overweight, BMI = 25-29.9 kg/m$^2$; obesity, BMI > 30 kg/m$^2$); age categories (below the median, ≤ 65 years old; above the median, > 65 years old), alcohol intake (g/day$^2$), and hypertension status. A false discovery rate (FDR) < 0.05 was considered statistically significant (up dash line).

**Figure 3**: Associations between 1-year changes in significant metabolite subnetworks and 1-year changes in cardiovascular risk factors. The models were adjusted for recruiting center (Alicante, Barcelona, Reus, Valencia), smoking status (former smoker, never smoker, smoker), type 2 diabetes status, sex, body mass index (BMI) categories (overweight, BMI = 25-29.9 kg/m$^2$; obesity, BMI ≥ 30 kg/m$^2$); age categories (below the median, ≤ 65 years old; above the median, > 65 years old), alcohol intake (g/day$^2$), and hypertension status. *$p$ < 0.05; **$p$ < 0.01. HDL, high-density lipoprotein; LDL, low-density lipoprotein; HOMA-IR, homeostatic model assessment of insulin resistance; HbA1c, glycated hemoglobin.

**Figure 4**: Effect of PREDIMED-Plus intervention on 1-year changes in alpha diversity indexes Chao1, Shannon, and Simpson. Effects of PREDIMED-Plus intervention tested with linear regression model adjusted for recruiting center (Alicante, Barcelona, Reus, Valencia), smoking status (former smoker, never smoker, smoker), type 2 diabetes status, sex, body mass index (BMI) categories (overweight, BMI = 25-29.9 kg/m$^2$; obesity, BMI ≥ 30 kg/m$^2$); age categories (below the median, ≤ 65 years old; above the median, > 65 years old), alcohol intake (g/day$^2$), hypertension status. $p < 0.05$ deemed as significant. CG, control group; IG, intervention group. Values indicated as mean ± SD.

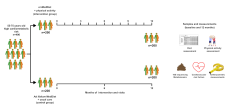
**Figure 5**: Effect of 1-year PREDIMED-Plus interventions on fecal microbiota taxa abundances. Multivariable association between group*time and fecal microbiota taxonomic features tested with generalized linear models adjusted for recruiting center (Alicante, Barcelona, Reus, Valencia), smoking status (former smoker, never smoker, smoker), type 2 diabetes status, sex, body mass index (BMI) categories (overweight, BMI = 25-29.9 kg/m$^2$; obesity, BMI ≥ 30 kg/m$^2$); age categories (below the median, ≤ 65 years old; above the median, > 65 years old), alcohol intake (g/day$^2$), hypertension status. Participants' IDs are set as random effect parameters. Multiple testing corrections were performed with the Benjamini-Hochberg procedure. Features with FDR < 0.25 were reported. Label "1" indicates group*time = intervention*1-year, "0" otherwise.

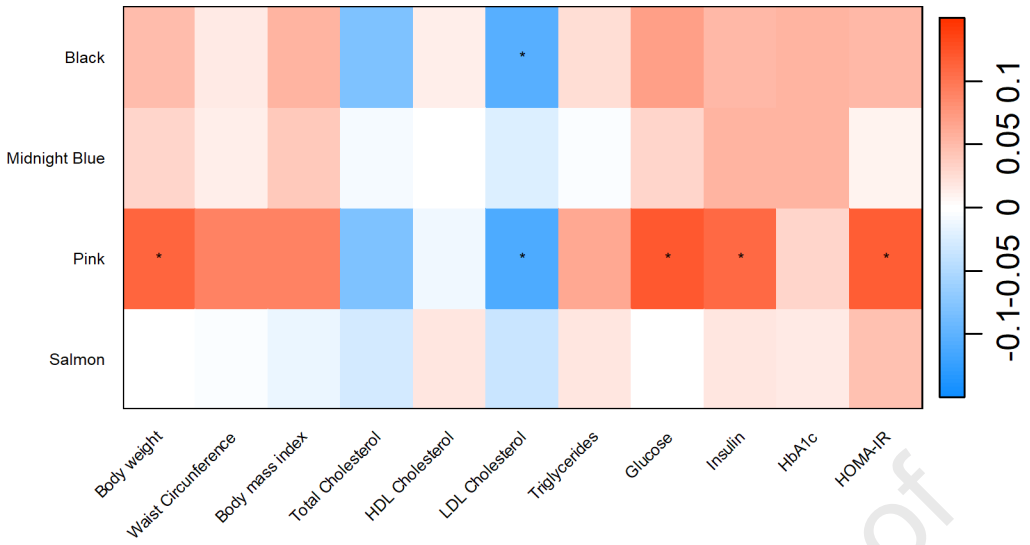
**Figure 6**: Association between 1-year changes in alpha diversity indexes and 1-year changes in fecal metabolites subnetworks. Association tested with linear regression models adjusted for the study group, recruiting center (Alicante, Barcelona, Reus, Valencia), smoking status (former smoker, never smoker, smoker), type 2 diabetes status, sex, body mass index (BMI) categories (overweight, BMI = 25-29.9 kg/m$^2$; obesity, BMI ≥ 30 kg/m$^2$); age categories (below the median,

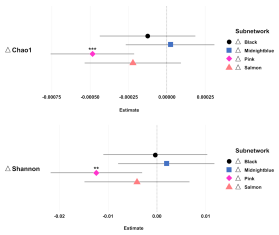
≤65 years old; above the median, >65 years old), alcohol intake (g/day2), and hypertension status. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

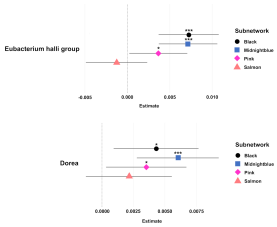
**Figure 7**: Association between differential abundant taxonomic features and metabolites subnetworks. Association tested with linear mixed models adjusted for study group*time, recruiting center (Alicante, Barcelona, Reus, Valencia), smoking status (former smoker, never smoker, smoker), type 2 diabetes status, sex, body mass index (BMI) categories (overweight, BMI = 25-29.9 kg/m$^2$; obesity, BMI ≥ 30 kg/m$^2$); age categories (below the median, ≤ 65 years old; above the median, > 65 years old), alcohol intake (g/day$^2$), and hypertension status. Participants' IDs are set as random effect parameters. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

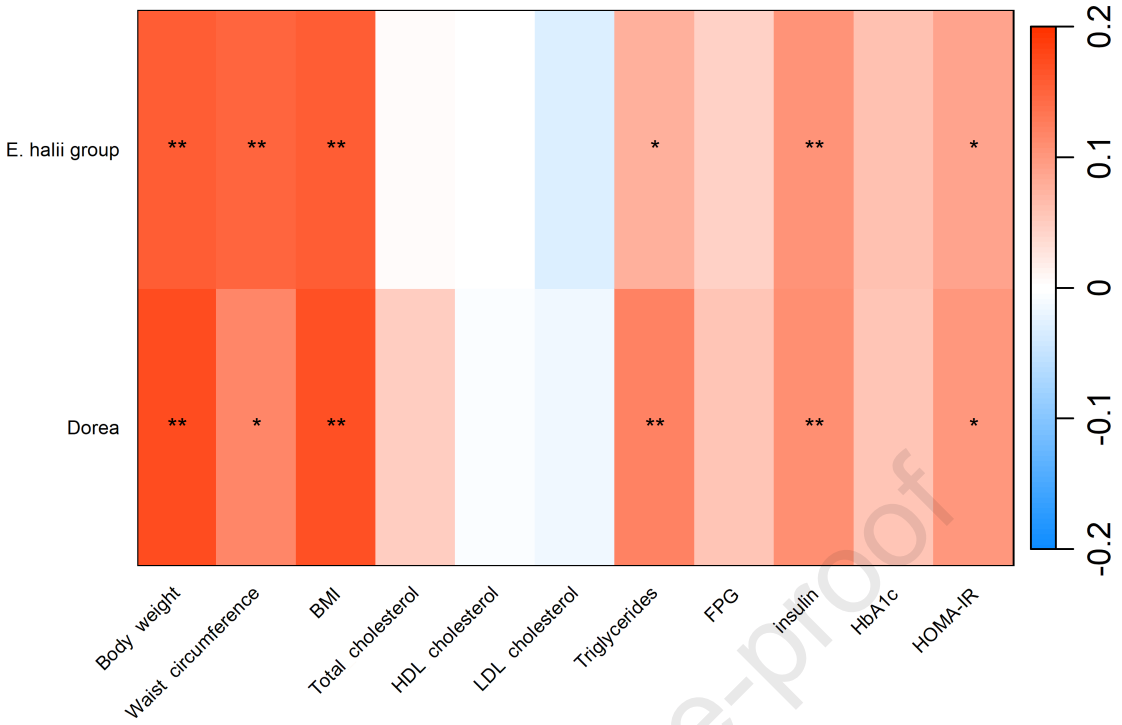
**Figure 8**: Heatmap showing the association between intervention-related differential abundant features and changes in cardiovascular risk factors. Association tested with linear mixed models adjusted for study group*time, recruiting center (Alicante, Barcelona, Reus, Valencia), smoking status (former smoker, never smoker, smoker), type 2 diabetes status, sex, body mass index (BMI) categories (overweight, BMI = 25-29.9 kg/m$^2$; obesity, BMI ≥ 30 kg/m$^2$); age categories (below the median, ≤ 65 years old; above the median, > 65 years old), alcohol intake (g/day$^2$), and hypertension status. Participants' IDs are set as random effect parameters. For each cell, colors indicate the association coefficient with cardiovascular risk factors and asterisks denote significance. *$p < 0.05$; **$p < 0.001$. HDL, high-density lipoprotein; LDL, low-density lipoprotein; FPG, fasting, plasma glucose; HbA1c, glycated hemoglobin; HOMA-IR, homeostatic model assessment of insulin resistance.

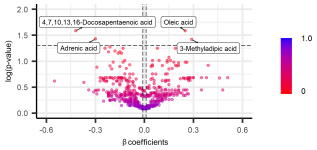


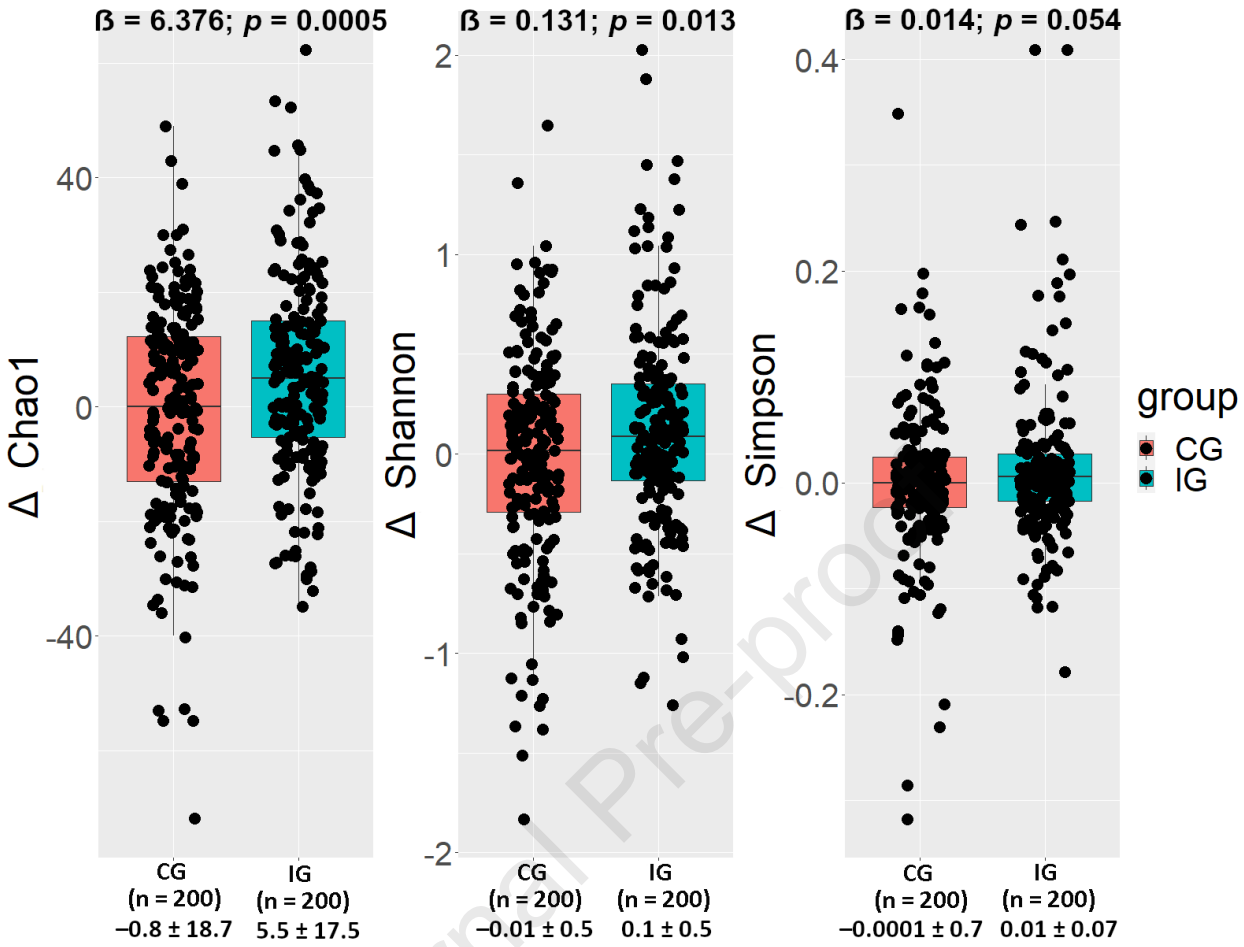


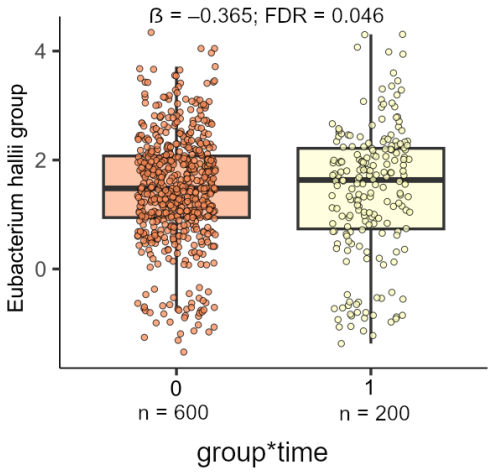


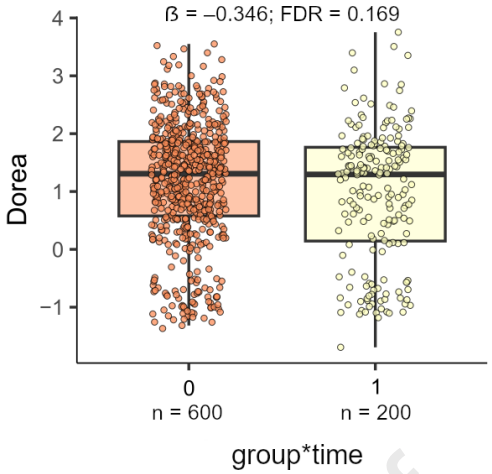










**Declaration of interests**

☐ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☒ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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