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Original article

Acute and chronic improvement in postprandial glucose metabolism by a diet resembling the traditional Mediterranean dietary pattern: Can SCFAs play a role?

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SUMMARY

Background & aims: Postprandial metabolic abnormalities are considered important and independent risk factors for cardiovascular diseases. However, the effects of the Mediterranean diet on postprandial metabolism and the mechanism underpinning the effects on clinical variables have not been exhaustively explored. Therefore, the aims of the present study were to evaluate the acute and medium-term effects (8 weeks) on postprandial glucose and lipid metabolism of a diet resembling a typical Mediterranean diet (Med-D) compared to a western-type diet (Control-D), and the mechanisms underlying those effects.

Methods: Twenty-nine overweight/obese individuals of both genders, aged 20–60 years, were enrolled and randomly assigned to two isoenergetic dietary interventions: 1) a Med-D (n = 16), and 2) a Control-D (n = 13). Adherence to the dietary interventions was assessed by a 7-day food record. A meal test resembling the assigned diet was performed at baseline and after 8 weeks of intervention. Blood samples at fasting and over 4-h after the meal were collected to assess metabolic parameters and short chain fatty acid (SCFA) levels. Fecal samples were also collected to evaluate the microbiota composition.

Results: Glucose and insulin responses were significantly reduced at baseline after the Med test meal compared to the Control meal ($p < 0.05$) and this effect was strengthened after 8 weeks of intervention with the Mediterranean diet ($p < 0.05$); together with an improvement in OGIS. At the end of the intervention, postprandial plasma butyric acid incremental area under the curve (IAUC) was significantly increased in the Med-D group ($p = 0.019$) and correlated inversely with plasma insulin IAUC and directly with oral glucose insulin sensitivity (OGIS) (r: −0.411, $p = 0.046$ and r: 0.397, $p = 0.050$ respectively). These metabolic changes were accompanied by significant changes in gut microbiota, such as an increase in the relative abundance of *Intestinimonas butyriciproducens* and *Akkermansia muciniphila* ($p < 0.05$) in the Med-D compared to Control-D group.

Conclusions: Our study provides strong evidence that a diet resembling the traditional Med-D improves postprandial glucose metabolism and insulin sensitivity. Furthermore, the study highlights a possible involvement of gut microbiota metabolites - such as butyric acid, and of dietary fiber as a precursor - in improving glucose metabolism and insulin sensitivity.

1. Introduction

Epidemiological and intervention studies have provided evidence that a dietary pattern resembling the traditional features of

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the Mediterranean Diet is associated with numerous health benefits [1,2], in both Mediterranean and non-Mediterranean countries [1,3].

Specifically, epidemiological studies show that greater adherence to a Mediterranean dietary pattern is associated with a lower risk of developing type 2 diabetes, in both healthy subjects and individuals at high cardiovascular risk [4,5], as well as with lower risk of all-cause mortality [6,7].

Some intervention studies, such as the PREDIMED, indicate that a Mediterranean type of diet reduces the risks of cardiovascular diseases and type 2 diabetes [8,9]. These advantages seem to be mediated by the beneficial influence on some cardiovascular risk factors such as body weight, blood pressure, fasting lipid and glucose metabolism. Higher postprandial plasma triglyceride, glucose and insulin levels are considered important and independent risk factors in dyslipidemic and diabetic subjects as well as in individuals with normal fasting blood triglyceride and glucose levels [10–13].

Some clinical trials have investigated the effects of single nutrients provided by a Mediterranean diet, such as monounsaturated fatty acids or dietary fibers, on postprandial lipemia and glucose metabolism in acute [14,15] and in medium term conditions [16,17]. However, the effects of the Mediterranean diet as a whole dietary pattern on postprandial metabolism (i.e. glucose, insulin and lipid postprandial responses) have not been exhaustively explored. Indeed, a Mediterranean type of diet includes many foods, with a frequency of consumption that can be summarized as a high intake of extravirgin olive oil, legumes, fruits, vegetables, nuts, and fish, moderate intake of dairy products, and a low consumption of meat and processed meat products. As such, this dietary pattern provides many nutrients and antioxidant compounds, including unsaturated fats, dietary fibers, and various phytochemicals whose health benefits are extensively recognized [2].

Only one study on the medium-term effects of the Mediterranean diet as a whole focused on postprandial lipemia and adopted diets slightly restricted in energy, thus influencing lipid metabolism through body weight loss [18].

Another rather relevant issue – albeit poorly understood – is the mechanism through which diet, particularly the Mediterranean diet, could affect clinical outcomes. To this regard, there is growing interest in the possible role of microbiome changes and specifically their effects on the production of short chain fatty acids – acetate, propionate, and butyrate – as possible modulators of glucose and lipid metabolism [19,20]. Adherence to a Mediterranean diet has been associated with specific signatures in microbiota composition, mainly related to increased fiber-degrading microbial taxa, which have also been associated to increased fecal levels of SCFAs [21]. However, intervention studies are the most appropriate tool to shift from association to causality and acquire robust information on the diet-microbiome inter-relationships [22]. For this reason, we have recently performed a randomized clinical trial to assess the effect of a Mediterranean dietary intervention on microbiota, metabolome and clinical variables in healthy overweight and obese subjects with unhealthy lifestyle [23].

This study was performed in a subgroup of Meslier and coworkers' cohort [23] with the hypothesis to evaluate 1) the acute effects on postprandial glucose and lipid metabolism of a test meal resembling a typical Mediterranean lunch compared to a western diet-type lunch; 2) the medium-term effects (8 weeks) on postprandial glucose and lipid metabolism of a diet resembling a typical Mediterranean diet (Med-D group) compared to a western type diet (Control-D group); 3) the mechanisms underpinning those effects.

2. Materials and methods

2.1. Participants

Forty-nine overweight/obese individuals (BMI between 25 and 35 kg/m^2) of both genders, aged 20–60 years, among those attending the clinical nutrition center, and among the employees of the University of Naples Federico II, were assessed for eligibility for this specific study. Thirty-three individuals were recruited according to the inclusion/exclusion criteria previously reported [23]. Briefly, inclusion criteria were: healthy subjects of age 20–65 years, BMI$\geq$24 kg/m^2, who habitually consumed no more than 2 portions a day of wholegrain cereals and/or foods enriched with dietary fiber, no more than 3 servings of fruit and vegetables per day, did not take probiotics and functional foods and/or food supplements of any kind, and with low level of physical activity (sedentary lifestyle). Exclusion criteria were: gastrointestinal disorders of any kind, pregnancy or breastfeeding, previous abdominal surgery, hypertriglyceridaemia (triglycerides> 300 mg/dL), hypercholesterolemia (cholesterol> 220 mg/dL), hypertension, pharmacological treatments of any type at enrollment and in the 2 months prior to the study, habitual diet rich in fruit and vegetables, high level of physical activity, consumption of wine or alcohol equivalent beverage greater than 3 glasses of wine per day, contemporary participation in other studies. Three candidates declined to participate before starting the nutritional intervention for personal reasons; one subject allocated to the control group dropped out after two weeks for family-related problems. Overall, twenty-nine overweight/obese were randomized to the two dietary interventions and completed the study (Supplemental Fig. 1).

This study was registered on ClinicalTrials.gov (Number: NCT03071718), was conducted according to the recommendations of the Declaration of Helsinki and was approved by the Ethics Committee of Federico II University of Naples (Protocol number: 108/16). Signed, written informed consent was obtained from each participant.

2.2. Study design

The study was based on a randomized, controlled, parallel group design and consisted of a 2-week run-in period–during which participants were stabilized on their own diet, followed by an 8-week nutritional intervention period. At the end of the run-in period, participants were randomly assigned to the Med-D or Control-D group. Participants in the Med-D group followed a diet that was isocaloric compared to their habitual diets but had the features of the traditional Mediterranean diet, whereas participants in the Control-D group maintained their habitual diets, which had the typical features of a western diet (by selection) [23]. Randomization was carried out stratifying for gender (men vs women) and body mass index (BMI) (25–30 and 30–35 kg/m^2) using a random allocation software. Allocation was carried out by personnel not involved in the study; therefore, investigators and dieticians were aware of the participants' group allocation only after the randomization process.

During the study, participants were asked not to modify the level of physical activity that had been recorded at the beginning of the study and assessed every 2 weeks thereafter.

At baseline and at 4 and 8 weeks during the intervention, participants underwent clinical investigations, including measurements of body weight through the same calibrated beam balance scale; waist and hip circumferences; and blood pressure, in the supine position in a standardized way after 5–10 min rest with an automatic sphygmomanometer (OMRON M3, OMRON Healthcare Europe B.V. Scorpius 33, 2132 LR, Hoofddorp, Olanda). At baseline

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and every 4 weeks since the beginning of the nutritional intervention, blood samples in the fasting state and fecal samples were collected from each participant and analyzed as recently described [23]. In addition, participants underwent blood sampling over 4 h after a test-meal resembling the Mediterranean or the Western lunch, according to the diet assigned.

2.3. Experimental diets and test meals

Participants in the Med-D group consumed an individualized diet that maintained the daily energy and macronutrient intake of each individual's habitual diet and guaranteed a dietary pattern typical of the Mediterranean Diet. Contrarily, participants in the Control-D group maintained their habitual diet. The diets of the participants in Med-D group were formulated to increase their individual intake of dietary fiber, plant vs animal proteins, and monounsaturated and polyunsaturated fats vs saturated fats. Therefore, the diets of participants in the Med-D group were designed to have fruit and vegetables (at least 5 portions, ~500 g/day) and nuts (30 g/day), refined cereal products replaced with wholegrain products (at least 2 portions, ~200 g/day between wholegrain pasta, bread and breakfast cereal), meat and derived meat products with legumes and fish (at least 2 portions, ~300 g/week of fish and 3 portions, ~300 g/week of legumes), butter and any other habitual condiments with extravirgin olive oil. Participants were encouraged to consume meat, dairy products, and eggs only once a week. On the contrary, subjects in the Control-D group were instructed to keep their habitual diet unvaried during the intervention and did not consume extra virgin olive oil.

To improve adherence to the Med and Control diets, the main food products in both diets were supplied to participants in amounts enough to cover their household consumption for the whole study period. Moreover, adherence was reinforced by dietitians through phone call and weekly counselling.

At baseline and at the end of the nutritional intervention, participants spent a day at the Clinical Research Centre where their metabolic profile was evaluated at fasting and in postprandial conditions after a meal resembling the composition of their intervention diets. In particular, individuals randomized to the Med-D group received a Mediterranean test meal (resembling a Mediterranean lunch) both at baseline and after the 8 weeks, whereas those randomized to the Control-D group consumed a control test meal.

The Med-D test meal was composed of dried beans (80 g), cod (50 g), rocket (100 g), wholemeal bread (120 g), extravirgin olive oil (29 g), and an orange (150 g); the Control test meal was composed of rice (80 g), parmesan (30 g), bresaola (60 g), white bread (30 g), olive oil (21 g), and a banana (160 g). The two meals were prepared in the metabolic kitchen by a dietitian with standardized amounts of all foods in order to make the two meals similar in energy and macronutrient composition, but different in animal and vegetable protein sources, saturated fat, total fiber content, and glycemic index and load (Supplemental Table 1).

2.4. Dietary assessment

Adherence to diets was assessed using a 7-d food record filled in by participants during the run-in period, before starting the intervention and at weeks 4 and 8 of the intervention.

All 7-d food records were analyzed by a computerized program (Metadieta software) using the food database of the Italian National Institute for Food and Nutrition [24,25].

2.5. Laboratory analyses

Blood samples were drawn after a 12-h overnight fast and over 4 h after the test meal, from an antecubital vein for the measurements of plasma glucose, insulin, lipid profile, and short chain fatty acids (SCFAs). Plasma insulin concentrations were measured by an enzyme-linked immunosorbent assay (ELISA) for the specific determination of biologically active insulin (DAKO Insulin, DAKO Diagnostics, Ely, UK). Plasma glucose, cholesterol, and triglycerides were assayed by enzymatic colorimetric methods (ABX Diagnostics, Montpellier, France; Roche Molecular Biochemicals, Mannheim, Germany; Wako Chemicals GmbH, Neuss, Germany, respectively) on a Cobas Mira autoanalyzer (ABX Diagnostics, Montpellier, France). HDL-cholesterol was isolated from plasma by a precipitation method with a sodium phosphotungstate and magnesium chloride solution and measured by the same enzymatic colorimetric method utilized for total cholesterol. The LDL-cholesterol concentration at fasting was calculated according to the Friedewald formula [26]. Insulin sensitivity in the postprandial period was evaluated by the OGIS index.

SCFAs (acetic, propionic, butyric acids) were extracted from serum and determined by Gas-Chromatography/Flame Ionization Detector (GC/F.I.D.) Dani (Analitica Instruments S.p.A. Milan—Italy) using a megabore column compatible with aqueous solvent according to Remesey and Demigne [27]. The samples before GC analysis, were deproteinized by addition of metaphoshoric acid. In these conditions, proteins positively charged act as polycations coprecipitating with metaphosphoric acid.

2.6. Evaluation of the microbiota composition

Fecal samples (about 1 g) were fully homogenized in STE buffer (100 mMNaCl, 10 mMTris-Cl pH 8.0, 1 mM EDTA pH 8.0) and centrifuged (500×*g*, 1 min) to pellet debris. The supernatant was centrifuged (12000×*g*, 2 min) and the pellet was used for DNA extraction by using a PowerFecal DNA isolation kit (Mo Bio Laboratories, Inc., Carlsbad, CA). The V3—V4 region of the 16S rRNA gene was amplified by using primers and PCR conditions recently described [28]. PCR products were purified with the Agencourt AMPure XP beads (Beckman Coulter) and quantified using a Plate Reader AF2200 (Eppendorf). The Illumina 16 S metagenomic sequencing library preparation protocol was used to perform library multiplexing, normalization and pooling. The sequencing was carried out on a MiSeq platform with the MiSeq Reagent kit v2, resulting in 2 × 250-bp paired-end reads. Raw sequences were demultiplexed, and forward and reverse reads were joined by using FLASH [29]. Joined reads were trimmed and filtered using Prinseq [30] (Phred quality score < 33 and reads < 250 bp were discarded). High quality reads were processed applying the QIIME [31] pipeline as recently described [28]. Alpha diversity of the samples was measured by the Shannon diversity index.

All laboratory analyses were performed blind in respect to the assigned treatment.

2.7. Sample size, calculations and statistical analysis

The primary endpoint was postprandial insulin response. Based on our previous study [32], thirty individuals had to be studied to detect a 30% difference in insulin response between the two groups, with 0.05 significance level and 80% power (type II error = 0.2), assuming a 10% drop-out rate.

Data are expressed as mean ± standard deviation (M±SD) unless otherwise stated for continuous variables, and number or percentage for categorical values, as appropriate.

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Energy intake and nutrient composition at the end of the run-in period and during the intervention were calculated from the food records; the intakes during the intervention were expressed as mean of the two food records filled-in at 4 and 8 weeks. Postprandial incremental AUCs (IAUC) were calculated by the trapezoidal method.

Assessment of postprandial insulin sensitivity (OGIS) was calculated from the meal glucose tolerance test (MGTT) over 3-h according to Mari et al. [33] using an Excel spreadsheet for 3-h OGTT.

Differences between the two groups at baseline and at the end of the intervention were evaluated by the unpaired t-test. Moreover, the differences found at the end of the intervention were adjusted for baseline values when appropriate (generalized linear model). Differences between values at baseline and after 8 weeks of intervention were evaluated by the paired sample t-test separately for the two groups. A repeated measures ANOVA was performed to examine the effects of meal and time and meal × time interaction effect on postprandial glucose and insulin responses. In this analysis, postprandial values measured every 30 min over an interval of 240 min were included as levels of the within-subject "time" factor, and Med-meal and Control-meal were included as levels of the within-subject "meal" factors. Spearman correlation analysis was used to test the relations between the nutrient composition of the diet at the end of the intervention and metabolic outcomes (Med-D and Control-D). Multivariate regression analysis was then performed to evaluate the relation between each nutrient per se and metabolic outcomes. Finally, a Spearman correlation analysis was also used to test the relation between SCFA plasma levels and metabolic outcomes in both the whole study population and the two intervention groups separately (Med-D and Control-D), and between microbial features and metabolic outcomes as well. Moreover, non-parametric Kruskal–Wallis and pairwise Wilcoxon tests were carried out to find OTUs (Operational taxonomic units) differentially abundant between the Med-D and Control-D groups. The level of statistical significance was set at $P < 0.05$. Analyses were performed using the Statistical Package for the Social Sciences (SPSS) for Windows (21.0 version; IBM) and the R environment (https://www.r-project.org).

3. Results

Twenty-nine subjects (16, 7M/9 F, in the Med-D group and 13, 7M/6 F in the Control group) completed the nutritional intervention. The general characteristics of the participants, their anthropometric data and blood pressure values, at baseline and at the end of the nutritional intervention are reported in Table 1. At baseline, the Med-D and Control-D groups did not differ significantly for age, BMI, body weight, waist and hip circumferences, systolic and diastolic blood pressure levels (Table 1). As expected, and according to the protocol, body weight, BMI, and waist and hip circumferences, did not change during the intervention period in either group and the same finding was observed for systolic and diastolic blood pressure.

3.1. Dietary compliance

Similarly to the findings obtained in the whole population and described by Meslier and co-workers [23], the two subgroups in Med-D and Control-D involved in this study had similar diets at baseline and showed a high compliance to the nutritional interventions they were assigned to (Table 2). Indeed, compared to the Control-D, participants in Med-D group increased their intake in plant proteins, unsaturated fats, dietary fiber and sugars from fruits, while they decreased the intake of animal proteins and saturated fats. These changes were independent from energy intake and macronutrient intake that did not change over intervention.

3.2. Fasting and postprandial lipid and glucose metabolism

At baseline, Med-D and Control-D groups did not differ in terms of fasting plasma cholesterol, LDL-chol, HDL-chol and triglycerides (Table 3). After 8 weeks, LDL-chol was significantly lower in the Med-D group than in the Control-D group (114 ± 38 vs 127 ± 33 mg/dl, $p = 0.041$), triglycerides tended to be lower (101 ± 45 vs 117 ± 46 mg/dl, $p = 0.070$), while HDL-chol levels were not different (Table 3).

Postprandial triglycerides response was not different between the Med-D and Control-D groups at baseline and was not influenced by the two dietary interventions (Table 3).

Fasting and postprandial plasma glucose and insulin before and at the end of the intervention are shown in Fig. 1, either as single time points or IAUC.

Fasting values were similar. Conversely, at baseline, postprandial plasma glucose levels were significantly lower after the Med test meal compared to the Control one ($F = 3.245$, $p = 0.009$ for time × meal interaction, repeated measures ANOVA; $p < 0.05$ at 60, 90 and 120 min, paired sample t-test) but IAUC was not significantly different. Postprandial plasma insulin levels at baseline were significantly lower after the Med test meal compared to the Control one ($F = 3.308$, $p = 0.004$ for time × meal interaction, repeated measures ANOVA). They were reduced at all time points ($p < 0.05$) with a significant reduction also in the IAUC ($p < 0.0001$).

Table 1
Sex, age, anthropometric data and blood pressure of study participants.

	Med-D Group	Control-D Group	P-value (Med-D vs Control-D Group)	Med-D Group	Control-D Group	P-value (Med-D vs Control-D Group)
	Baseline			After 8-wk of intervention		
	n = 16	n = 13		n = 16	n = 13	
Sex (% of men)	7 (43.8%)	7 (53.8%)	0.320	–	–	
Age (years)	41.6 ± 12.3	45.9 ± 13.0	0.363	–	–	
Body weight (kg)	82.2 ± 11.7	81.8 ± 11.8	0.914	82.3 ± 12.0	81.3 ± 12.0	0.824
BMI (kg/m^2)	28.9 ± 2.3	29.3 ± 3.5	0.703	28.9 ± 2.4	29.2 ± 3.6	0.831
Waist Circumference (cm)	97.1 ± 8.9	97.9 ± 7.7	0.800	97.2 ± 8.9	97.8 ± 7.4	0.852
Hip Circumference (cm)	110.1 ± 6.4	109.1 ± 8.0	0.698	110.1 ± 6.7	109.3 ± 8.1	0.769
Systolic blood pressure (mm/Hg)	110.0 ± 13.2	117.2 ± 14.4	0.177	107.6 ± 8.2	112.6 ± 10.4	0.114
Diastolic blood pressure (mm/Hg)	69.5 ± 10.3	73.2 ± 8.3	0.311	69.3 ± 7.4	68.8 ± 8.2	0.421
						M±SD

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Table 2
Energy and nutrient composition of the diet at baseline and at the end of intervention.

	Med-D Group	Control-D Group	P-value (Med-D vs Control-D Group)	Med-D Group	Control-D Group	P-value (Med-D vs Control-D Group)
	Baseline			After 8-wk of intervention		
	n = 16	n = 13		n = 16	n = 13	
Total Energy (kcal/day)	1857 ± 404	1879 ± 582	0.904	1842 ± 454	1816 ± 574	0.893
Total Proteins (% of TE)	16.7 ± 2.9	16.2 ± 3.5	0.678	15.6 ± 2.1	15.2 ± 3.4	0.706
Proteins (animal sources) (% of TE)	10.1 ± 3.4	9.2 ± 3.6	0.470	5.8 ± 2.3[a]	8.6 ± 4.0	0.024
Proteins (plant sources) (% of TE)	5.7 ± 1.2	5.1 ± 1.1	0.174	9.0 ± 1.6[a]	4.6 ± 0.8	<0.0001
Plant/Animal proteins ratio	0.6 ± 0.3	0.6 ± 0.2	0.695	1.8 ± 0.9[a]	0.6 ± 0.3	<0.0001
Total Fat (% of TE)	34.1 ± 3.9	34.5 ± 4.9	0.799	34.6 ± 6.1	34.7 ± 5.4	0.959
SFA (% of TE)	9.7 ± 2.5	8.8 ± 2.0	0.271	6.7 ± 1.4[a]	9.2 ± 1.9	<0.0001
MUFA (% of TE)	15.1 ± 2.1	14.9 ± 4.2	0.858	17.9 ± 3.8[a]	13.6 ± 2.6	0.002
PUFA (% of TE)	4.3 ± 1.0	3.9 ± 0.9	0.280	6.2 ± 1.6[a]	3.8 ± 0.9	<0.0001
Carbohydrates (% of TE)	46.8 ± 4.4	47.1 ± 5.9	0.902	48.1 ± 5.6	49.0 ± 5.8	0.691
Sugar (% of TE)	14.4 ± 3.7	14.0 ± 4.5	0.791	17.2 ± 3.1	13.8 ± 4.1	0.015
Fiber (g/1000 Kcal)	11.7 ± 2.4	10.0 ± 2.3	0.076	19.3 ± 3.1[a]	8.1 ± 2.3[a]	<0.0001 M±SD

[a] vs Baseline, in the Med-D group and Control-D group, Paired sample T-Test.

Table 3
Fasting plasma cholesterol, triglycerides, HDL-c and LDL-c, and postprandial triglycerides IAUC at baseline and after 8-wk in the two experimental groups.

	Med-D Group	Control-D Group	P-value (Med-D vs Control-D Group)	Med-D Group	Control-D Group	P-value[a] (Med-D vs Control-D Group)
	Baseline			After 8-wk of intervention		
	n = 15[b]	n = 13		n = 15[b]	n = 13	
Fasting values						
Total Cholesterol (mg/dl)	190 ± 43	196 ± 35	0.685	179 ± 43	196 ± 40	0.071
LDL-c (mg/dl)	123 ± 38	128 ± 33	0.762	114 ± 38	127 ± 33	0.041
Triglycerides (mg/dl)	98 ± 43	101 ± 37	0.862	101 ± 45	117 ± 46	0.070
HDL-c (mg/dl)	46 ± 7	48 ± 11	0.697	44 ± 6	46 ± 10	0.812
Postprandial values (Incremental Area)						
Triglycerides (mg/dl*4 h)	7332 ± 5867	7905 ± 5951	0.804	9699 ± 5911	10,115 ± 5427	0.854 M±SD

[a] Data were corrected for baseline values.
[b] One subject was excluded because using phytosterol supplements.

After 8 weeks of dietary intervention, plasma glucose response remained significantly lower after the Med-D test meal compared to the Control one ($F = 3.612$, $p = 0.001$ for time × meal interaction, repeated measures ANOVA; $p < 0.05$ at all time points, paired sample t-test) and, in this case, also IAUC was significantly lower ($p = 0.033$). Plasma insulin response at the end of the intervention remained significantly lower after the Med-D test meal ($F = 3.501$, $p = 0.001$ for time × meal interaction, repeated measures ANOVA; $p < 0.05$ at all time points, paired sample t-test) as well as IAUC ($p = 0.001$). In addition, comparing IAUCs before and after the Mediterranean dietary intervention, postprandial glucose IAUC was not significantly different before and after the Mediterranean Diet. On the contrary, postprandial insulin IAUC was significantly lower at the end of the intervention compared to baseline conditions ($p = 0.002$).

Postprandial insulin sensitivity index (OGIS) was not significantly different at baseline while it was significantly improved after 8 weeks on Med-D compared to the Control-D ($p = 0.008$) (Fig. 2).

After 8 weeks of intervention, the incremental area of blood glucose and insulin, and OGIS remained statistically significant even after correction for baseline response ($p = 0.029$, <0.0001, and 0.006, respectively).

Correlation analysis showed that after 8 weeks of intervention in the whole group, the dietary intake of fiber and the plant/animal protein ratio were inversely associated with plasma glucose and insulin responses, expressed as IAUC ($p < 0.05$). Dietary fiber intake was also directly associated with OGIS ($p < 0.05$). The intake of saturated fatty acids was not significantly associated with plasma glucose response and OGIS, but a significant direct association was found with plasma insulin response, expressed as IAUC ($p < 0.05$) (Supplemental Table 2). Regarding plasma lipids, only the intake of saturated fatty acids was directly and significantly associated with total plasma cholesterol and LDL-chol ($p < 0.05$) (Supplemental Table 2).

To evaluate the relationship between each component of the diet per se on plasma glucose and insulin response, we performed a multivariate regression analysis with fiber and plant/animal protein ratio as covariates (for insulin response, also saturated fatty acids were added as covariate). Only fiber intake remained significantly and inversely associated with plasma glucose and insulin IAUCs (ß = −0.478, $p = 0.014$; ß = −0.570, $p = 0.002$, respectively).

3.3. Fasting and postprandial SCFAs levels

Fasting and postprandial plasma levels of acetic and propionic acids were not different either at baseline or at the end of intervention (Table 4). The same finding was found for fasting plasma levels of butyric acid.

The response of butyric acid to the test meals was not different at baseline, while postprandial plasma butyric acid levels

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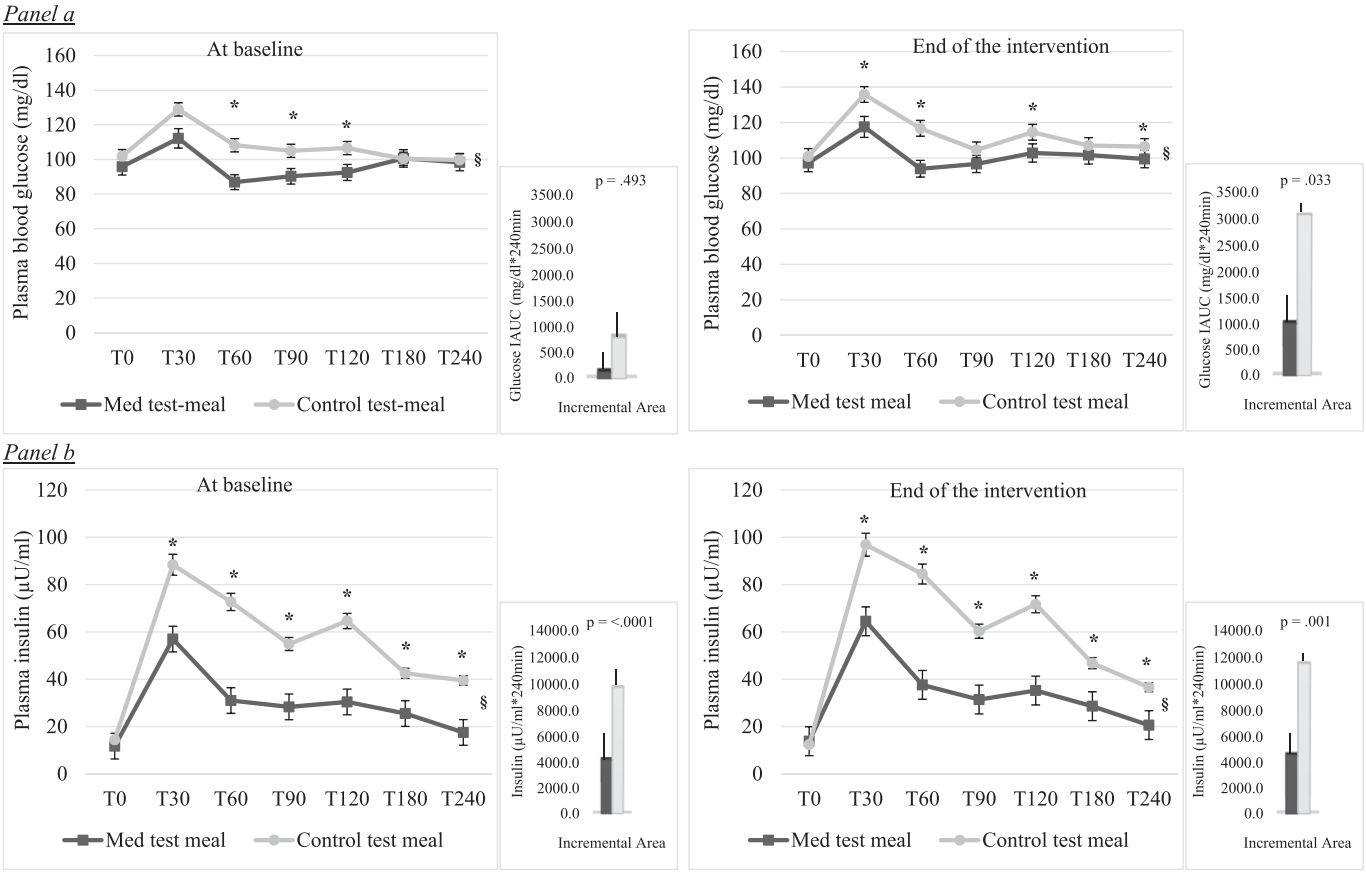

Fig. 1. Fasting and postprandial plasma glucose (Panel a) and insulin (Panel b) responses, either as single time points or IAUC, at baseline and at the end of the intervention. * indicates $p < 0.05$ by unpaired sample T-Test. § indicates $p < 0.05$ for time × meal interaction, repeated measures ANOVA.

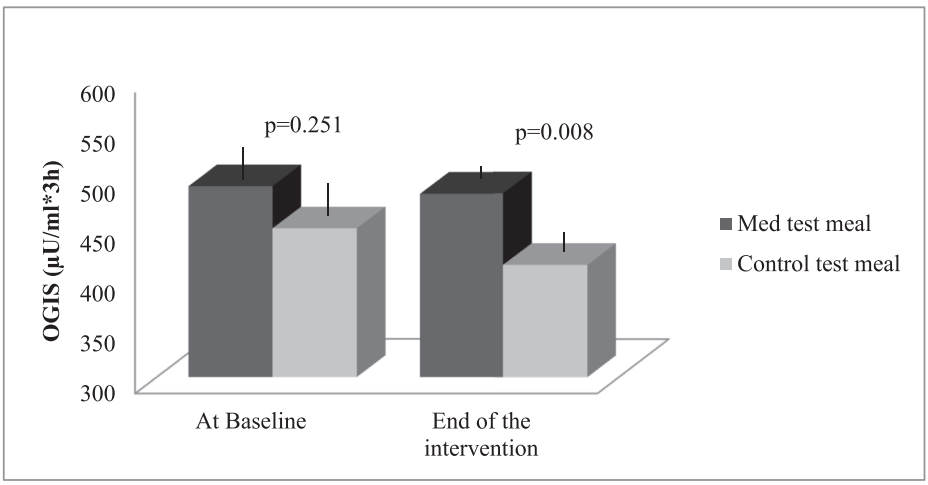

Fig. 2. Postprandial insulin sensitivity index (OGIS) at baseline and at the end of the intervention.

significantly increased at the end of the intervention only in the Med-D group, with a higher IAUC compared with the Control-D group (1530 ± 591 µmol/L vs −295 ± 144 µmol/L, p = 0.019). Furthermore, butyric acid IAUC at the end of the intervention was inversely correlated with plasma insulin IAUC and directly with OGIS in the whole group of participants (r: −0.411, p = 0.046 and r: 0.397, p = 0.050 respectively) as well as in the Med-D group (r: −0.544, p = 0.039 and r: 0.449, p = 0.045 respectively) but not in the Control-D group (r: −0.309, p = 0.385 and r: 0.376, p = 0.082 respectively).

3.4. Effects of dietary intervention on gut microbiota composition

We investigated the changes in microbiota composition in this specific group of subjects before and after the intervention with the Mediterranean diet by comparing the Med-D and Control-D groups. A significant increase in alpha diversity in the Med-D compared to Control-D group was observed at the end of treatment ($p < 0.05$) (Fig. 3). Significant variations in microbial taxa occurred as result of the intervention (Table 5). Interestingly, among other things, we found a significant decrease in the relative abundance of

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Table 4
Fasting and incremental area of SCFAs levels[b] at baseline and at the end of the intervention.

	Med-D Group	Control-D Group	P-value (Med-D vs Control-D Group)	Med-D Group	Control-D Group	P-value[a] (Med-D vs Control-D Group)
	Baseline			After 8-wk of intervention		
	n = 14	n = 13		n = 14	n = 13	
Fasting values						
Acetic acid (μmol/L)	261 ± 95	299 ± 157	0.441	258 ± 110	272 ± 114	0.750
Propionic acid (μmol/L)	27 ± 12	27 ± 11	0.927	32 ± 12	31 ± 12	0.707
Butyric acid (μmol/L)	30 ± 10	24 ± 10	0.126	29 ± 10	30 ± 15	0.743
Postprandial values (Incremental Area)						
Acetic acid (μmol/L*4 h)	−4991 ± 6691	−7379 ± 2883	0.774	−7650 ± 5173	−3889 ± 6211	0.645
Propionic acid (μmol/L*4 h)	−726 ± 419	670 ± 333	0.921	−728 ± 716	−6668 ± 5922	0.250
Butyric acid (μmol/L*4 h)	−142 ± 554	792 ± 423	0.229	1530 ± 591	−295 ± 144	0.019
						M±SE

[a] Data were corrected for baseline values.
[b] Data on 27 individuals (one did not perform the test meal, and another was excluded for very high butyric acid levels (>60 μmol/L) at fasting.

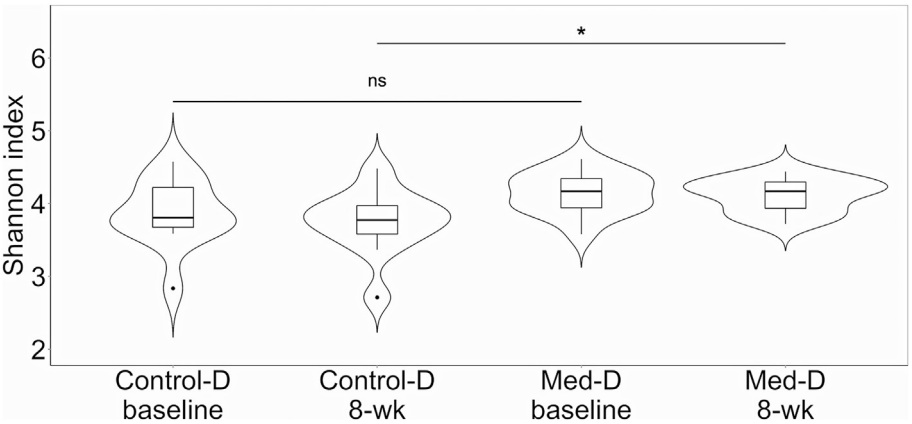

Fig. 3. Violin plot showing alpha diversity (Shannon Index) variation across samples in Med-D and Control-D groups at different timepoints. * Indicates $p < 0.05$ by unpaired Wilcoxon test. ns, not significant.

Table 5
Relative abundances of significant contrasting OTUs between Med-D and Control-D groups. Data are expressed as mean ± standard deviation. P value refers to variation at the specific timepoint compared to baseline in Med-D vs Control-D by unpaired Wilcoxon test. Status indicates in which diet those OTUs are more abundant. OTU, operational taxonomic unit.

OTUs	Control-D		Med-D		pvalue	Status
	baseline	Weeks 8	baseline	Weeks 8		
Firmicutes; *Ruminococcaceae*; *Ruminococcus*; *Ruminococcus faecis*	0.024 ± 0.031	0.05 ± 0.071	0.05 ± 0.037	0.021 ± 0.025	0.002	Control-D
Firmicutes; *Lachnospiraceae*; *Coprococcus*; *Coprococcus comes*	0.189 ± 0.16	0.244 ± 0.176	0.204 ± 0.089	0.112 ± 0.119	0.003	Control-D
Firmicutes; *Lachnospiraceae*; *Lachnoclostridium*; *Lachnoclostridium hathewayi*	0.004 ± 0.004	0.003 ± 0.005	0.084 ± 0.311	0.01 ± 0.019	0.005	Control-D
Firmicutes; *Lachnospiraceae*; unclassified; *Clostridium sphenoides*	0 ± 0	0 ± 0.001	0.002 ± 0.002	0 ± 0.001	0.009	Control-D
Actinobacteria; *Bifidobacteriaceae*; *Bifidobacterium*; *Bifidobacterium breve*	0.001 ± 0.003	0.002 ± 0.006	0.003 ± 0.008	0.001 ± 0.002	0.015	Control-D
Firmicutes; *Ruminococcaceae*; *Oscillospira*; *Oscillospira* [*Flavonifractor*] *plautii*	0.001 ± 0.002	0.002 ± 0.004	0.001 ± 0.003	0.001 ± 0.003	0.017	Control-D
Firmicutes; *Ruminococcaceae*; *Oscillospira*; *Oscillospira* [*Clostridium*] *viride*	0.027 ± 0.027	0.031 ± 0.033	0.053 ± 0.051	0.025 ± 0.023	0.03	Control-D
Firmicutes; *Lachnospiraceae*; *Blautia*; *Ruminococcus torques*	0.059 ± 0.064	0.125 ± 0.169	0.13 ± 0.156	0.048 ± 0.041	0.031	Control-D
Firmicutes; *Streptococcaceae*; *Streptococcus*; *Streptococcus gallolyticus*	0 ± 0	0 ± 0.001	0.022 ± 0.078	0 ± 0.001	0.033	Control-D
Firmicutes; *Lachnospiraceae*; [*Ruminococcus*]; *Clostridium glycyrrhizinilyticum*	0 ± 0	0 ± 0.001	0.002 ± 0.006	0 ± 0	0.033	Control-D
Firmicutes; *Lachnospiraceae*; *Dorea*; *Coprococcus comes*	0 ± 0.001	0.001 ± 0.002	0.001 ± 0.002	0 ± 0	0.038	Control-D
Firmicutes; *Lachnospiraceae*; *Lachnoclostridium*; *Clostridium hathewayi*	0.001 ± 0.002	0 ± 0.001	0.05 ± 0.175	0.01 ± 0.009	0.045	Control-D
Firmicutes; *Eubacteriaceae*; *Eubacterium*; *Eubacterium sulci*	0.006 ± 0.009	0.003 ± 0.005	0 ± 0.001	0.01 ± 0.012	0.002	Med-D
Firmicutes; *Lachnospiraceae*; *Coprococcus*; *Anoxystipes fissicatena*	0.034 ± 0.04	0.021 ± 0.018	0.022 ± 0.037	0.034 ± 0.034	0.009	Med-D
Firmicutes; *Clostridiaceae*; *Clostridium*; *Clostridium sporosphaeroides*	0.002 ± 0.005	0.001 ± 0.001	0.002 ± 0.003	0.007 ± 0.01	0.018	Med-D
Firmicutes; *Lachnospiraceae*; *Tyzzerella*; *Clostridium lactatifermentans*	0.003 ± 0.005	0.001 ± 0.002	0.001 ± 0.002	0.002 ± 0.006	0.022	Med-D
Firmicutes; *Ruminococcaceae*; unclassified; *Intestinimonas butyriciproducens*	0.386 ± 0.699	0.202 ± 0.382	0.19 ± 0.207	0.459 ± 0.539	0.025	Med-D
Firmicutes; *Lachnospiraceae*; [*Ruminococcus*]; *Clostridium glycyrrhizinilyticum*	0.001 ± 0.002	0 ± 0	0 ± 0	0 ± 0.001	0.027	Med-D
Firmicutes; *Lachnospiraceae*; unclassified; *Ruminococcus gauvreauii*	0.002 ± 0.004	0.001 ± 0.002	0 ± 0	0.001 ± 0.002	0.03	Med-D
Firmicutes; unclassified; unclassified; *Catabacter hongkongensis*	0.001 ± 0.004	0 ± 0	0.003 ± 0.011	0.005 ± 0.014	0.033	Med-D
Firmicutes; *Oscillospiraceae*; *Oscillibacter*; *Oscillibacter valericigenes*	0.004 ± 0.006	0.002 ± 0.004	0.009 ± 0.016	0.012 ± 0.023	0.034	Med-D
Firmicutes; *Lachnospiraceae*; unclassified; *Clostridium hathewayi*	0.004 ± 0.005	0.002 ± 0.002	0.006 ± 0.007	0.013 ± 0.016	0.035	Med-D
Verrucomicrobia; *Verrucomicrobiaceae*; *Akkermansia*; *Akkermansia muciniphila*	0.058 ± 0.089	0.028 ± 0.044	0.014 ± 0.021	0.116 ± 0.346	0.035	Med-D
Bacteroidetes; *Rikenellaceae*; *Alistipes*; *Alistipes Senegalensis*	0.048 ± 0.063	0.037 ± 0.06	0.048 ± 0.029	0.079 ± 0.068	0.046	Med-D
Firmicutes; *Lachnospiraceae*; *Blautia*; *Blautia glucerasea*	0.214 ± 0.106	0.149 ± 0.103	0.211 ± 0.164	0.222 ± 0.114	0.046	Med-D
Bacteroidetes; *Prevotellaceae*; *Prevotella*; *Prevotella oralis*	1.019 ± 3.054	0.231 ± 0.631	0.619 ± 2.316	0.992 ± 3.701	0.049	Med-D

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Ruminococcus torques, Coprococcus comes, Streptococcus gallolyticus and *Flavonifractor plautii* ($p < 0.05$) and a significant increase in the relative abundance of *Intestinimonas butyriciproducens* and *Akkermansia muciniphila* ($p < 0.05$) in the Med-D compared to Control-D group after 8 weeks of intervention. The increase in butyric acid observed was positively correlated to the relative abundance of *Bacteroides xylanisolvens* and *Roseburia hominis* (r: 0.26, $p = 0.05$ and r: 0.34, $p = 0.015$ respectively).

4. Discussion

The most relevant and novel results of this study, which was performed in overweight/obese subjects, are: 1) a test meal resembling a Mediterranean lunch is able to improve acute postprandial glucose response and, in particular, postprandial insulin response; 2) an 8-week consumption of a diet resembling the traditional Mediterranean Diet – based on wholegrain, legumes, vegetables, fruit, fish, nuts, extra virgin olive oil, and low in refined cereal products, meat and animal fat – further improves post-meal glucose and insulin responses and increases post-meal insulin sensitivity index.

Furthermore, the study suggests the involvement of gut microbiota metabolites – in particular butyric acid – in improving glucose metabolism and insulin sensitivity. Finally, it confirms the beneficial effects of the Mediterranean diet on fasting lipids, in particular LDL-cholesterol, also in people without metabolic abnormalities and with quite normal lipid levels.

It should be emphasized that in our study the improvement of cardio-metabolic risk factors induced by the Mediterranean diet was observed as compared to a Control diet balanced in nutrients, with a saturated fat content below 10%, a high content of monounsaturated fat and low added sugar, suggesting that the beneficial effects of the Mediterranean diet could be even stronger if the control diet had been a more radical Western diet. Based on the nutrient composition reported above, the main beneficial effects observed in the Mediterranean diet could be due to the increasing consumption of plant-based foods and fish. However, we cannot exclude that part of the benefits induced by the Mediterranean diet on cardiometabolic risk factors may be due also to polyphenols contained in extravirgin olive oil and plant-based foods. Furthermore, these data were obtained in free-living conditions using commonly available food items and without the use of food supplements, thus increasing the generalizability of our findings.

The possible effects of the Mediterranean diet have been studied in terms of both cardiovascular risk factors and cardiovascular events [3,6]. However, its possible impact on postprandial glucose and insulin responses is still unknown although postprandial abnormalities, in particular of glucose metabolism, might contribute to cardiovascular risk. To this regard, our study provides an important contribution, as it shows that the consumption of a Mediterranean type of meal was able to reduce acute glycemic and insulinemic responses. In addition, this benefit was strengthened in the medium term, leading to a clinically relevant decrease in glucose and insulin responses (57% and 67%, respectively). These results suggest an improved insulin action in the medium term, as demonstrated by the increased post-meal insulin sensitivity index (OGIS). Moreover, they are in line with the scientific evidence indicating the Mediterranean diet as a dietary pattern able to 1) reduce the risk of developing type 2 diabetes in healthy subjects and in individuals at high cardiovascular risk [8], 2) improve insulin resistance in the absorptive state in subjects with the metabolic syndrome and 3) exert favorable effects on glucose control in diabetic patients [34].

Worthy of note is that our intervention was based on the adoption of a Mediterranean diet as a whole by the participants, therefore our findings are strictly related to the whole dietary regimen. However, according to the multivariate regression analysis, dietary fiber intake remained significantly and inversely associated with plasma glucose and insulin responses, suggesting a key role of this dietary component on glucose and insulin improvement. The effect of dietary fiber, mainly for viscous fiber, can be due to a delayed digestion of carbohydrates and glucose absorption in the small intestine. It is likely that in our study the composition of the Med diet meal, higher in dietary fiber than the control meal (29.0 g vs 5.7 g) and containing 50% of dietary fiber from legumes (a source of viscous dietary fiber), might account for the low post-meal glucose and insulin responses observed acutely. The additional medium-term benefits of the Mediterranean diet on postprandial glucose metabolism and insulin sensitivity seem to involve the gut microbiota and associated metabolites [35,36]. In fact, we observed some interesting shifts in the gut microbiota composition, including an increase in species richness and evenness, which is correlated with improved health, since a reduced microbiota diversity is usually associated with several types of diseases, including obesity [37]. In addition, a desired increase in fiber-degrading microbial species such as the butyrate-producing *I. butyriciproducens* [38] and the next-generation beneficial microbe *A. muciniphila* [39] was achieved. On the other hand, a decrease in the levels of the potential proinflammatory *R. torques* [40,41], and of *S. gallolyticus* [42] and *F. plautii* associated with colorectal cancer [43] were observed. Finally, relative less *C. comes* were also detected, a species recently found to be enriched in the gut of obese individuals [44]. Overall, a Mediterranean based dietary treatment might contribute in restoring gut eubiosis condition in treated subjects.

In parallel with the changes in gut microbiota, the Med-D induced an increase in post-meal butyric acid levels, inversely correlated with plasma insulin response and directly with OGIS, strongly suggesting that the improved glucose metabolism and insulin sensitivity by Med diet could be also mediated by the activity of butyric acid. Butyric acid, along with propionic and acetic acids, are produced from dietary fiber by fermentation in the large intestine and they are partially absorbed to reach different tissues. Consistently, the Med-D intervention caused an increase in fiber-degrading bacteria and the increased concentrations of butyrate were positively correlated to the levels of the xylanolytic *B. xylanisolvens* [45] and to the butyrate producing *R. hominis* [46], further suggesting an effective interplay between diet, microbiota and metabolome. Generally, circulating propionic acid is associated with possible beneficial effects on glucose metabolism and insulin sensitivity. Indeed, in one of our previous studies, a 12-wk wholegrain wheat-based diet increased fasting plasma propionate, which was in turn associated with lower postprandial insulin concentrations [47].

In the present study, plasma propionate levels did not vary significantly either at fasting or in the postprandial period, whereas postprandial plasma butyric acid levels were increased after the Med Diet. We hypothesized that the different findings between the two studies were explained by the high variety of dietary fibers in the overall Mediterranean diet used in this study compared to the wholegrain dietary fiber, which is mainly insoluble.

To the best of our knowledge, this is the first time that postprandial plasma butyric acid levels are considered. In any case, especially on the basis of in vitro and animal studies, a more recent

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hypothesis is that also butyric acid may play a key role in the regulation of glucose metabolism with a possible action either at the level of insulin secretion or insulin sensitivity. In fact, it has been reported that butyrate might activate G protein-coupled receptors (GPCRs) in the gut with production of the glucagon-like peptide 1 (GLP-1), known to increase insulin secretion [48]. Regarding the improvement of insulin sensitivity, butyrate could act decreasing ectopic lipid deposition and inflammation in liver and peripheral tissues through an increase in fatty acids β-oxidation [49]. In addition, butyrate could improve gut integrity with subsequent reduction in systemic inflammation and oxidative stress leading to improvement in insulin sensitivity [49].

In addition to providing novel evidence on postprandial glucose metabolism, our study indicated that the Med diet is able to reduce LDL cholesterol levels also in normolipidemic overweight/obese individuals, reinforcing the data already obtained in subjects with higher LDL cholesterol levels [50]. The effects on triglycerides were less evident, with a trend to a reduction of fasting levels and no change in postprandial response. These results might be due to the very low fasting triglyceride levels of the participants and, as to the postprandial data, also to the fact that the test meal was quite low in total fat (35 g) – one of the main determinants of postprandial triglycerides response.

The main strengths of our study are that 1) it is a controlled intervention study performed in free-living conditions, utilizing a Mediterranean dietary pattern followed for centuries in Mediterranean regions. This, of course, increases its feasibility and the possibility to be implemented on a large scale and for very long period of time; 2) it has been focused on postprandial glucose metabolism, an aspect generally neglected; 3) it shows a close link between metabolic and gut microbiota results.

The study also presents some limitations: 1) an 8-week dietary intervention cannot be considered a real long-term experiment, although it is certainly sufficient to induce changes in lipid profile, glucose metabolism and insulin sensitivity; 2) it was performed in a small number of participants and 3) in overweight/obese individuals without strong metabolic alterations; therefore, the results obtained might not be generalized to other populations. However, this group of individuals represents a large part of the general population and the clinical results obtained are important in a context of metabolic and cardiovascular disease prevention.

In conclusion, the results of this study on a subgroup of participants of the study described by Meslier and co-workers [23] provides strong evidence that a diet resembling the traditional Med diet improves both cardio-metabolic risk factors in fasting conditions, and postprandial glucose metabolism and insulin sensitivity. Therefore, the health benefits of a Mediterranean diet for prevention of CVD and type 2 diabetes could be also mediated by its postprandial effect on glucose metabolism. Our study also highlights the implications of microbial butyric acid, and of dietary fiber as a precursor, in mediating some of the health effects of the Med diet. However, additional mechanistic studies are required to fully understand the potential role of changes in gut microbiota through the diet in the prevention of metabolic diseases and the possible role of SCFA. The benefits of the Mediterranean diet are due to the combined action of several components of plant origin, particularly dietary fiber, which could exert pleiotropic effects on different cardio-metabolic risk factors.

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Author contributions

Conceptualization: RG, PV, DE, AAR; Data curation: MV, ML; Laboratory analysis: ML, DL, AM, DS; Formal analysis: MV and ML; Funding acquisition: DE; Investigation: MV, RG, GDP; Methodology: RG, PV, DE, AAR; Project administration: PV, DE, AAR; Resources: MV, RG, PV, DE, AAR; Supervision: RG, PV, DE, AAR; Roles/Writing - original draft: MV, RG, ML, PV, DE, AAR; Writing - review & editing: PV, DE, AAR.

Conflict of interest

The authors declare that they have no conflicts of interest to disclose.

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Appendix A. Supplementary data

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