RESEARCH ARTICLE

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Effects of the Mediterranean Diet or Nut Consumption on Gut Microbiota Composition and Fecal Metabolites and their Relationship with Cardiometabolic Risk Factors

*Serena Galié, Jesús García-Gavilán, Lucía Camacho-Barcía, Alessandro Atzeni, Jananee Muralidharan, Christopher Papandreou, Pierre Arcelin, Antoni Palau-Galindo, David Garcia, Josep Basora, Alejandro Arias-Vasquez, and Mònica Bulló**

Scope: To examine whether a Mediterranean Diet (MedDiet) compared to the consumption of nuts in the context of a habitual non-MedDiet exerts a greater beneficial effect on gut microbiota and fecal metabolites; thus, contributing to explain major benefits on cardiometabolic risk factors.
Methods and Results: Fifty adults with Metabolic Syndrome are randomized to a controlled, crossover 2-months dietary-intervention trial with a 1-month wash-out period, following a MedDiet or consuming nuts (50 g day^{-1}). Microbiota composition is assessed by 16S rRNA gene sequencing and metabolites are measured using Nuclear Magnetic Resonance (NMR) and liquid chromatography coupled to triple quadrupole mass spectrometry (LC-qTOF) platforms in a targeted metabolomics approach. Decreased glucose, insulin and the homeostatic model assessment of insulin resistance (HOMA-IR) is observed after the MedDiet compared to the nuts intervention. Relative abundances of *Lachnospiraceae NK4A136* and an uncultured genera of Ruminococcaceae are significantly increased after the MedDiet compared to nuts supplementation. Changes in *Lachnospiraceae NK4A136* are inversely associated with insulin levels and HOMA-IR, while positively and negatively with changes in cholate and cadaverine, respectively.
Conclusions: Following a MedDiet, rather than nuts, induces a significant increase in *Lachnospiraceae NK4A136* and improves the metabolic risk. This genera seems to affect the bile acid metabolism and cadaverine which may account for the improvement in insulin levels.

1. Introduction

The role of specific foods or dietary components in shaping gut microbiota and fecal metabolites and their impact on human health is widely recognized. However, beyond the traditional view of the health effects of single foods, regardless of the dietary context in which they are consumed, the interest of full dietary patterns becomes an area of growing interest.

Consumption of plant-based foods has been associated with both lower cardiometabolic risk factors and a diverse microbiota profile, with a greater abundance of probiotic species compared to the intake of animal-based foods.[1,2] Within plant-based foods, nuts, a complex matrix of nutrients rich in fiber, unsaturated fatty acids and other phytochemical compounds, have demonstrated a favorable impact on gut microbiota. Regular consumption of almonds and pistachio was related to an increased amount of butyrate producers in humans.[3] Similarly, supplementation of

S. Galié, J. García-Gavilán, L. Camacho-Barcía, A. Atzeni, J. Muralidharan, C. Papandreou, A. Palau-Galindo, M. Bulló
Department of Biochemistry and Biotechnology
Faculty of Medicine and Health Sciences
University RoviraiVirgili (URV)
Reus, Spain
E-mail: monica.bullo@urv.cat

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S. Galié, J. García-Gavilán, L. Camacho-Barcía, A. Atzeni, J. Muralidharan, C. Papandreou, P. Arcelin, M. Bulló
IISPV
Institute of Health Pere Virgily
Reus Spain

S. Galié, J. García-Gavilán, L. Camacho-Barcía, A. Atzeni, J. Muralidharan, C. Papandreou, M. Bulló
CIBER Fisiopatología de la Obesidad y Nutrición (CIBEROBN)
Instituto de Salud Carlos III
Madrid, Spain

P. Arcelin, A. Palau-Galindo
SAGESSA
ABS Reus V. Centre d'Assistència Primària Marià Fortuny
Reus Spain

D. Garcia
ABS Alt Camp Oest. Centre d'Atenció Primària Alcover
Spain

almonds and walnuts in rats was associated with increased *Lactobacillus spp.* and *Bifidobacterium spp.*[4] or *Roseburia*,[5] respectively. Changes in gut microbiota related to circulating urolithins,[6] urine hippurate, p-cresol sulfate and dimethylamine,[7] and fecal secondary bile acids[8] have been reported after nuts consumption.

Nuts are typically included in Mediterranean Diet (MedDiet), a dietary pattern widely recognized as a powerful nutritional strategy to improve cardiometabolic health. The MedDiet is also rich in other types of foods such as whole grains, vegetables, legumes, fruits, and olive oil that, at least individually, seems to be also related to a diverse microbiome profile. Higher adherence to MedDiet has been positively associated with changes in specific bacteria and derived metabolites.[9] However, few studies have examined the effect of the MedDiet on gut microbiota composition and fecal metabolites together with their potential benefits on cardiometabolic health. An ancillary analysis of the CORDIOPREV study (CORonary Diet Intervention with Olive oil and cardiovascular PREVention) has demonstrated the restoration of some microbiota species after 2 years following a MedDiet in obese adults with Metabolic Syndrome.[10] More recently, a 12-month MedDiet intervention in elderly subjects displayed changes in specific taxa associated with lower frailty, improved cognitive function, and lower inflammation.[11] Although the effects of consuming either a MedDiet or including nuts in an habitual non-MedDiet on gut microbiota composition is poorly explored, less is known about dietary modulation of fecal metabolites which could help to provide a functional readout of microbial activity.[12]

Therefore, we examined whether following a MedDiet modifies gut microbiota composition and fecal metabolomics profile as well as cardiometabolic risk factors compared to a non-MedDiet supplemented with nuts. We also analyzed whether changes in gut microbiota and fecal metabolites are associated with changes in cardiometabolic risk factors.

2. Results

A total of 50 participants were randomized to the two dietary interventions. Of them, 38 were finally included in the analyses due to the fact that six dropped out for personal reasons and other six were excluded because of the unavailability of gut microbiota data (Figure S2, Supporting Information). No significant differences in participants' baseline characteristics were observed between the two interventions (**Table 1**). The mean ± SD for the 17-items MedDiet score was 11.37 ± 2.39 [increase, 4.49 (95% CI, 3.5–5.5] after MedDiet and 9.18 ± 2.41[increase, 0.5 (95% CI, -0.05 to 1.0)] at the end of nuts supplementation; between group differences 3.48 (95% CI, 2.41–4.56) $p < 0.001$. No significant differences in total energy and macronutrients intake were observed between interventions. Following a MedDiet resulted in a higher increase in fruit and fish and a decrease in alcohol and potatoes consumption compared to the non-MedDiet supplemented with nuts period. As expected, nuts consumption was significantly increased in the nuts intervention group (Table S2, Supporting Information). Significant differences in changes in glucose, insulin and in the homeostatic model assessment of insulin resistance (HOMA-IR) were observed between the MedDiet and the nuts supplementation periods (Table 1).

2.1. Effect of Dietary Interventions on Gut Microbiota Composition

There were no significant differences neither in estimated alpha-diversity indices (Figures S3, S4, and S5, Supporting Information) nor in beta-diversity (Table S3, Supporting Information). Additionally, no clear discrimination of microbial composition within each intervention was found in Non-metric Multi-Dimensional Scaling (NMDS) plots based on Bray-Curtis dissimilarity (Figure S6, S7, and S8, Supporting Information). The ratio of relative abundances between Bacteroidetes and Firmicutes was not significantly different between or within the interventions (data not shown). Differences in the gut microbiota composition revealed significant enrichment in *Lachnospiraceae NK4A136* genera and uncultured genera of Ruminococcaceae family after the MedDiet intervention compared to the non-MedDiet + nuts (linear discriminant analysis (LDA) score of 2.0 and $p < 0.05$, **Figure 1**). The within-group analysis revealed a significant increase in *Roseburia* and *Oxaobacter* while *Ruminococcaceae UCG014* and *Lactococcus* decreased (LDA score of 2.0 and $p < 0.05$, **Figure 2**). No significant changes were observed after the MedDiet intervention.

2.2. Effect of Dietary Interventions on Fecal Metabolites

Positive associations were found between changes in four metabolites' concentrations and the MedDiet intervention, (**Figure 3**) with the highest effect on homocitrulline followed by acetate, cadaverine and malate changes. Inverse associations were also observed between changes in nine metabolites and the nuts supplementation intervention. The highest effect of nuts intervention was found for changes in tryptophan followed by taurine, hyodeoxycholic acid (HDCA), methionine sulphoxide, serotonin, cholate, alanine, glycerol and valine.

2.3. Associations Between Gut Microbiota Composition, Fecal Metabolites, and Cardiometabolic Risk Factors

Increases in relative abundances of *Lachnospiraceae NK4A136* were significantly associated with decreases in insulin and

J. Basora
Tarragona-Reus Research Support Unit
Jordi Gol University Institute for Primary Care Research
Tarragona 43202, Spain
A. Arias-Vasquez
Department of Psychiatry
Radboudumc
Cognition and Behaviour
Donders Institute for Brain
Nijmegen, GA 6525, The Netherlands
A. Arias-Vasquez
Department of Human Genetics
Radboudumc
Cognition and Behaviour
Donders Institute for Brain
Nijmegen, GA 6525, The Netherlands

Table 1. Study participants' baseline and changes of anthropometric and biochemical parameters.

Characteristics	All	Mediterranean Diet		Nuts		MedDiet vs. nuts (changes)	
	Baseline (38)	Baseline	Change	Baseline	Change	*p* value (treat)[1]	*p* value (treat*per)[1]
Age (years)	51.37 (49.19, 53.55)	51.37 (49.19, 53.55)	–	51.37 (49.19, 53.55)	–	–	–
Weight (kg)	85.1 (81.54, 88.66)	84.71 (79.71, 89.70)	−0.79 (−1.37, −0.21)	85.5 (80.3, 90.7)	0.02 (−0.48, 0.51)	0.131	0.414
Waist circumference (cm)	101.83 (98.77, 104.9)	102.71 (99.24, 106.19)	−1.35 (−2.62, −0.08)	100.96 (95.83, 106.08)	1.07 (−2.01, 4.15)	0.583	0.900
SBP (mmHg)	134.88 (131.12, 138.64)	134.74 (129.76, 139.71)	−0.78 (−4.54, 2.98)	135.03 (129.26, 140.8)	−1.88 (−6.23, 2.47)	0.836	0.978
DBP (mmHg)	84.87 (82, 87.74)	84.87 (81.51, 88.23)	−0.82 (−2.81, 1.18)	84.87 (80.11, 89.63)	−2.45 (−5.71, 0.81)	0.583	0.900
Total cholesterol (mg dL^{-1})	210.21 (200.09, 220.33)	210.21 (196.66, 223.76)	−7.37 (−14.16, −0.57)	210.21 (194.81, 225.61)	−3.66 (−10.51, 3.19)	0.686	0.414
LDLc (mg dL^{-1})	132.53 (123.99, 141.07)	133.32 (122.11, 144.52)	−5.79 (−12.38, 0.8)	131.74 (118.55, 144.93)	−3.5 (−9.74, 2.74)	0.836	0.414
HDLc (mg dL^{-1})	50.58 (47.04, 54.11)	51.32 (46.01, 56.62)	−1.16 (−3.24, 0.93)	49.84 (45.05, 54.64)	0.16 (−1.51, 1.82)	0.283	0.978
VLDLc (mg dL^{-1})	27.11 (23.64, 30.57)	25.58 (20.54, 30.62)	−0.42 (−3.2, 2.36)	28.63 (23.84, 33.42)	−0.66 (−4.00, 2.69)	0.991	0.900
Triglycerides (mg dL^{-1})	135.97 (118.6, 153.35)	128.63 (103.39, 153.87)	−2.42 (−16.11, 11.27)	143.32 (119.22, 167.42)	−3.13 (−19.9, 13.63)	0.991	0.900
Glucose (mg dL^{-1})	101.05 (96.76, 105.34)	101.53 (95.36, 107.70)	−4.05 (−7.48, −0.63)	100.58 (94.45, 106.7)	1.97 (−0.93, 4.88)	0.032	0.900
Insulin (mcUI mL^{-1})	12.54 (10.54, 14.54)	12.99 (10.64, 15.35)	−1.65 (−3.3, 0)	12.08 (8.79, 15.38)	2.61 (−0.05, 5.26)	0.032	0.414
HOMA−IR	3.16 (2.61, 3.71)	3.28 (2.63, 3.93)	−0.56 (−1.05, −0.06)	3.04 (2.13, 3.95)	0.88 (0, 1.76)	0.032	0.414
Lymphocytes (x10^3 μL^{-1})	2.20 (2.02, 2.38)	2.16 (1.94, 2.38)	−0.13 (−0.35, 0.09)	2.24 (1.95, 2.52)	−0.01 (−0.12, 0.10)	0.448	0.900
IL−6 (pg mL^{-1})	2.93 (2.29, 3.57)	2.65 (1.76, 3.53)	0 (−0.62, 0.62)	3.23 (2.3, 4.17)	−0.15 (−0.62, 0.32)	0.836	0.414
Zonulin (ng mL^{-1})	40.16 (38.22, 42.1)	40.4 (38.82, 41.97)	1.31 (−0.88, 3.51)	39.92 (36.31, 43.52)	1.07 (−1.32, 3.46)	0.991	0.978
17−items MedDiet score	7.13 (6.49, 7.77)	6.95 (6.22, 7.67)	4.13 (3.28, 4.98)	7.32 (6.26, 8.38)	0.26 (−0.99, 1.52)	<0.016	0.414

All values are given as means (95% CI). Changes in anthropometric and biochemical parameters between dietary interventions were analyzed by using linear mixed-models analysis of variance with intervention groups and periods modelled as fixed factors, baseline values as covariates and subjects as random effect. DBP, diastolic blood pressure; HDLc, high density lipoprotein cholesterol; HOMA-IR, homeostatic model assessment of insulin resistance; LDLc, low density lipoprotein cholesterol; MedDiet, Mediterranean Diet; SBP, systolic blood Pressure; VLDLc, very low-density lipoprotein cholesterol. [1]Adjusted with the Benjamini-Hochberg False Discovery Rate method.

HOMA-IR (**Table 2**). Similar results were found after adjusting analyses for adiposity measures except for the association with HOMA-IR (Table S, Supporting Information). No associations were found between genera differentially abundant after the nuts intervention and cardiometabolic risk factors. Regarding fecal metabolites, decreased concentrations of fecal HDCA, cholate-bile acid, cholic acid (CA) and β-aminoisobutyric acid (BAIBA) were associated with increased glucose and insulin circulating levels (**Table 3** and Table S5, Supporting Information). Increased concentrations of fecal cadaverine and acetate and decreased concentrations of methionine and serotonin were associated with increased insulin levels (Table 3). Decreased BAIBA was also associated to increased HOMA-IR. Associations between changes in the relative abundance of genera and fecal metabolites changing during the interventions are shown in **Table 4**. Increased *Lachnospiraceae NK4A136* observed in the MedDiet intervention was positively associated with increases in the concentrations of cholate-bile acid and negatively with cadaverine. Also, increases in *Ruminococcaceae UCG014* were positively associated with cholate-bile acid and HDCA, whereas increases in *Lactococcus*, were inversely associated with HDCA and serotonin. In contrast, increases in *Roseburia* and *Oxalobacter*, were positive and negatively associated to changes in cadaverine, respectively.

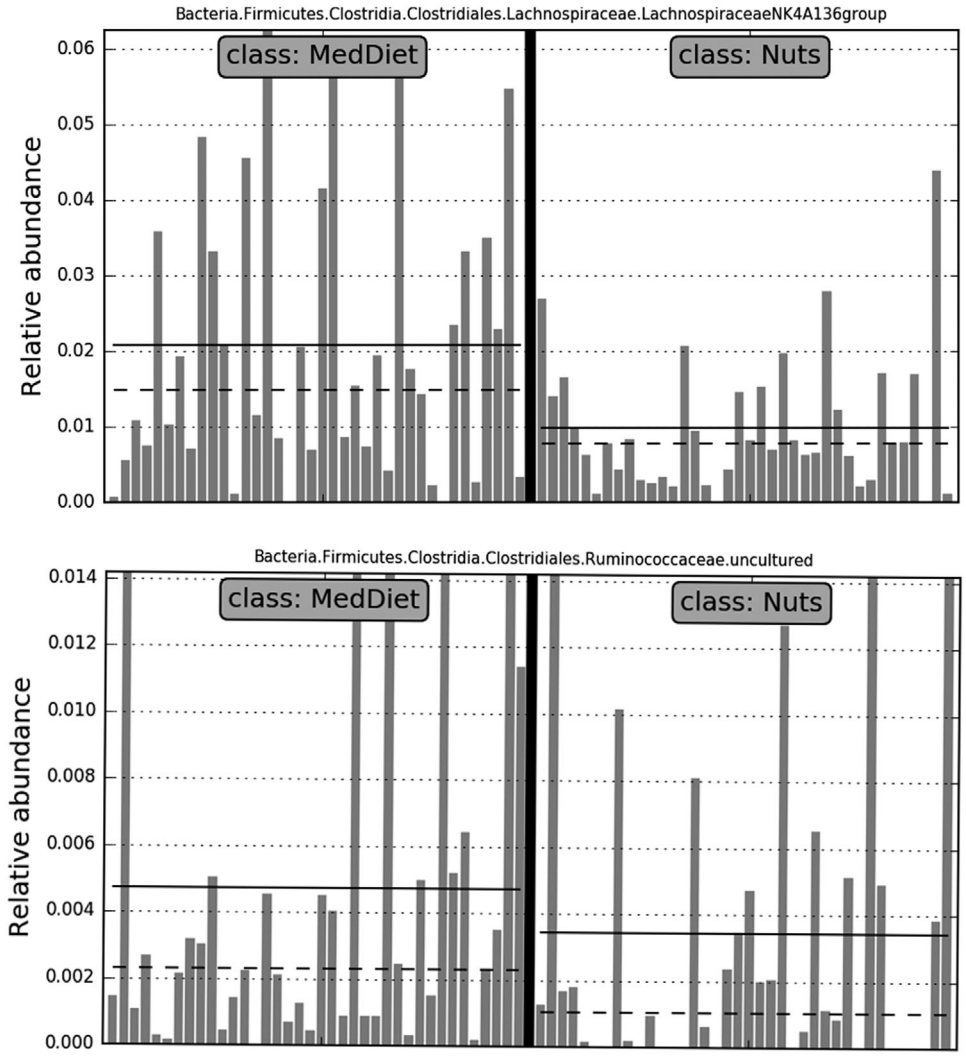

Figure 1. Plots of LEfSe results for between-dietary interventions comparisons of significant relative abundances at genus level. The two histograms reproduce the differential composition in changes of relative abundances for each of the two genera detected as statistically and biologically different between MedDiet and nuts interventions. The horizontal continue and dotted black line, respectively, represent the mean and median of changes in relative abundances for that selected genera.

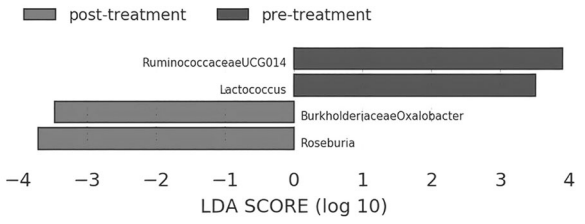

Figure 2. Plot of LEfSe results for relative abundances comparisons within the nuts intervention at genus level. LEfSe scores (in absolute value) can be interpreted as the degree of consistent difference in relative abundances between genera before (indicated as pre-treatment) and after (indicated as post-treatment) the nutritional intervention. The histograms, thus, identifies which clades among all those detected as statistically and biologically differential explain the greatest differences between communities of these two classes.

3. Discussion

In the present study we demonstrated for the first time that participants with Metabolic Syndrome following a Mediterranean dietary pattern, as opposed to a non-MedDiet diet supplemented with nuts, significantly changed specific microbiota genera and fecal metabolites that could partially explain the reduction of glucose, insulin levels, and HOMA-IR. Our findings imply that the quality of the diet may account for the metabolic benefits of the MedDiet, independent of changes in adiposity.

Strong evidence suggests that following a MedDiet or consuming nuts exerts beneficial effects on metabolic risk markers.[13,14] Results of our study highlight the importance of a full dietary pattern like the MedDiet instead of a specific healthy food on improving metabolic health. In line with previous studies, our findings suggest that medium-term dietary interventions do not induce major changes on alpha and beta-diversity indices.[11]

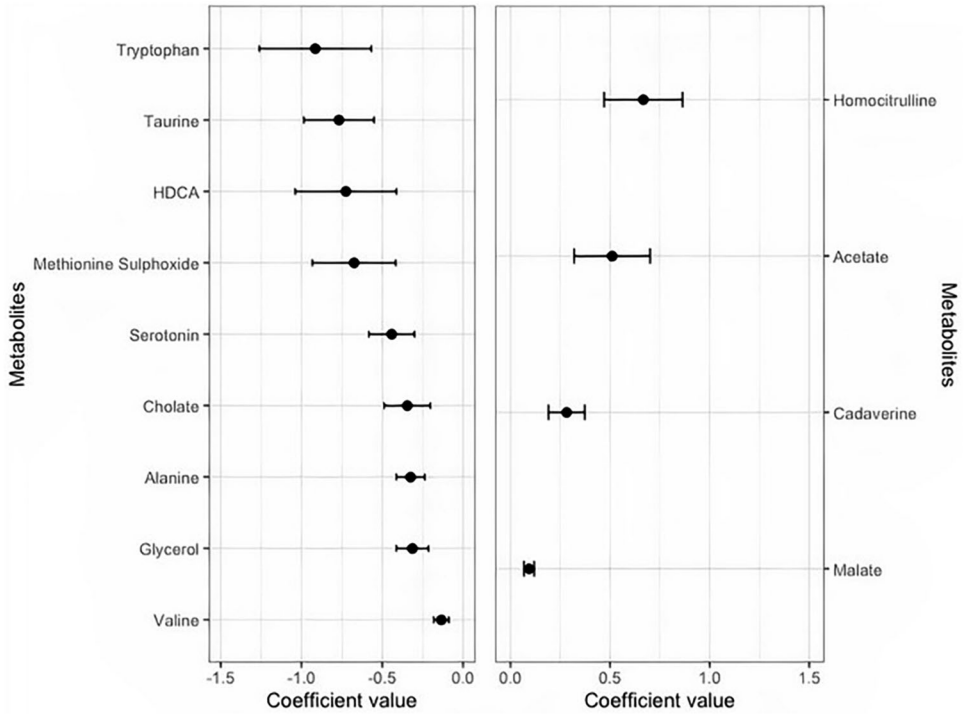

Figure 3. Changes in fecal metabolites ranked from the lowest to the highest elastic net positive or negative regression coefficients for the Mediterranean dietary intervention.

Table 2. Associations between changes in relative abundances of *Lachnospiraceae NK4A136* and uncultured genera of Ruminococcaceae with changes in cardiometabolic risk factors.

	Lachnospiraceae NK4A136						*Ruminococcaceae uncultured*					
	Fixed effects			Random effect			Fixed effects			Random effect		
	B coeff	SE	*p*-value	ICC grp	SD	*p*-value	B coeff	SE	*p*-value	ICC grp	SD	*p*-value
Glucose (mg dL^{-1})	−0.0003	0.0002	0.176	0.097	9.73e−03	0.143	−0.0001	0.0001	0.181	0.112	9.70e−03	1
Insulin (mcUI mL^{-1})	−0.0008	0.0003	0.017	−0.061	9.71e−03	0.119	0.0000	0.0001	0.966	−0.071	2.35e−07	1
HOMA-IR	−0.0023	0.0011	0.036	−0.066	9.70e−03	0.129	−0.0001	0.0003	0.848	−0.077	2.36e−07	1

Generalized linear regression model for the changes in relative abundances of *Lachnospiraceae NK4A136 group* and *Ruminococcaceae uncultured genera*. HOMA-IR, homeostatic model assessment of insulin resistance; ICC grp, intra-class coefficient of correlation; SD, standard deviation; SE, standard error.

However, following a MedDiet compared to a non-MedDiet supplemented with nuts, displayed a significant increase in *Lachnospiraceae NK4A136* genera and uncultured genera of the Ruminococcaceae family. Other genera from the Lachnospiraceae family have been previously associated to a higher MedDiet adherence.[15–17]

The MedDiet intervention was also associated with increased fecal short chain fatty acid (SCFA) acetate and the organic acid malate, together with decreased fecal bile acids (HCDA and cholate) supporting some[11,15] but not all[17] previous findings. Although we failed to find any significant association between changes in *Lachnospiraceae NK4A136* and the fecal SCFAs, this genus belongs to a family of anaerobic bacteria involved in the fermentation of plant polysaccharides to SCFA. A study conducted in Type 2 Diabetes (T2D) mice treated with a flavonoid-rich extract demonstrated an increase in this genera abundance.[18] This genus was positively correlated with fecal acetate, butyrate and total SCFAs in children.[19] Inconsistencies exist in the literature about the role of fecal SCFAs on metabolic health. In a recent cross-sectional study among 160 adults circulating rather than fecal SCFA were associated with peripheral insulin sensitivity.[20] Our study showed positive associations of changes in fecal acetate and butyrate with changes in insulin levels and this is in accordance with the findings of a recent cross-sectional analysis of 441 community-dwelling adults in which fecal acetate and butyrate were significantly correlated with insulin levels but not glucose and HOMA-IR.[21] An increase in *Lachnospiraceae NK4A136* in mice has been correlated with a decrease in glucose levels, improved glucose tolerance and reduced inflammatory status by activation of the IRS1/PI3K/AKT and inhibition of the JNK1/2

Table 3. Associations between changes in fecal metabolites and changes in cardiometabolic risk factors.

Cardiometabolic risk factors	Metabolites	Coefficient	Metabolites	Coefficient
Glucose			Cholate	−0.059
			HDCA	−0.076
Insulin	Acetate	0.528	Methionine	−0.167
	Cadaverine	0.491	Serotonin	−0.414
			HDCA	−0.427
			Cholate	−0.463

HDCA, hyodeoxycholic acid.

Table 4. Fecal metabolitessignificantly associated with cardiometabolic risk factors ranked from the highest to the lowest elastic net positive or negative regression coefficients for genera significantly changed across and within interventions.

MedDiet vs. nuts	Nuts			
Lachnospiraceae NK4A136	*Ruminococcaceae UCG014*	*Lactococcus*	*Roseburia*	*Oxalobacter*
Cholate	Cholate	HDCA	Cadaverine	Cadaverine
0.134	0.459	−0.398	0.368	−0.271
Cadaverine	HDCA	Serotonin		Cholate
−0.333	0.322	−0.807		−0.430
				HDCA
				−0.491

HDCA, hyodeoxycholic acid.

insulin pathway.[18] Similarly, to these findings, we also observed that increased *Lachnospiraceae NK4A136* genus was associated with decreased insulin levels and HOMA-IR. The reduction in fecal bile acids after the MedDiet, reflects the replacement of foods from animal origin with plant-based foods. Beyond their role to facilitate the intestinal absorption of dietary fat, bile acids may act as signaling molecules to control several metabolic pathways including regulating glucose metabolism[22] through the regulation of multiple receptors. This could explain the inverse association between changes in fecal bile acids with glucose and insulin levels. At the same time, fecal bile acids are elevated in patients with type 2 diabetes and uncontrolled hyperglycemia, while they are decreased upon insulin treatment, supporting a modulatory role of insulin on bile acid metabolism.[23] Interestingly, increases in *Lachnospiraceae NK4A136* were associated with increases in fecal cholate. Previous studies have revealed positive correlations between Lachnospiraceae but also Ruminococcaceae families with secondary fecal bile acids.[24] These correlations arTe likely due to the ability of these taxa to perform 7α-dehydroxylation from primary to secondary bile acids to provide an energy advantage to the bacteria. Therefore, it could be speculated that in a dynamic situation with increasing *Lachnospiraceae NK4A136*, the positive association we found between this genus and the fecal cholate was due to an increased reabsorption of cholate at intestinal level to convert to secondary bile acids. Changes in *Lachnospiraceae NK4A136* were also inversely associated with cadaverine, which has been found to promote insulin secretion.[25] In our study, increased cadaverine was associated with increased insulin levels. Whether increases in *Lachnospiraceae NK4A136* abundance play a role in the link between the MedDiet and improvements in insulin levels by decreasing fecal cadaverine concentrations needs further investigation.

Significant increases in *Roseburia* and *Oxalobacter* genera were observed after nuts consumption. Our results are similar to those observed in two different walnuts feeding studies conducted either in a healthy population or in subjects at risk for cardiovascular disease showing enrichment of *Roseburia* after 3- or 6-weeks of intervention.[8,26] An increase in *Oxalobacter* was also observed after nuts consumption in our study. Nuts are rich in oxalate, the main substrate for *Oxalobacter formigens*, and consumption of almonds has been related to a higher concentration of gastric oxalate.[27] Simultaneously, we found a significant decrease in *Ruminococcaceae UCG014 group* and *Lactococcus* after nuts supplementation. *Ruminococcaceae UCG014 group* increased after a methionine-choline deficient diet in mice[28] and after a high-fat-diet supplemented with quercetin and resveratrol[29] in rats, but no evidences are available in human studies.

Our study has limitations. First, the relatively small sample size and the high variability in gut microbiota composition among individuals made it challenging to find a microbial profile characterized by more genera discriminating both dietary interventions. Also, the crossover design of this study does not allow to completely discard a residual carry-over effect (despite the lack of statistical significance after testing the carry-over effect over the main outcomes) as well as the potential bias deriving from the unfeasible blinding. Furthermore, we have to take into consideration the known "Anna-Karenina Principle" in human microbiota, which essentially points out that individual gut microbiota composition under specific medical conditions is not able to vary in a common direction after an external stimulus like dietary intervention is applied.[30] So, the lack of a distinct microbial diversity between intervention groups observed in our study could be related to the absence of a common gut microbial behavior. Another important limitation is the use of 16S rRNA sequencing which, in contrast to shotgun metagenomics, does not allow to identification of bacterial species. Finally, conducting a targeted metabolomics analysis limits the perspective to discover new metabolites potentially associated with the identified genera and future untargeted metabolite profiling could be a complementary approach. Our study has also strengths that deserve to be mentioned such as the crossover, randomized, controlled design, able to balance the intra-individual variability in gut microbiota. Also, the longtime of the wash-out period avoided the potential carry-over effect.

In conclusion, the present study documented for the first time that following a MedDiet rather than the consumption of nuts in the context of a non-MedDiet, induced a significant increase in *Lachnospiraceae NK4A136* abundance and improved the metabolic risk profile. This genus seems to affect the fecal bile acids metabolism and cadaverine which may account for improvements in insulin and glucose metabolism. Further intervention studies are needed to understand the effects of different healthy dietary patterns on gut microbiota composition and functionality for the prevention and/or management of cardiometabolic diseases.

4. Experimental Section

Study Design and Population: METADIET was a randomized, controlled, crossover 2-months dietary-intervention trial, with a 1-month wash-out period. Eligible participants were community-dwelling subjects aged 25–60 years with a body mass index (BMI) between 25 and 35 kg m^{-2} and Metabolic Syndrome according to the ATPIII diagnosis criteria.[31] Exclusion criteria were: T2D; secondary obesity or related pathologies; non-controlled hypertension; LDL-cholesterol >160 mg dL^{-1}; triglycerides >400 mg dL^{-1}; 17-item MedDiet score >9;[32] regular intake of nuts (≥90 g $week^{-1}$); several chronic diseases (inflammatory, infectious, chronic obstructive pulmonary, neoplasia, endocrine, or hematological diseases); leucocytes >11 × 10^9; specific pharmacological treatments (anti-inflammatory, corticoids, hormones or antibiotics); changes in body weight (>5 kg in the last 3 months); alcohol or drug abuse and consumption of prebiotics, probiotics or laxatives. Subjects who met the inclusion criteria were randomly assigned to one of the two sequence intervention periods using a computer-generated random-number table. After 1 month of "wash-out" period, participants crossed the interventions for the other 8 weeks. Half of the participants followed a MedDiet intervention for 8 weeks, while the other half continued with their habitual non-MedDiet supplemented with 50 g day^{-1} of mixed nuts (almonds, hazelnuts, and walnuts, provided free) (Figure S1, Supporting Information). The trial was registered in the ISRCTN (ISRCTN88780852) on the 7th of April 2017, https://doi.org/10.1186/ISRCTN88780852. Written informed consent was obtained for all participants.

Dietary Interventions: Several face-to-face interviews with trained dietitians were scheduled at the beginning, after 15 days, at 1 month and at the end of each intervention period. During the MedDiet intervention period participants were encouraged to adhere to the 17-item MedDiet score used in the PREDIMED Plus study.[32] Participants received written material and all recommendations to follow the MedDiet, emphasizing in the consumption of at least two servings of vegetables and three fruits per day; ≥3 servings of legumes, ≥5 servings of whole-grain cereals/pasta, ≥3 servings of fish/seafood per week and the use of extra virgin olive oil as the main culinary fat. They were also instructed to reduce the consumption of red meat and processed foods ≤1 serving $week^{-1}$, use of butter and margarines (<1 $week^{-1}$), white bread (≤1 day^{-1}) and sugary beverages or sugar-sweetened fruit juices (<1 $week^{-1}$). Participants were provided with biweekly menus and seasonal recipes to facilitate the adherence to the MedDiet intervention. During the nuts intervention period, dietitians did not provide any other dietary advice rather than the consumption of 50 g day^{-1} of mixed nuts that were provided by free, and written culinary advices to include nuts in regular meals with soups, creams or as side food.

Nutritional data were collected in each sampling visit using 3-day dietary records, nutrient and energy intakes were calculated using Spanish food composition tables.[33,34] Adherence to the interventions was assessed by the validated 17-item MedDiet score[32] and counting the empty nuts-packaging returned in several visits along the intervention.

Anthropometry and Blood Pressure: Weight, height, and waist circumference were determined with calibrated scales and a wall-fixed stadiometer, BMI was then calculated. Blood pressure was measured in duplicate using a validated semiautomatic oscillometer (Omron HEM-705P, Holland).

Biological Samples, Collection, and Storage: Fasting blood samples were collected at baseline and at the end of each intervention period. Glucose, lipid profile and lymphocytes were measured using standard enzymatic automated methods. LDL-cholesterol was estimated using the Friedewald formula in subjects with triglycerides <400 mg dL^{-1}. Circulating insulin, IL-6 and zonulin levels were measured by commercial ELISA (Deltaclon SL, Spain and ImmunDiagnostik, Germany, respectively). The HOMA-IR was estimated.[35] Participants were instructed to collect stool samples in hermetic sterile-flasks and freeze them immediately at -20 °C. Frozen samples were delivered to the laboratory within 1–2 days after collection and stored in different aliquots at -80 °C.

16S rRNA Gene Sequencing and Data Processing: Fecal DNA extraction was performed with QIAmpPowerFecal DNA kit (Qiagen, Germantown). A first additional 5-min lysis step using FastPrep-24-5G Homogenizer (MP Biomedicals) was conducted. The 16S rRNA gene region was amplified with Ion Metagenomics kit (Life Technology, Carlsbad, California) by two separated PCR reactions using two primer sets: V2, V4, V8 and V3, V6-7, V9. 50–100 ng of combined amplicons were processed to obtain DNA libraries using Ion Plus Fragment Library kit and Ion Xpress Barcodes Adapters, 1–64 (Life Technology, Carlsbad, California). Adapter-ligated and nick-repaired libraries were purified by using CleanNGS kit (CleanNA, Waddinxveen, Netherlands). The libraries were amplified (Ion Plus Fragment Library kit) and quantified with Bioanalyzer (Agilent Technologies, Santa Clara, California) and the kit Agilent DNA 7500 Reagents (Agilent technology, Santa Clara, California). Finally, equimolar amounts of all the libraries (60 µM) were sequenced in four different runs with Ion 520 and Ion 530 Kit-Chef (Life Technologies, Carlsbad, California, EUA) and a 530 chip for sequentiation (Ion Torrent platform). The sequencing data were pre-processed with an adapted in-house script[36] in order to split only forward reads of each sample data into six subsets of six hypervariable regions. Reads from V4 region were used for this study. Quality control, length filtering at 280 bp and denoising of sequences with DADA2 pipeline other than taxonomy assignment were performed in QIIME2 software version 2019.4 using the latest version of Silva database (Silva 132) as 16SrRNA gene classifier. Finally, the study used a priori cut off value of 10% of prevalence at genus level on the absolute abundances of the amplicon sequence variant (ASV) table obtained from QIIME2, in order to remove ASVs with a prevalence ≤10% within and between samples. The study then transformed the filtered ASV table in relative abundances in all the samples, irrespective of treatment and sequence of intervention by using phyloseq package in R (version 1.34.0).

Fecal Metabolomics Analysis: Fecal samples were lyophilized previous to the metabolomics analysis. The 94 metabolites are listed in Table S1, Supporting Information. Nuclear Magnetic Resonance (NMR) and liquid chromatography coupled to triple quadrupole mass spectrometry (LC-qTOF) were used for the fecal metabolome analysis in a targeted approach. NMR was used to profile metabolites including SCFAs, alcohols and organic acids. 10–15 mg of lyophilized dry fecal matter was homogenized and separated into aqueous phase, insoluble compounds and protein and cellular debris. 200 µL of fecal aqueous phase and 400 µL of PBS in D2O (pH = 7.4, 0.05 M, TSP 1.48 mM for diluted concentration of 1 mM) were placed into a 5 mm o.d. NMR tube. 1H-NMR spectra were recorded at 300 K on an Advance III 600 spectrometer (Bruker, Germany) operating at a proton frequency of 600.20 MHz using a 5 mm PBBO broadband gradient probe. The acquired NMR was compared to references of pure compounds from the metabolic profiling AMIX spectra database (Bruker), HMDB, and Chenomx databases for metabolite identification. The study assigned metabolites by 1H–1H homonuclear correlation (COSY and TOCSY) and 1H–13C heteronuclear (HSQC) 2D-NMR experiments and by correlation with pure compounds run in-house. After pre-processing, specific 1H-NMR regions identified in the spectra were integrated using the AMIX 3.9 software package. LC-qTOF was used to determine bile acids, amino acids and its derivatives. The chromatographic separation of bile acids was performed on a Kinetex EVO C18 (150 × 2.1 mm) column and bile acid species were assigned by direct comparison with commercial standards. The chromatographic separation of amino acids was performed on a ACQUITY UPLC HSS T3 Column, and assigned by direct comparison with commercial standards. The chromatographic behavior and presence of possible interferences were based on the methodology previously described.[37]

Statistical Analyses: The sample size was estimated to detect a difference of three-fold changes in microbiota genus (*Roseburia, Oscillospira* and *Prevotella*) between the dietary interventions, accepting an alfa risk of 0.05 and a beta risk of 0.1 in a bilateral contrast.[38] A total of forty-five participants were required considering 10% withdrawal. Normality was checked by Lilliefors test. Descriptive data of participants were presented as means and 95% CI or medians with 25–75% interquartile range for quantitative variables, and as percentages for categorical variables. Changes in anthropometric, biochemical and nutritional data were analysed by using linear mixed-model analysis of variance with intervention groups and periods modelled as fixed factors, baseline values as covariates and subjects as a random effect. To account for multiple testing, the study adjusted *p* for

treatment and p for treatment * period of the crude and multivariable-adjusted associations with the use of the Benjamini-Hochberg false discovery rate (FDR) procedure. To check for possible carry-over effect, the study adopted a linear model of regression analysis of variance with intervention group and period modelled as fixed factors and subjects as random effect. For the microbiome analysis, α-diversity indices were calculated in R with "phyloseq" package (version 1.30.0). Adonis test was performed in R with "Adonis" function ("vegan" package, version 2.5-6) using β-diversity calculated as Bray-Curtis dissimilarity within and between interventions. NMDS plots of Bray-Curtis dissimilarity were generated. Bacteroides/Firmicutes ratio was calculated with bfratio function in "microbiome" package in R (version 1.9.19) and paired Wilcoxon test was applied to compare this ratio between and within interventions. The relative abundances of taxa at genus level were subjected to a linear discriminant analysis effect size analysis (LEfSe, http://huttenhower.sph.harvard.edu/galaxy/).[39] LEfSe determines the features, such as operational taxonomic units, most likely to explain differences between classes by coupling standard tests for statistical significance (Kruskal Wallis and Wilcoxon tests) together with additional analysis to measure the magnitude of the observed phenomenon by ranking the biological consistency and effect size relevance.[39] LDA scores of 2 and a p-value for Wilcoxon test of 0.05 were used to identify genera that were enriched with respect to baseline timepoints in each dietary intervention or to the other diet in the comparison of genus abundances at final time point. Fitted linear mixed-effects models of regression analyses, with the selected genera from LEfSe analysis and the cardiometabolic risk factors significantly changed as fixed terms and subjects as random effect was also conducted by using lmer function of "lme4" package in R, version 1.1-2.6. An additional test to evaluate the p-value of the random effect was conducted with "rand" function in "lmerTest" package of R (version 3.1-3). To address potential confounding effects of weight and waist circumference changes on the association between genera and cardiometabolic risk factors the study conducted a sensitivity analysis by adding them as covariates. From a total of 94 fecal metabolites profiled, one metabolite was removed due to the high number of missing values (>20%) and metabolites with less than 20% missing values were imputed using the random forest imputation approach[40] ("missForest" function of "randomForest" R package version 4.6-14). The concentrations of metabolites were approximated to a normal distribution with the rank-based inverse normal transformation. Due to the high dimensionality and collinear nature of the data, logistic regression with elastic net penalty (alpha = 0.5) was implemented in the "glmnet" (R package, version 3.0-2) to select the metabolites that are associated with the dietary interventions. The same package was used and logistic regression with elastic net penalty was implemented for estimating the associations of changes in metabolites identified in the previous model (independent variables) with significant changes in cardiometabolic risk factors (each cardiometabolic risk factor was the dependent variable and treated as dichotomic variable according to the median). The associations between higher metabolites' concentrations (independent variables) and changes in microbial genera (each genera was the dependent variable) were analyzed implementing elastic net linear regression models. For this purpose, the study used changes in relative abundances of the genera significantly increased or decreased after dietary interventions which were normalized using "clr" function of the R package "compositions" (R package, version 1.40-4). All analyses were performed using R, version 3.6.2. All tests were two-sided, and significance was defined as $p < 0.05$.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

Data available by request to the authors.

Keywords

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