Original article

Impact of a Moderately Hypocaloric Mediterranean Diet on the Gut Microbiota Composition of Italian Obese Patients

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Abstract: Although it is known that the gut microbiota (GM) can be modulated by diet, the efficacy of specific dietary interventions in determining its composition and diversity in obese patients remains to be ascertained**.** The present work aims to evaluate the impact of a moderately hypocaloric Mediterranean diet on the GM of obese and overweight patients (OB). The GM of 23 OB patients (F/M= 20/3) was compared before (T0) and after 3 months (T3) of the nutritional intervention (NI). Fecal samples were analyzed by Illumina MiSeq sequencing of the 16S rRNA gene. At baseline, the GM characterization confirmed the typical obesity-associated dysbiosis. After 3 months of NI, patients presented a statistically significant reduction of the body weight and fat mass, along with changes in the relative abundance of many microbial patterns. In fact, we observed an increased abundance in several Bacteroidetes taxa (i.e. Sphingobacteriaceae, *Sphingobacterium*, *Bacteroides* spp., *Prevotella stercorea*) and depletion of many Firmicutes taxa (i.e. Lachnospiraceae members, Ruminococcaceae and *Ruminococcus*, Veillonellaceae, *Catenibacterium*, *Megamonas)*. In addition, the phylum Proteobacteria showed an increased abundance, while the genus *Sutterella*, within the same phylum, decreased after the intervention. Metabolic pathways, predicted by bioinformatic analyses, showed a decrease in membrane transport and cell motility after NI. The present study extends our knowledge of the GM profiles in OB, highlighting the potential benefit of a moderate caloric restriction in counteracting the gut dysbiosis.

Keywords: gut microbiota, obesity, weight loss, Mediterranean diet, 16S rRNA, High-throughput sequencing

1. Introduction

The worldwide prevalence of obesity nearly tripled between 1975 and 2016. In 2016, more than 1.9 billion people aged 18 years and over were overweight and of these, over 650 million were obese [1]. In the same year, more than a third of the Italian population was overweight (35.3%) and one person in ten was obese (9.8%) [2].

Overweight is defined as a BMI (weight in kilograms/height^2 in meters) of 25 to 29.9 kg/m^2, obesity as a BMI of >30 kg/m^2. Increased BMI has been associated with an increased risk of all-cause mortality [3,4] and specific causes of mortality including cancer, cardiovascular, and respiratory deaths [4].

Obesity is a chronic disease characterized by a multifactorial etiology including genetic, behavioral, environmental, psychological, social, and cultural factors that result in a positive energy balance that promotes excessive fat deposition [5].

The gut microbiota (GM), the community of microorganisms colonizing the gastrointestinal tract, is required for the development and homeostasis in adult life [6]. In fact, compositional changes of GM have been linked with metabolic disorders, including obesity and metabolic syndrome, and functional gastrointestinal disorders [7].

Several scientific pieces of evidence recognize that the metabolic activity of the intestinal microbiota can play an important role in the pathogenesis of obesity. Different mechanisms through which GM can promote the deposition of fat have been hypothesized, including the suppression of the fasting-induced adipose factor and the reduction of AMPK activation, with an increase of lipoprotein lipase activity, the extraction of energy from fiber, the changes in intestinal permeability and the consequent increase in LPS and chronic low-grade inflammation, and the metabolism of bile acids [4,8,9].

Considering the modifiable nature of the GM, and the driven role of the diet in determining its composition [10], the effect of many weight-loss interventions on GM composition was investigated in the last decade. A recent systematic review on this topic showed that both restrictive diets (very low energy or low-carb) and bariatric surgery (BS) interventions decreased the microbiota abundance, and generally reduced the butyrate producers *Lactobacillus* spp. and *Bifidobacterium* spp. [11].

To date, the elaboration of effective strategies aimed to obtain weight loss accompanied by the restoring of the gut microbial composition represents a still open challenge. In fact, if on the one hand beneficial modulation of GM can facilitate both the improvement of metabolic outcomes and weight-control in the long term, on the other, negative impacts on the already compromised gut microbial balance can have a deleterious effect on the colon. This consideration could be on the basis of the higher relapse rate after drastic diets [12].

In the present study, we investigated the potential role of a nutritional intervention (NI) based on moderate caloric restriction (duration= 3 months) in modulating the GM composition and diversity in a group of overweight and obese patients (OB).

2. Materials and Methods

2.1 Patients recruitment and samples collection

Institutional review boards and human subject committees at participating institutions approved the study (Prot.PG/2020/2973). All subjects gave written informed consent in accordance with the Declaration of Helsinki.

We recruited 23 OB patients, consecutively enrolled among obese outpatients at the Obesity Center of the University hospital of Cagliari (Italy). Subjects of both sexes aged at least 18 years were included. The inclusion criteria for the OB group were a BMI≥25 and being "diet-free". We defined as "diet-free" the patients that did not follow any specific diet within the last 12 months before the recruitment, in order to characterize the GM during their usual dietary habits. Exclusion criteria were the following: therapy with antibiotics, proton pump inhibitors, or metformin in the last 3 months; the use of prebiotics, probiotics, or dietary supplements in the last 3 months; the presence of Intestinal Bowel Disease; the history of cancer; the presence of psychiatric disorders. A group of 46 healthy normal-weight subjects (NW), matched for gender and age, was recruited as control at baseline.

Stool samples from each subject were collected at outpatient facilities and delivered to the laboratory within 3 hours. Fresh samples were stored at -80°C until further processing. Samples from OB patients were collected both at baseline, before the starting of the NI (T0), and after three months of NI (T3).

2.2 Anthropometric and nutritional assessment

All the anthropometric measurements were collected on the same day as sample collection. The height expressed in cm was measured with a stadiometer, after removal of the shoes, with the participant's head being in the "Frankfort plane". The body weight, expressed in kilograms, was measured with an impedance scale (TANITA BC-420), also used for the analysis of the body composition. The impedance analysis was executed at room temperature, with a fasted state (for at least 2 hours) and with the last moderate-intense physical activity performed at least 24 hours previously. The following parameters of body composition were extracted and collected for the present study: bodyweight expressed in kilograms, BMI, fat mass expressed in kilograms and percentage, muscle mass expressed in kilograms, basal metabolic rate (BMR). BMI was calculated by the ratio of weight in kilograms and height in meters squared. Waist circumference, expressed in centimeters, was measured using recommendations of the Airlie conference [13].

A weighted 3-day food intake record (3d-FR, including 2 days of the week and 1 weekend day) was collected on the same day of sample collection. The analysis was performed with the Winfood ® software (version 3.0) and the following parameters were obtained: average daily caloric intake expressed in Kcal, daily percentage of macronutrients intake (carbohydrates, lipids and, proteins), daily percentage of saturated lipids intake (on the total lipids intake), and daily intake of fiber in grams. Overall dietary habits were evaluated through the Mediterranean Diet Score (MedDietScore, range 0–55) that assessed adherence to the Mediterannean diet [14], where higher scores indicate higher compliance.

2.3 Nutritional intervention

Patients followed a prescribed diet, with a daily caloric intake equivalent to their BMR (±10%), as detected by the impedance analysis. The diet consisted of a 7-day meal plan (three meals and two snacks), with the indications of the food and their weighted intake expressed in grams and had a balanced composition in macronutrients (carbohydrates 55%, lipids 25%, protein 20%; fiber ≥25grams/day), as recommended by LARN guidelines [15]. Vegetables, fruit, cereals, fish, and pulses, typical of the Mediterranean style, were inserted into the diet. Patients received the recommendation to use extra-virgin olive oil as a seasoning and to increase the consumption of whole grains, avoiding added sugars and industrial foods [14].

2.4 Microbial DNA extraction and 16S rRNA Gene-Based Illumina MiSeq sequencing

DNA extraction, purification, and quantification by real-time PCR were performed as previously described **[16]**. In particular, quantitative PCR was performed using the primers pair 5'-CCTACGGGNGGCWGCAG-3' (forward) and 5'-GACTACHVGGGTATCTAATCC-3' (reverse), using genomic DNA from *E. coli* ATCC25922 as a reference to prepare the standard curve. The protocol of library preparation and sequencing has been described in detail elsewhere **[16]**. Barcoded amplicon libraries for the bacterial community analysis were generated using primers targeting the V3 and V4 hypervariable regions of the bacterial 16S rRNA gene and Nextera XT index kit (Illumina, inc.).

2.5 Bioinformatics and Statistics

Analysis of the data generated on the Miseq System was carried out using the BaseSpace 16S Metagenomics App (Illumina), whereas operational taxonomic units (OTUs) mapping to the Greengenes database (V.13.8) was performed using the Quantitative Insights Into Microbial Ecology (QIIME) platform (V.1.8.0), clustered into 97% identity using a two-step open-reference operational taxonomic unit (OTU).

Alpha diversity was generated with the script alpha_rarefaction.py in QIIME to obtain the Shannon index. Alpha diversities were compared by using the Wilcoxon test for paired data. Beta

diversity was generated in R-vegan, using Bray-Curtis distance. The statistical significance of Beta diversity was determined with Permutational Multivariate Analysis of Variance (PERMANOVA) (R-vegan, function adonis).

The analysis of the taxonomic levels was performed in R software v.3.5.2. At baseline, a comparison between OB and NW was performed considering only bacteria present in at least 25% of our samples and with a relative abundance ≥0.1% in cases and/or controls, by using the Kruskal-Wallis test (KW) followed by false discovery rate (FDR) adjustment. Linear Discriminant Analysis Effect Size (LEfSE) was employed for the identification of biomarkers, including only bacteria identified as significant in the KW test, after FDR correction. The comparison between gut microbiota profiles before and after the intervention was performed by using the Wilcoxon test for paired data. Only bacteria present in at least 25% of our samples and with a relative abundance ≥0.1% before or after intervention were considered. All the p-values were adjusted for FDR. Q-values < 0.05 were considered as statistically significant. Anthropometric measurements and nutritional data before and after NI were compared using a t-test for paired data.

Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) [17] was performed on Galaxy computational tool to infer metagenome composition in the samples. QIIME pipeline was used for OTUs picking from data generated on the Illumina platform. After the OTUs normalization by copy number, metabolic pathways were predicted and classified by the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database orthologs, at hierarchical level 3. OTUs contributing to the abundance of functional categories in each sample was detected by the function "metagenome contribution by higher category", using KEGG's classification type. The statistical significance of differences in metabolic pathways before and after NI was analyzed for all metabolism pathways that presented mean differences (the mean after NI minus the mean before NI) of at least 0.1% after the intervention, using Wilcoxon test for paired data (R software v.3.5.2) with Benjamini and Hochberg correction (FDR cutoff <0.05).

3. Results

Among the OB patients, 3 (13%) had BMI between 25 and 29.9 kg/m^2 (overweight), 8 (35%) had BMI between 30 and 34.9 kg/m^2 (class I obesity), 10 (43%) had BMI between 35 and 39.9 (class II obesity) kg/m^2, and 2 (9%) had BMI ≥ 40 kg/m^2 (class III obesity). Most of the included patients presented metabolic dysfunctions: 13 patients (57%) had hypertension, 8 (35%) patients had dyslipidemia, and 5 (22%) patients were diagnosed with diabetes or insulin resistance. Four patients (17%) were active smokers. The nutritional anamsesis pointed out an excessive consumption of processed meat, industrial food, and sugary drinks, and a low consumption of fruit, vegetables, and pulses, as well as the absence of whole grains. Table 1 summarizes the clinical characteristics of the included patients.

At the time of the second sample collection, at T3, the 23 OB patients presented a statistically significant decrease in body weight (-7.5%), BMI (-4,5%), waist circumference (-5,6%), and fat-mass (-13,5%), without variations in the muscle mass. In addition, the analysis of the 3-d FR, reporting the nutritional intake in the 3-days before the second sample collection, showed a decrease in the caloric intake compared with baseline, indicating a good adherence to the diet. Moreover, at T3, OB patients showed a greater adherence to the Mediterranean diet, though not significant, presenting a similar mean value than NW (T3=32 ± 5; NW=33 ± 6).
The anthropometric measurements and the nutritional intake before and after intervention are shown in Table 2.

3.1 Gut microbiota diversity and composition

3.1.1 Gut microbiota diversity

The OB patients presented no significant difference in the Shannon index compared with controls (OB= 3.41 ± 0.54, NW= 3.46 ± 0.36, p-value= 0.834). Similarly, at T3, the 23 patients did not present a significant change in the Shannon index (3.39±0.66, p-value=0.761).
On the other hand, the PERMANOVA analysis of Beta diversity showed significant separation between OB and NW at T0 (p-value=0.002, F= 4.916, R^2= 0.068), as illustrated in Figure 1a. Noteworthy, the GM of OB did not segregate from that of NW after the NI (Figure 1a, p-value=0.122).

3.1.2 Gut microbiota composition

The enrolled OB patients were characterized by a Firmicutes/Bacteroidetes ratio more than twice compared to controls (OB= 4.73 ± 5.26, NW= 1.75 ± 1.82, p-value=0.007). After the NI, we observed a reduction in the ratio Firmicutes/Bacteroidetes (M±SD after NI=2.42 ± 2.86). However, the change was not significant (p-value=0.128).

Based on the relative abundance of the gut bacteria, the LefSe algorithm was applied to the taxa significant at the KW test and confirmed after the FDR adjustment (Figure 1b): a total of 19 biomarkers were identified as being associated with obesity and overweight, with the phylum Firmicutes being at the top of the rank. Three Firmicutes families were also enriched in OB (Gemellaceae, Streptococcaceae, and Thermicanaceae; q-values= 0.0026, 0.0039, and 0.0305, respectively), along with the genera *Megamonas*, *Gemella*, *Streptococcus*, *Megasphaera*, *Veillonella*, *Thermicanus*, and *Catenibacterium* (q-values between 0.0006 and 0.0412) and the species *Megamonas funiformis*, *Megasphaera hominis*, and *Eubacterium biforme* (q-values= 0.0305, 0.0005, and 0.0286, respectively). In addition, 2 genera of Gammaproteobacteria, known for the pro-inflammatory activity (*Enterobacter*, *Serratia*; q-values= 0.0092 and 0.0112, respectively) were more abundant in OB. Oppositely, 19 biomarkers were associated with leanness, with Bacteroidetes at the top, followed by many corresponding taxa: we found an increase in the families Flavobacteriaceae, Porphyromonadaceae and Sphingobacteriaceae (q-values= 0.0173, 0.0095, 0.0005), the genera *Rikenella*, *Flavobacterium*, *Parabacteroides*, and *Pedobacter* (q-values between 9.9E-04 and 0.0393), and several species within these genera. We repeated the same analysis at T3, in order to detect any reversion of the signatures identified as associated with OB at baseline: the differences with NW controls were fewer in terms of both number (38 significant results at T0, 22 significant results at T3) and strength (Linear Discriminant Analysis values ranged between -6 and + 6 at T0, and between -4 and + 4 at T3). As shown in Figure 1c, the phyla Firmicutes and Bacteroidetes were no longer significant, as well as some taxa within these two phyla; in particular, the family Sphingobacteriaceae and *Sphingobacterium shayense*, the genus *Parabacteroides* and *Bacteroides* spp., in the phylum Bacteroidetes, and Streptococcaceae, *Megamonas*, and *Megamonas funiformis*, in the phylum Firmicutes. In addition, *Desulfovibrio piger*, within the phylum Proteobacteria, was associated with OB at baseline, but not after the NI.

The relative abundance of several microbial patterns was significantly modified after the NI (Figure 2), as indicated by the paired analysis. When considering the FDR adjustment, we obtained a total of 24 significant results. The mean and standard deviation and the corresponding p-values and q-values for each significant result are shown in Table 3.

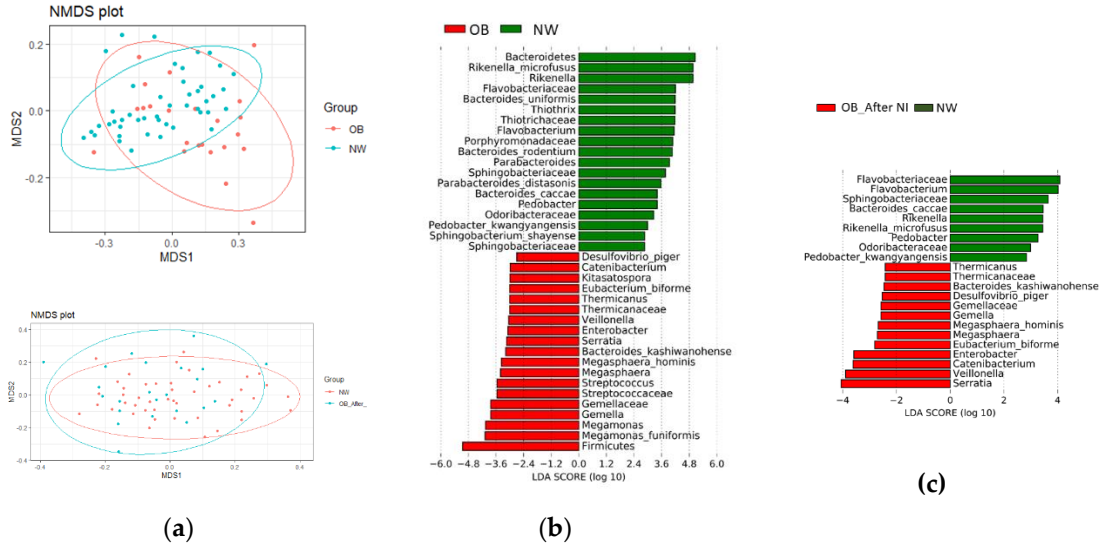
At the phylum level, we obtained an increase in Proteobacteria (q-value= 0.001), while the genus *Sutterella* (q-value=0.002), within the same phylum, decreased significantly. One family within the phylum Bacteroidetes was enriched (Sphingobacteriaceae, q-value=0.003), along with its species *Sphingobacterium shayense* (q-value=0.003). We also observed an increase in *Bacteroides uniformis* and *Prevotella stercorea* (q-values= 0.036 and 0.001, respectively), within the same phylum. On the other hand, 2 families within the phylum Firmicutes were depleted (Ruminococcaceae and Veillonellaceae q-values= 0.001 and 0.004), as well as *Ruminoccocus* and *Ruminoccus* spp. (q-values between 0.001 and 0.012). In addition, the abundance of several Firmicutes taxa belonging to the family Lachnospiraceae

changed significantly, with an increase in *Coprococcus eutactus* (q-value=0.005), as well as a decrease in *Roseburia*, *Roseburia faecis*, and *Pseudobutyrivibrio xylanivorans* (q-values between 0.001 and 0.026). Besides, the relative abundance of the Firmicutes taxa *Megamonas* and *Megamonas funiformis* (q-values= 0.046 and 0.038, respectively) decreased after the intervention. However, an increase in the Firmicutes genera *Catenibacterium* and *Veillonella* (q-values= 0.049 and 0.004, respectively) was also observed, together with the increase in *Sedimentibacter hydroxybenzoicus* and *Veillonella montpellierensis*, at the species level (q-values= 0.004 and 0.001, respectively).

3.1.3 Predicted metabolic pathways

After the NI, a significant difference in seven metabolic pathways was found: one pathway related to membrane transport (ABC transporters), one pathway associated with transporters, two pathways related to cell motility ("flagellar assembly", "bacterial motility proteins"), and one pathway associated with sporulation decreased. On the other hand, the pathways "lipopolysaccharides biosynthesis proteins" and "membrane and intracellular structural molecules" increased after the intervention (Figure 3).

3.2 Figures, Tables, and Schemes



(**a**) (**b**) (**c**)

Figure 1 (**a**) Overweight and obesity are associated with altered Beta diversity at baseline but not after a hypocaloric Mediterranean diet followed for three months. Three-dimensional scatter plot, generated using the Non Metric Multidimensional Scaling (NMDS) conducted on the community Bray-Curtis distance matrix, showing a significant separation between OB patients (OB) and normal-weight controls (NW) at baseline (upper figure, p-value=0.002). After the nutritional intervention (NI), the gut microbiota of OB did not segregate from that of NW (lower figure, p-value=0.122); (**b**) Obese and overweight patients present distinct microbial signatures at baseline. Results are ranked by the Linear Discriminant Analysis value (LDA score): bacteria in red were more expressed in OB, while bacteria in green were more abundant in NW; (**c**) The NI reversed some of the microbial patterns identified at baseline.

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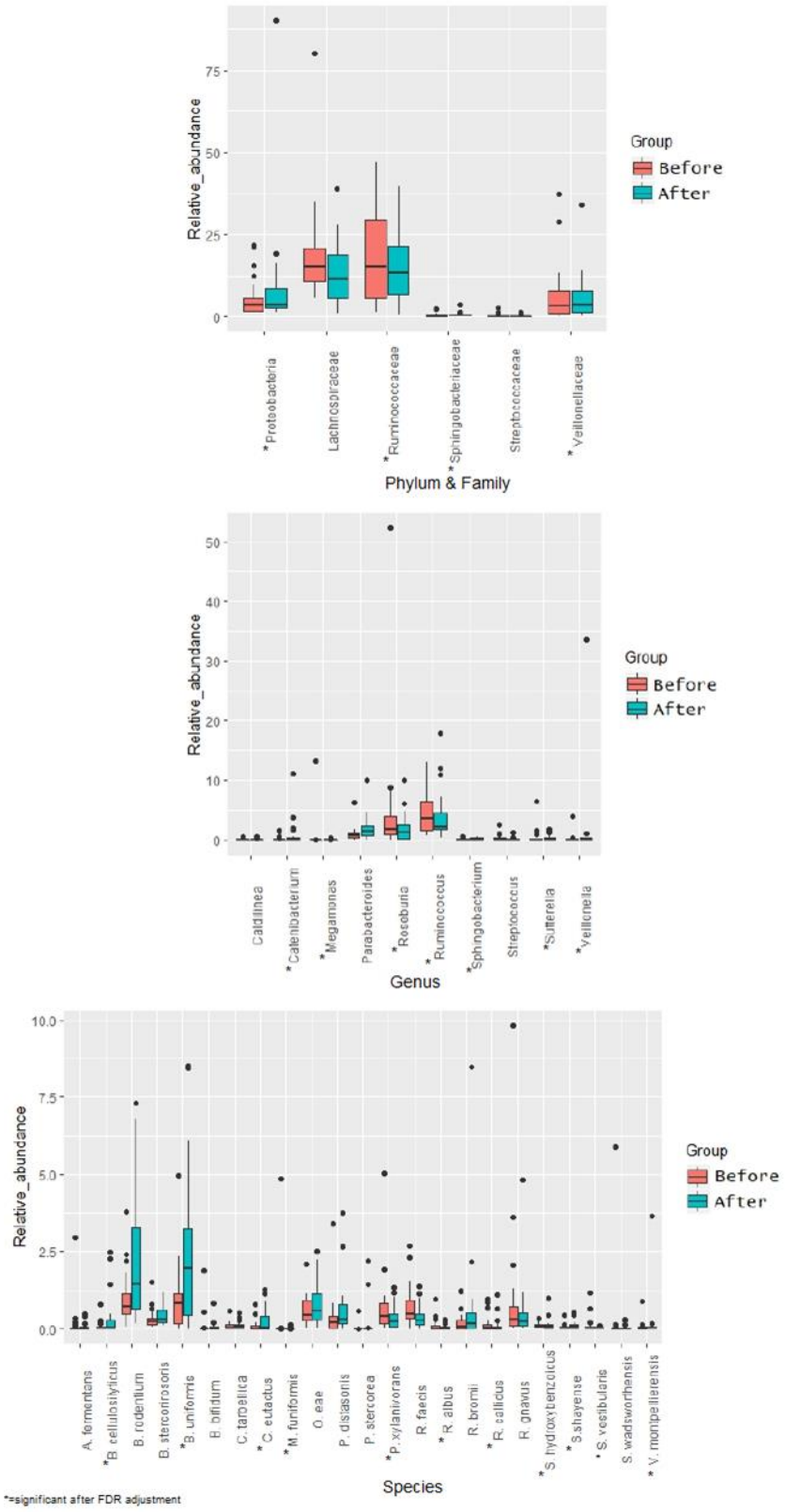

Figure 2. Statistically significant differences in bacterial relative abundance in obese and overweight patients after the nutritional intervention, at the phylum & family, genus, and species levels, respectively. The significance level was obtained by performing the Wilcoxon test for paired data. P-values were adjusted for FDR (FDR<0.05). A change in twenty-four taxa was found when considering the FDR adjustment. The interquartile ranges (IQRs) and boxes, medians (lines in the boxes), and lowest and highest values for the first and third quartiles are plotted in the figures. Each group is

identified by colors, as indicated on the right side of the figure (Before intervention= pink, After intervention= light blue). Every sample is represented by a black dot.

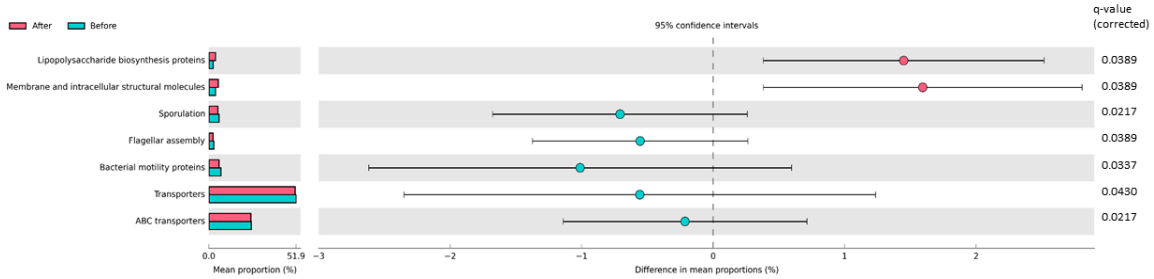

Figure 3. Statically significant differences in predicted metabolic pathways in obese and overweight patients after the nutritional intervention (NI). The OTUs table generated by QIIME for the bacterial communities was analyzed by using the PICRUSt algorithm. Seven metabolic pathways were significantly different after the NI. Pathways that were more abundant after NI are on the positive side (pink circle with 95% CI). Pathways that were less abundant after NI are on the negative side (light-blue circle with 95% CI). The q-values represent the Benjamini Hochberg's FDR-corrected p-value (Wilcoxon test for paired data). Mean proportions are shown in stacks (before NI= light-blue, after NI= pink). The difference in mean proportions indicates the mean proportion after NI minus the mean proportion before NI.

Table 1. Clinical characteristics of the OB patients

Clinical parameter	M (SD)
Sex, (Females/Males)	20/3
Age, M (SD)	54 (9)
Overweight, N (%)	3 (13)
Class 1 obesity, N (%)	8 (35)
Class 2 obesity, N (%)	10 (43)
Class 3 obesity, N (%)	2 (9)
Hypertension, N (%)	13 (57)
Dyslipidemia, N (%)	8 (35)
Insulin Resistance, N (%)	3 (13)
Type II Diabetes, N (%)	2 (9)
Current Smoking status (Yes), N (%)	4 (17)
Former Smoking status (Yes), N (%)	5 (22)
Current Alcohol consumption (None), N (%)	9 (39)
Current Alcohol consumption (Rare), N (%)	8 (35)
Current Alcohol consumption (Moderate), N (%)	6 (26)

M= mean, N=number, SD= Standard deviation

Table 2. Anthropometric measurements and nutritional intake before and after the nutritional intervention.

Clinical parameter	Before	After	p-value*
Weight (Kg), M (SD)	89.5 (19.3)	82.8 (17.0)	0.015
Waist circumference (cm), M (SD)	108 (14)	102 (16)	0.040
Body mass index, M (SD)	35.2 (4.3)	33.6 (4.5)	0.001
Fat mass (Kg)	37.8 (10.2)	32.7 (8.2)	0.0002
Muscle mass (Kg)	47.2 (14.0)	47.6 (9.8)	0.493
Daily caloric intake (Kcal), M (SD)	1727 (552)	1341 (298)	0.007
Carbohydrates intake (%), M (SD)	50 (6)	50 (8)	0.578
Lipids intake (%), M (SD)	33 (6)	29 (9)	0.196
Saturated lipids intake/ Total lipids intake (%), M (SD)	39 (6)	35 (8)	0.129
Daily proteins intake (grams/day), M (SD)	71 (21)	64 (13)	0.384
Daily fibers intake (grams/day), M(SD)	14 (6)	17 (6)	0.234
MedDietScore	29 (5)	32 (5)	0.665

* The difference after the NI was tested using a t-test for paired data. M= mean, N=number, SD= Standard deviation

Table 3. Significant changes in the bacterial relative abundance after the nutritional intervention

Phylum	Family	*Genus*	*Species*	p-value	q-value
Actinobacteria	Bifidobacteriaceae	*Bifidobacterium*	*bifidum*	0.043	0.196
Bacteroidetes	Bacteroidaceae	*Bacteroides*	*cellulosilyticus*	0.006	**0.039**
			rodentium	0.012	0.059
			stercorirosoris	0.016	0.091
			uniformis	0.005	**0.036**
	Tannerellaceae	*Parabacteroides*		0.016	0.093
			distasonis	0.019	0.097
	Prevotellaceae	*Prevotella*	*stercorea*	3,09E-05	**0.001**
	Sphingobacteriaceae			1,62E-04	**0.003**
	Sphingobacteriaceae	*Sphingobacterium*		1,26E-03	**0.011**
			shayense	1,62E-04	**0.003**
Chloroflexi	Caldilineaceae	*Caldilinea*		0.045	0.196
			tarbellica	0.045	0.196
Firmicutes	Acidaminococcaceae	*Acidaminococcus*	*fermentans*	0.036	0.099
	Erysipelotrichaceae	*Catenibacterium*		0.007	**0.049**
	Lachnospiraceae			0.042	0.194
	Lachnospiraceae	*Coprococcus*	*eutactus*	0.001	**0.005**
		Pseudobutyrivibrio	*xylanivorans*	5,23E-05	**0.001**
		Roseburia		0.004	**0.026**
		Roseburia	*faecis*	0.000	**0.001**
Firmicutes	Selenomonadaceae	*Megamonas*		0.007	**0.046**
		Megamonas	*funiformis*	0.005	**0.038**
	Ruminococcaceae			2.62E-04	**0.003**
	Ruminococcaceae	*Oscillospira*	*eae*	0.048	0.196
		Ruminococcus		2.70E-05	**0.001**
			albus	0.003	**0.012**
			bromii	0.039	0.186
			callidus	0.001	**0.008**
			gnavus	0.042	0.196

Phylum	Family	*Genus*	*Species*	p-value	q-value
	unclassified Tissierellia	*Sedimentibacter*	*hydroxybenzoicus*	0.000	**0.004**
	Streptococcaceae			0.009	0.051
		Streptococcus		0.015	0.073
		Streptococcus	*vestibularis*	3,09E-05	**0.001**
	Veillonellaceae			2.95E-04	**0.004**
	Veillonellaceae	*Veillonella*		3.73E-04	**0.004**
			montpellierensis	1.27E-04	**0.001**
Proteobacteria				2.70E-05	**0.001**
	Sutterellaceae	*Sutterella*		0.001	**0.012**
			wadsworthensis	0.018	0.093

Continuation of Table 3

Phylum	Family	*Genus*	*Species*	MD	↑/↓
Actinobacteria	Bifidobacteriaceae	*Bifidobacterium*	*bifidum*	-0.04	↓
Bacteroidetes	Bacteroidaceae	*Bacteroides*	*cellulosilyticus*	0.268	↑
			rodentium	1.35	↑
			stercorirosoris	-0.095	↓
			uniformis	1.52	↑
	Tannerellaceae	*Parabacteroides*		0.996	↑
			distasonis	0.341	↑
	Prevotellaceae	*Prevotella*	*stercorea*	0.109	↑
	Sphingobacteriaceae			0.185	↑
		Sphingobacterium		-0.067	↓
			shayense	0.062	↑
Chloroflexi	Caldilineaceae	*Caldilinea*		0.015	↑
			tarbellica	0.015	↑
Firmicutes	Acidaminococcaceae	*Acidaminococcus*	*fermentans*	0.05	↑
	Erysipelotrichaceae	*Catenibacterium*		0.706	↑
	Lachnospiraceae			-5.902	↓
		Coprococcus	*eutactus*	0.175	↑
		Pseudobutyrivibrio	*xylanivorans*	-0.334	↓
		Roseburia		-2.8	↓
		Roseburia	*faecis*	-0.361	↓
	Selenomonadaceae	*Megamonas*		-0.039	↓
			funiformis	-0.207	↓
	Ruminococcaceae			-3.75	↓
	Ruminococcaceae	*Oscillospira*	*eae*	0.197	↑
		Ruminococcus		-0.203	↓
			albus	-0.066	↓
			bromii	0.457	↑
			callidus	-0.039	↓
			gnavus	-0.441	↓
	unclassified Tissierellia	*Sedimentibacter*	*hydroxybenzoicus*	0.041	↑
	Streptococcaceae			-0.311	↓
		Streptococcus		-0.316	↓
		Streptococcus	*vestibularis*	-0.096	↓
	Veillonellaceae			-0.989	↓
	Veillonellaceae	*Veillonella*		1.379	↑
			montpellierensis	0.136	↑
Proteobacteria				3.942	↑
	Sutterellaceae	*Sutterella*		-0.201	↓
			wadsworthensis	-0.205	↓

MD= Mean difference of bacterial relative abundance after the NI. Results were obtained by the Wilcoxon test for paired data performed on R software (v3.5.2). Q-values =p-values adjusted for Benjamini and Hochberg's false discovery rate (FDR) correction test for multiple comparisons (FDR<0.05). ↓=significantly reduced after the NI, ↑=significantly increased after the NI.

4. Discussion

The present work compared the GM of 23 overweigth and obese patients before and after 3 months of NI aimed to lose weight. At the time of the second sample collection, the patients presented a significant decrease in body weight, waist circumference, and, fat mass. The patients followed a moderately low-calorie diet, which allowed preserving of the muscle mass. In fact, an efficient dietary approach should focus not only on changes in the total body weight but also on body composition, considering that the reduction of body weight can be driven not only by a loss of fat mass but also by alterations in both the fat-free mass and fluid. However, the decrease in muscle mass can be connected with lowered resting energy expenditure/metabolism, fatigue, declines in neuromuscular function, and increased risk for injury [18]. Furthermore, the diet was based on the Mediterranean regime in free-living conditions. The Mediterranean diet is known not only to be efficacious for weight loss and cardiovascular risk level reduction in OB individuals, but also to positively modulate GM composition and diversity [19–21]. The lost in body weight as well as the improvement in body composition, as detected by the impedance analysis, suggested a good adherence to the diet, as confirmed by the analysis of the food diaries, reporting the nutritional intakes during the 3 days prior to the second sample collection (T3).

At baseline, the GM characterization showed an alteration in several microbial patterns in OB compared to NW, which can be summarized by the increased ratio Firmicutes/Bacteroidetes, with the trend being confirmed also at the lower taxonomic levels, and by the raise in Gammaproteobacteria. The increased ratio Firmicutes/Bacteroidetes and the identified microbial taxa within these two phyla can be associated with increased hydrolysis of non-digestible polysaccharides, and with increased nutrients absorption [22], resulting in increased calories production. In addition, the increased relative abundance of Firmicutes has been found to raise the number of lipid droplets and the export of fatty acids to the liver in the animal model [22,23]. Concerning the identified alterations within the phylum Bacteroidetes, and in particular the depletion in Flavobacteriaceae, Porphyromonadaceae, and Sphingobacteriaceae, findings from the animal model have demonstrated the capacity of life-style interventions, based on specific diets or increased physical activity, to restore the normal contents in the gut [24,25]. However, the mechanisms explaining the Bacteroidetes modulation of the bodyweight remain still unclear, though a recent experiment in mice has suggested the role of secondary bile acid-activated FXR signaling in the liver, and succinate-activated intestinal gluconeogenesis [44]. Finally, lipopolysaccharides from Gram-negative bacteria, including enterobacteria, cause an increase in intestinal permeability, leading to low-grade inflammation, called endotoxemia, frequently observed in obese subjects [26]. At T3, the comparison with NW controls demonstrated the reversion of some of the signatures previously identified, in particular among the taxa belonging to the phyla Firmicutes and Bacteroidetes. Worth mentioning, the GM of OB patients did not longer segregate from that of NW controls.

When conducting the paired analysis, a total of 24 significant results (one at the phylum level, 3 at the family level, 7 at the genus level, 13 at the species levels) was obtained after FDR adjustment. Moreover, we noticed a decreased trend in the ratio Firmicutes/Bacteroidetes and an increased trend in the alpha-diversity, although these results were not statistically significant.

Regarding the gut microbial alterations within the phylum Bacteroidetes, we observed an increased abundance in the family Sphingobacteriaceae and its member *S. shayense*, and a raise in *B. uniformis*. Both these three taxa were identified as being negatively associated with obesity at baseline. Remarkably, the administration of *B. uniformis* strains improved metabolic and immune dysfunction associated with intestinal dysbiosis in obese mice [27]. We also observed depletion of Firmicutes taxa known for being associated with obesity (i.e. Veillonellaceae, *Megamonas*, and *Megamonas funiformis*, three taxa that express propionate production pathways) [22–24].

Walker et al. investigated the impact of 3 different diets in modulating the GM composition of 14 obese men. Volunteers were provided successively with a control diet, diets high in resistant starch (RS) or non-starch polysaccharides (NSPs), and a reduced carbohydrate weight loss (WL) diet, over 10 weeks [28]. The findings of Walker et al. suggested that the supplementation with RS can balance the reduction of the family Lachnospiraceae and/or its members, caused by the WL diet. Interestingly, this reduction was found also after different types of hypocaloric diets (high protein, fiber-rich, or with prebiotics supplementation) in other studies [29–31].

Similarly, we found a decrease in Lachnospiraceae members (*Roseburia*, *Roseburia faecis*, *Pseudovibrio xylanivorans*), though an increase in *Coprococcus eutactus*, within the same family, was also reported. Members of this family can hydrolyze starch and other sugars to produce butyrate and other Short-Chain Fatty Acids (SCFAs) and play a central role in the mechanisms of bacterial cross-feeding [38]. Especially, the genus *Roseburia* is among the most involved in the control of gut inflammatory processes, atherosclerosis, and maturation of the immune system [38,39]. However, it should be noted that despite the well-known benefits provided by this family, Lachnospiraceae was positively associated with metabolic diseases in humans and animal models [36,40–43]. At the same time, higher SCFAs production (acetate, propionate, and butyrate) was associated with gut dysbiosis and obesity in a recent study with large sample size [32], and the finding was confirmed in a random effect meta-analysis published last year [33].

Taken together, the changes in GM composition after the intervention suggest a decrease in SCFAs producing bacteria (Lachnospiraceae and Veillonellaceae, *Ruminoccocus* spp., and *Megamonas*). It is unclear whether the beneficial effect of SCFAs is somehow compromised in obese subjects, or whether the effect is simply not strong enough to compensate for incorrect lifestyle habits diet or genetic predisposition [34]. SCFAs are known to activate G protein-coupled receptors (GPCRs), including GPR41 and GPR43, expressed in human adipocytes, colon epithelial cells, and peripheral blood mononuclear cells [35]. It has been proposed that in the case of the obese condition, the binding of SCFAs to G-protein coupled receptors at the intestinal level might be attenuated, leading to increased intestinal energy harvesting and hepatic lipogenesis [33].

Noteworthy, we found an increase in *Oscillospira eae* after the NI, though not confirmed after FDR adjustment. The genus *Oscillospira* has been associated with low BMI/leanness in several studies [36,37]. These bacteria metabolize glucoronate, a sugar found on the cell surface and in the extracellular matrix of most human tissues [38]. The degradation of host glucoronate by *Oscillospira* causes an energy expenditure for the host that may explain its association with leanness [37].

Cancello et al. evaluated the efficacy of a short-term dietary intervention on the GM of elderly Italian women with obesity: after 15 days of hospitalization following a hypocaloric Mediterranean diet, they noticed a decrease in pro-inflammatory bacteria, along with a moderate weight loss and improved metabolic function. In line with our study, the diet provided a daily energy deficit equal to 250 kcal. The study showed the efficacy of a balanced diet with moderate caloric restriction, even of short duration, in improving gut health, reversing the GM dysbiosis found at baseline [39].

When comparing our findings with those of weight-loss strategies different than diet, BS should be mentioned. This intervention is based on surgery on the stomach and/or intestine, aimed to lose weight. BS is an option for obese individuals whit a BMI above 40, or a BMI between 35-40 in presence of comorbidities [40].

Several studies showed an increased relative abundance of the phylum Proteobacteria after BS [41–45]. Interestingly, this phylum was increased after the nutritional intervention in the current work. However, after BS, a raise in the class Gammaproteobacteria and in the pro-inflammatory genera *Escherichia*, *Klebsiella* and, *Enterobacter* was also found [41–46], while they were not increased after the NI in the present work. Instead, we observed, in the same phylum, a decrease in the *Sutterella*

genus, which can degrade IgA antibodies and have been recently linked with gastrointestinal diseases [17]. However, an increase in the relative abundance of the phylum Verrucomicrobia or its members, an increase in members of the phylum Bacteroidetes assigned to the genus *Alistipes* , as well as a general decrease in members of the phylum Firmicutes were also reported after BS [41,42,44–46]. The decrease in Firmicutes members is in common with this work. In addition, Firmicutes taxa within the genera *Veillonella* were shown to be increased after BS [46] and after the NI in this study.

Regarding the predicted metabolic pathways, a decrease in membrane transport and cell motility was shown after the NI in the present work. Increased cell motility has been recently associated with obesity [47], and with the presence of metabolic syndrome [48]. In contrast with the present work, a raise in bacterial cell motility was observed in obese patients after BS [44]. We also observed an increase in "lipopolysaccharides biosynthesis proteins" and "membrane and intracellular structural molecules" after the NI. The lipopolysaccharides biosynthesis proteins were identified as associated with obesity in other recent studies [49,50], but the direction of the association remains unclear.

In conclusion, findings from the present study underline the potential benefit of a moderately restrictive nutritional approach based on the Mediterranean diet in counteracting the gut dysbiosis, commonly observed in obese and overweight patients. The generalizability of the findings could be limited by the small sample size. Regarding methodological issues, it should be pointed out that targeting 16S variable regions at the species level cannot achieve the taxonomic resolution reached by sequencing the entire gene (around 1500 bp). This limitation should be taken into account when interpreting the results at the species level.

Availability of data and materials

Our sequence data for the 16S rRNA gene was deposited in the European Nucleotide Archive (ENA) (https://www.ebi.ac.uk/ena), under the study accession number PRJEB39062 (http: ://www.ebi.ac.uk/ena/data/view/PRJEB39062).

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