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Impact of Extreme Obesity and Diet-Induced Weight Loss on the Fecal Metabolome and Gut Microbiota

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Scope: A limited number of human studies have characterized fecal microbiota and metabolome in extreme obesity and after diet-induced weight loss.

Methods and results: Fecal samples from normal-weight and extremely obese adults and from obese participants before and after moderate diet-induced weight loss are evaluated for their interaction with the intestinal adenocarcinoma cell line HT29 using an impedance-based in vitro model, which reveals variations in the interaction between the gut microbiota and host linked to obesity status. Microbiota composition, short chain fatty acids, and other intestinal metabolites are further analyzed to assess the interplay among diet, gut microbiota, and host in extreme obesity. Microbiota profiles are distinct between normal-weight and obese participants and are accompanied by fecal signatures in the metabolism of biliary compounds and catecholamines. Moderate diet-induced weight loss promotes shifts in the gut microbiota, and the primary fecal metabolomics features are associated with diet and the gut–liver and gut–brain axes.

Conclusions: Analyses of the fecal microbiota and metabolome enable assessment of the impact of diet on gut microbiota composition and activity, supporting the potential use of certain fecal metabolites or members of the gut microbiota as biomarkers for the efficacy of weight loss in extreme obesity.

1. Introduction

The high current prevalence of obesity represents a public health concern. Obesity is associated with various noncommunicable chronic diseases, all of which are linked to chronic inflammation, including cardiovascular diseases, type 2 diabetes and cancer. Genetic and lifestyle factors, such as dietary patterns and physical activity, together with other factors, such as socioeconomic, psychiatric, and/or metabolic disorders, are among the explanations given for the rise in obesity.[1] However, the combination of these factors does not fully explain the pathogenesis of obesity. A large body of evidence suggests that the gut microbiota is linked to low-grade chronic inflammation in parallel to the pathophysiology of obesity, type 2 diabetes and metabolic syndrome.[2] Shifts in gut microbiota composition and decreased gene richness are associated with impaired metabolism (insulin resistance, low-grade inflammation, and adipocyte

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hypertrophy), but also with the chance of success in the response to dietary interventions (in moderate obesity).[3,4] However, to date, few studies have addressed microbiota characterization in extreme obesity after diet-induced weight loss.[5,6] Moreover, it is clear that the gut microbiota participates in the metabolism of several compounds.[7] Emerging evidence supports the role of short chain fatty acids (SCFA) as molecules at the interface between the activity of the gut microbiota and the host metabolism, playing an important role in obesity.[8] In addition to modulation of colonic function, intestinal integrity, and motility, microbial SCFA affect the metabolism of several host organs (i.e., muscle, adipose, hepatic, and brain tissues).[8] In addition, increased levels of SCFA have been associated with gut dysbiosis, gut permeability, excess adiposity, and cardiometabolic risk factors.[9] Other intestinal compounds, such as amino acids, nucleotides, bile acids, phenolic compounds, fatty acids, neurotransmitters, or sterols, can originate from diet-microbiota interactions and/or from interactions of the microbiota with endogenous metabolites of the host, but their role in obesity has not been established to date. For these reasons, the combination of microbiome and metabolome analysis has helped to elucidate the molecular mechanisms of human obesity.[10,11] Several reports have addressed metabolic shifts associated with obesity and diet-induced weight loss in humans, primarily in serum samples.[11] However, the fecal metabolome, in spite of being considered a useful tool for understanding interactions among diet and human metabolism,[12] has rarely been explored to investigate the impact of dietary weight changes on metabolite profiles.

The aim of the present study was to evaluate the effects of extreme obesity and a moderate diet-induced weight loss on the gut microbiota and metabolomics profile. For this purpose, an in vitro model was used to evaluate the behavior of HT29 cell monolayers upon exposure to isolated microbiotas (IM) or fecal supernatants (FS) from normal-weight (NW) and extremely obese patients (OB) before and after diet-induced weight loss (OB.2). To this end, variations in HT29 cell monolayer trans-epithelial resistance (due to changes in morphology and/or attachment of the epithelial cells) during exposure to the microbiotas and supernatants were assessed. We further analyzed microbiota composition (16S rRNA gene profiling and qPCR) and characterized the fecal metabolome to identify interactions between diet and the gut microbiota in extreme obesity.

2. Results and Discussion

2.1. Interaction of Fecal Isolated Microbiotas and Supernatants with HT29 Cells

Potential differences in FS and IM collected from NW, OB, and OB.2 groups were evaluated using the RTCA in vitro impedance-based model using the HT29 intestinal cell line at proliferation and confluence states as an indicator of variations in the interaction pattern between the gut microbiota and the host.[13] The comparison of FS and IM between NW and OB groups showed that FS in the OB group differed significantly from that of the NW group in the response induced in HT29 cells at the confluence state (**Figure 1**) but not at the proliferation state (Figure S1, Supporting Information), with no significant differences being found for IM. Regarding the OB versus OB.2 group comparisons, IM but not FS demonstrated significant differences when tested in HT29 cells only at the confluence state (Figure 1) but not in the proliferation state (Figure S1, Supporting Information). These results reflect distinct behaviors of the intestinal cell line in response to IM and FS from NW and OB subjects and support previous data from our group, where the same in vitro test was employed to discriminate the response of intestinal epithelial cells to gut microbiotas from different human populations.[13,14] These differences in RTCA profiles induced by NW and OB samples are suggestive of different gut microbiota signatures that might be related to changes in their composition and metabolic activity; thus, these factors were further examined.

2.2. Impact of Extreme Obesity and Diet-Induced Weight Loss on Microbiota Composition

Microbiota composition was evaluated as relative proportions by 16S rRNA gene profiling and, for some representative microbial groups, as absolute levels by qPCR. To assess overall differences in microbial community structures, we determined ecological parameters based on α diversity (Chao I and Shannon indexes) with results obtained through 16S rRNA gene sequencing. OB subjects exhibited lower α diversity as measured by Chao1 index compared to NW subjects, but no significant differences were observed in microbial α diversity as a function of weight loss between OB and OB.2 samples (Table S1, Supporting Information). Analysis of the relative abundance of microbial taxa in NW and OB revealed a depletion of the Christensenellaceae family and the *Blautia* genus and an increase of the Alcaligenaceae family in OB with respect to NW individuals (**Figure 2**). The depletion of the *Blautia* genus was further supported by significantly lower levels of *Clostridium* cluster XIVa observed by qPCR (one of whose most abundant genera was *Blautia*) in OB with respect to NW subjects. Significantly lower levels of total bacteria and higher levels of the *Staphylococcus* genus were also observed by qPCR in OB compared to the NW group (Figure 2).

Our results confirm previous studies where the relationship between reduced α diversity and higher BMI have been reported[3,15] and are in keeping with a recent study performed in extremely obese participants, where ≈75% of subjects exhibited reduced gene richness.[16] In previous works, relative abundance of the Christensellaneaceae family in the human gut has been inversely correlated with host BMI in populations from different countries and has been associated with metabolic health status in studies performed on different diseases, including obesity and inflammatory bowel disease,[17] as well as with weight loss in extreme obesity without complications following a very low calorie diet.[5] Therefore, the highest relative abundance of the Christensellaneaceae family observed in this study in NW subjects with respect to OB individuals confirms previous findings by other authors suggesting that this family is associated with leanness. In contrast, *Blautia,* included in the *Clostridium*

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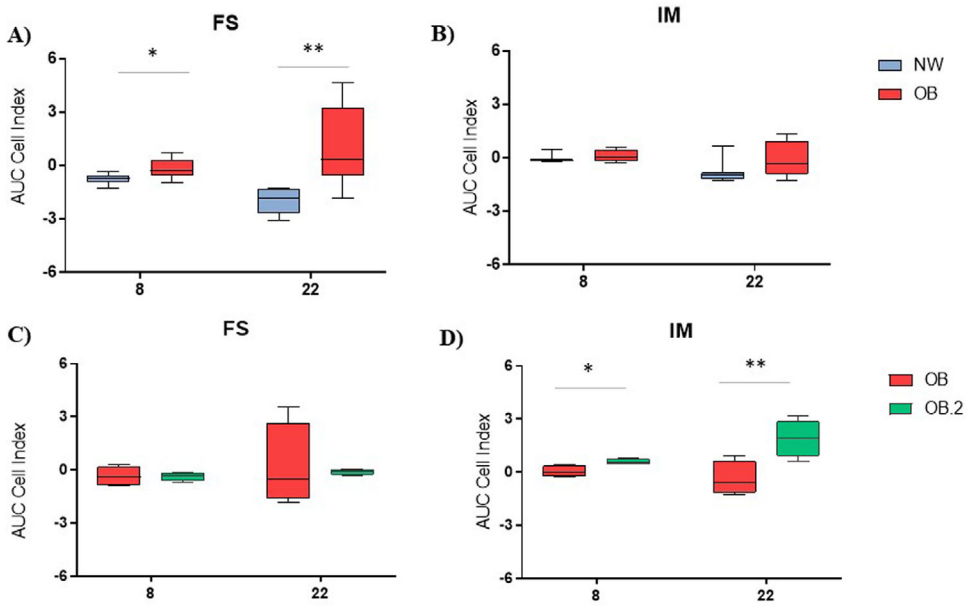

Figure 1. Real-time monitoring (RTCA) of interactions between the isolated microbiotas (IM) or fecal supernatants (FS) from normal-weight adults (NW, $n = 7$) and extremely obese patients (OB, $n = 9$) A,B) before (OB) and after weight loss (OB.2) C,D) ($n = 4$) with HT29 intestinal epithelial cells in a confluence state. The results are shown as median (interquartile rank) of the calculated area under the curve value (AUC) obtained at 8 and 22 h of interaction between IM or FS and HT29 cells. Statistically significant differences between OB and NW group with $p < 0.05$ and <0.01 are indicated with (*) and (**), respectively.

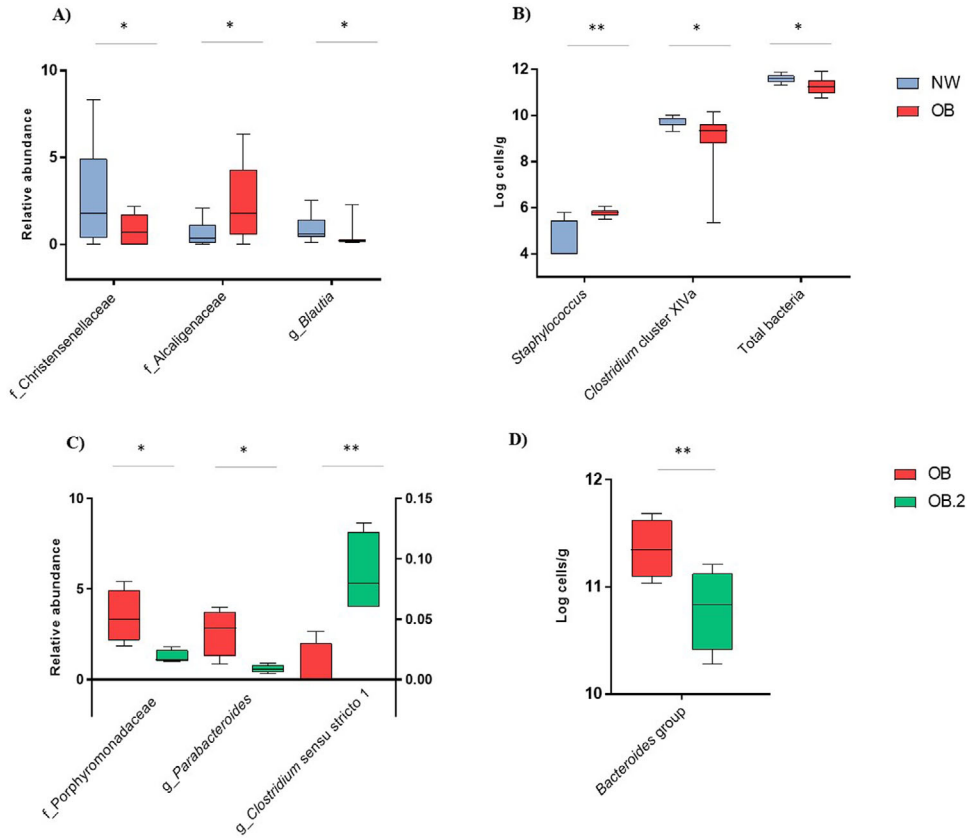

Figure 2. Relative abundance determined by 16S rRNA gene profiling (graph on the left) and absolute levels of bacterial groups determined by qPCR (graph on the right) in fecal samples from extremely obese (OB, $n = 9$) and normal-weight (NW, $n = 9$) subjects A,B) before (OB) and after weight loss (OB.2) ($n = 4$) C,D). Only statistically significant results are shown. $p < 0.05$ and <0.01 are indicated with (*) and (**), respectively.

Table 1. Absolute mM concentration (media ± SD) of SCFA quantified by CG-FID in extreme obesity (OB, $n = 9$) and normal-weight groups (NW, $n = 8$).

	OB	NW	*p*-Value
Acetic acid	73.659 ± 32.819	47.864 ± 22.940	0.084
Propionic acid	24.680 ± 12.799	13.979 ± 7.673	0.054
Iso-butyric acid	2.566 ± 0.721	2.338 ± 0.320	0.423
Butyric acid	24.901 ± 18.580	10.027 ± 8.569	0.053
Iso-valeric acid	3.965 ± 1.060	3.570 ± 0.637	0.374
Valeric acid	4.403 ± 1.073	3.135 ± 0.754	**0.011**
Caproic acid	2.292 ± 0.694	2.010 ± 0.855	0.074
Acetic/propionic acid ratio	3.222 ± 1.102	3.616 ± 0.904	0.437
Acetic/butyric acid ratio	4.001 ± 2.088	7.650 ± 5.899	0.102
Total SCFA	136.466 ± 59.339	82.921 ± 39.344	**0.047**

Significant values ($p < 0.05$) are bold faced.

XIVa cluster, is one of the most abundant genera in the human gut, producing butyric and acetic acids.[18] Reduction in the abundancy of members from this group has been associated with obesity,[19] and the relative abundance of *Blautia* has been reported to be inversely associated with visceral fat accumulation in a Japanese population.[20] The higher relative abundance of the Alcaligenaceae family (Proteobacteria) and higher counts of *Staphylococcus* genus (Firmicutes) found in our OB subjects are also in good agreement with previous research showing that gut microbiota dysbiosis during metabolic disorders is often linked to an increased prevalence of Proteobacteria,[21] whereas a positive association between Proteobacteria and *Staphylococcus* has also been previously reported in obesity.[22,23]

In the gut microbiota of OB subjects, the relative abundance of the Porphyromonadaceae family and the *Parabacteroides* genus, both of which belong to the Bacteroidetes phylum and *Bacteroides* group, decreased, whereas *Clostridium* sensu stricto1 genera (Firmicutes) was significantly increased ($p < 0.05$) after weight loss (OB.2) (Figure 2). Moreover, qPCR data showed that absolute levels of the *Bacteroides* group, which includes *Bacteroides-Prevotella-Porphyromonas* genera, was reduced after weight loss (OB.2), partially confirming our results of 16S rRNA gene sequencing (Figure 2). These microbiota findings are in line with a previous report showing a decrease in Bacteroidetes after weight loss induced by a very low-calorie diet, in contrast to the increase in levels of this phylum obtained when weight loss was the result of a gastric sleeve resection.[6] However, other available literature about weight loss interventions shows heterogeneous results and reports different outcomes for the Bacteroidetes phylum and other specific taxa.[24] Our data confirm a recent study indicating no change in microbial diversity after weight loss induced by diet in extremely obese subjects,[5] although previous reports have shown an increase in microbial richness following weight loss.[3,25] At this point, it is important to mention that in such studies, weight loss (>10%) was achieved by very low caloric restriction diets, whereas in our study, diets were less restrictive, and moderate weight decreases (<10%) were generally obtained. In addition, in most studies, individuals are not subcategorized by the degree of obesity, and as such, the extremely obese group remains understudied. Finally, methodological differences in microbiota analyses may also account for the discrepancies among different studies.

2.3. Fecal Short Chain Fatty Acid Profiles

As expected, acetate was the major SCFA in feces from both NW and OB subjects, followed by propionate and butyrate. Significantly higher molar concentrations were only found for total SCFA and valeric acid in the OB group compared to the NW group (**Table 1**). We also observed a tendency for increased levels of butyric ($p = 0.053$), propionic ($p = 0.054$), caproic ($p = 0.074$), and acetic ($p = 0.084$) acids in the OB group that did not reach statistical significance. This finding is in accordance with previous human reports and a recent meta-analysis showing higher levels of fecal SCFA are associated with obesity.[26] In contrast, we did not observe differences in fecal SCFA levels between OB and OB.2 samples (Table S2, Supporting Information), suggesting that moderate weight loss of the individuals participating in the present study had no impact on the production of such compounds by the intestinal microbiota. Although it has been suggested that weight loss may affect SCFA metabolism, current data derived from a limited number of studies are inconsistent. Moreover, a meta-analysis found that decreased levels of fecal SCFA after dietary weight loss are primarily related to reduced carbohydrate intake,[27] which was not the case for the diet prescribed in the extremely obese participants in our study.

2.4. Fecal Untargeted Metabolic Profiles

Data processing and peak filtering of FS from NW, OB, and OB.2 samples allowed us to detect 491 metabolites in 20 total samples analyzed. We then compared the fecal metabolic profiles of eight NW and eight OB subjects, as well as four matched fecal samples from selected obese individuals after OB.2.

2.4.1. Metabolomics Comparison between Normal-Weight and Obese Individuals

Overall metabolic differences between OB and NW individuals were first evaluated by PCA, where there was not good separation between the groups, suggesting that a substantial part of the variability is not related to body weight status (Figure S2, Supporting Information). Thirteen metabolites displayed significant

Table 2. Tentative metabolite identification whose concentration in fecal samples changed significantly ($p < 0.05$) in OB ($n = 8$) versus NW ($n = 8$) individuals.

Exact mass	Retention time [min]	Empiric formula	Tentative identification	Condition	Fold change	*p*-Value
239.0798	1.43	$C_{11}H_{13}NO_5$	N-acetyl-3-hydroxy-L-tyrosine (N-acetyl-DOPA)	Downregulated	0.521	0.014
328.2241	8.25	$C_{18}H_{32}O_5$	9,10-Dihydroxy-8-oxo12-octadecenoic acid	Upregulated	2.932	0.019
392.2906	8.73	$C_{24}H_{40}O_4$	Deoxycholic acid	Upregulated	4.444	0.022
277.1061	3.14	$C_{13}H_{15}N_3O_4$		Downregulated	0.405	0.022
436.2824	8.83	$C_{24}H_{40}O_6$		Upregulated	2.089	0.026
230.0794	2.11	$C_{11}H_{10}N_4O_2$		Downregulated	0.635	0.033
174.0890	6.17	$C_8H_{14}O_4$	Suberic acid	Downregulated	0.353	0.037
333.1536	2.10	$C_{13}H_{23}N_3O_7$		Upregulated	1.622	0.037
303.1789	2.66	$C_{13}H_{25}N_3O_5$	Tripeptide (Ala, Thr, Ile, Leu, Val)	Upregulated	3.674	0.038
174.0533	2.17	$C_7H_{10}O_5$	Dimethyl 2-oxoglutarate	Downregulated	0.531	0.039
498.3543	8.78			Upregulated	3.010	0.042
458.2640	9.01	$C_{23}H_{34}N_6O_4$		Upregulated	2.096	0.044
390.2769	8.72	$C_{24}H_{38}O_4$	12-Ketodeoxycholic acid	Upregulated	3.040	0.046

differences ($p < 0.05$) between NW and OB, seven of which were tentatively identified (**Table 2**). 12-Ketodeoxycholic acid and deoxycholic acid are secondary bile acids found in feces that are upregulated in OB participants.[28] Primary bile acids are produced in the liver from cholesterol, conjugated to glycine and taurine to form primary bile salts, transported to the gallbladder, and poured to the small intestine during digestion. Primary bile salts can be deconjugated by intestinal bacteria and transformed into primary bile acids in the intestine by microbial bile salt hydrolases, and then they can be converted to secondary bile acids by specific microorganisms through a 7α-dehydroxylation reaction.[29] The upregulation of 12-ketodeoxycholic and deoxycholic acids observed in this study is in agreement with the elevated intestinal levels of secondary bile acids that have been previously reported by other authors in obesity, type 2 diabetes, and non-alcoholic fatty liver disease associated with gut microbiota dysbiosis.[29–31] Moreover, higher levels of secondary bile acids have been reported in animal models of diet-induced obesity[32,33] and have been correlated with increased cancer risk, as well as digestive and cardiovascular complications.[34] Another compound that was upregulated in OB subjects was 9,10-dihydroxy-8-oxo-12-octadecenoic acid, an oxidized polyunsaturated omega-6 long-chain fatty acid derived from linoleic acid. Oxidized derivatives of linoleic acid have been correlated with inflammation in metabolic syndrome and cancer[35,36] and with changes in adrenal function associated with obesity.[37] For these reasons, upregulation of this compound in OB subjects could be in line with metabolic and physiological impairments generally recognized in this pathology. Tripeptide(s) tentatively formed by the amino acids alanine, threonine, isoleucine, leucine, and/or valine were also upregulated in obese individuals. Higher levels of tripeptides found in the feces of OB subjects might be related to changes in the absorption of peptides in the small intestine. It has been shown that PepT1, the primary intestinal di/tri-peptides transporter, is regulated by insulin and leptin and improves glucose homeostasis in animal models of insulin resistance and obesity.[38,39] The fecal metabolome of our OB subjects was also characterized by downregulation of dimethyl 2-oxoglutarate, a precursor of 2-oxoglutarate (alpha-ketoglutarate) that is an intermediate of the Krebs cycle. This metabolite is found in human feces and other body fluids and may be synthesized by the gut microbiota.[40] When 2-oxoglutarate is administered orally, it reduces body weight and modulates the gut microbiota in mice.[41] 2-Oxoglutarate mediates glucose and glutamine-stimulated insulin secretion in the pancreas[42] and participates in the urea cycle, amino acid transamination reactions, and the formation of GABA from glutamate. This metabolite has various physiological functions, including acting as an antioxidant or as an anticancer agent to enhance host-defense.[41] Thus, the downregulation of 2-oxoglutarate in OB individuals might be related to host metabolic impairment and/or gut dysbiosis associated with obesity. Suberic acid is a medium-chain dicarboxylic acid previously identified in human feces,[43,44] derived from oleic acid. The downregulation of suberic acid in fecal OB individuals might be related with either dietary habits and/or with changes in the metabolism of lipids associated with obesity. N-acetyl-3-hydroxy-L-tyrosine (also known as N-acetyl-L-DOPA) is an acetylated form of the catecholamine L-DOPA that is downregulated in our group of OB individuals and whose presence has been previously detected by other investigators in human feces.[45] Catecholamines are a series of neurotransmitters (L-DOPA, dopamine, norepinephrine, and epinephrine) synthesized from tyrosine in the kidney, brain, adrenal medulla, and all sympathetically innervated tissues. In addition to endogenous biosynthetic pathways, some catecholamines are also present in the diet, and some foods are particularly rich in L-DOPA, such as fava beans and other legumes.[46] It has also recently been reported that L-DOPA is converted into dopamine in the small intestine by several bacterial strains that express the tyrosine decarboxylase enzyme.[47] Downregulation of fecal levels of N-acetyl-L-DOPA in our OB individuals likely reflects alterations in the metabolism of tyrosine and derived catecholamines, including potential

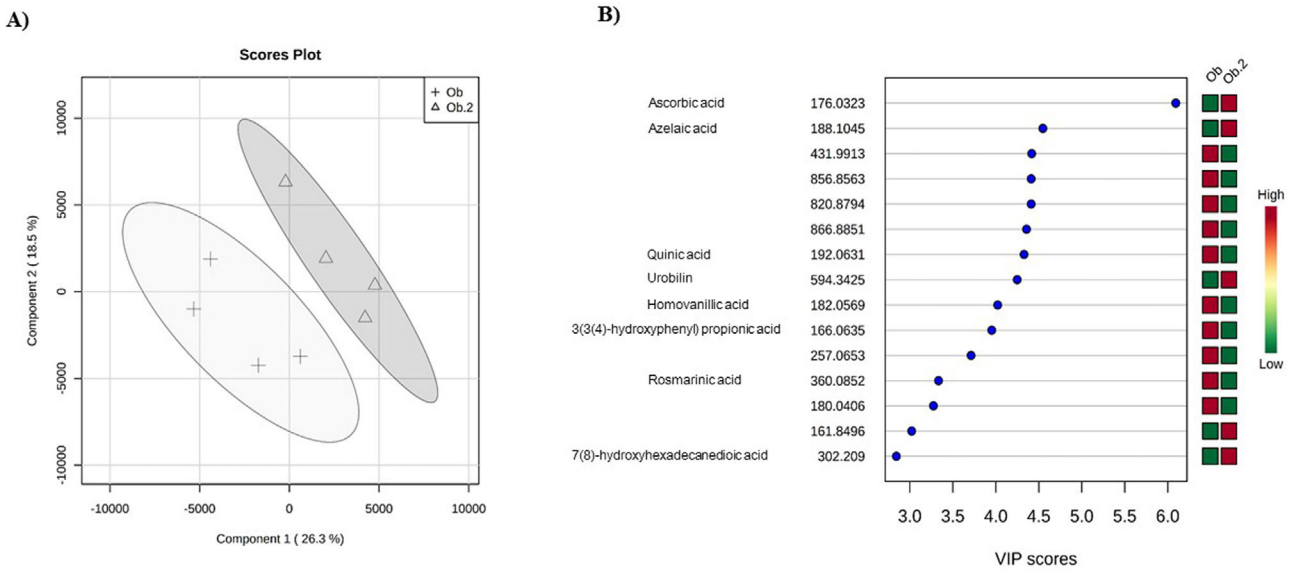

Figure 3. Fecal metabolite profiles of obese individuals before (OB) and after diet weight loss (OB.2) ($n = 4$). A) Discriminant analysis (PLS-DA). B) VIP values of PLS-DA projections for the 15 most discriminating metabolites.

alterations in the interaction of the gut microbiota with dietary components.

The abovementioned fecal metabolic results suggest that impaired metabolism of the host and the gut microbiota promoted by the obese status is likely the primary cause of the differences observed in the fecal metabolome between OB and NW subjects. Primary alterations observed in the present study include metabolism of biliary compounds and lipids, as well as energetics metabolism and catecholamine formation/turnover.

2.4.2. Metabolomic Comparison of Extremely Obese Individuals Before and After Moderate Weight Loss

We performed a partial least square discriminant analysis (PLS-DA) to identify the metabolites responsible for the separation between OB and OB.2 fecal samples (**Figure 3**). Fifteen metabolites were the primary contributors to discrimination in the PLS-DA model based on criteria of variable importance in the projections (VIP) > 2.50 (Figure 3), of which eight were tentatively identified (Table S3, Supporting Information). Among the more discriminating metabolites between OB and OB.2, 75% were food-derived metabolites (ascorbic acid, azelaic acid, quinic acid, rosmarinic acid, 3(3(4)-hydroxyphenyl) propionic acid, and 7(8)-hydroxyhexadecanedioic acid). The first-ranked compound was ascorbic acid (vitamin C), which is provided by the diet through intake of fruits and vegetables and whose concentration in the feces increased after weight reduction. Previous studies have also associated increased BMI with reduced blood concentrations of vitamin C[48] and with an increase in vitamin C intake in patients submitted to diet-induced weight loss.[49] Azelaic acid is a saturated medium-chain fatty acid that is also upregulated in OB.2 fecal samples and has been previously reported in the feces.[28,44] It is provided by dietary whole grains or can be produced endogenously during the catabolism of dietary oleic acid. Administration of azelaic acid to high fat diet-induced type 2 obese mice improves insulin sensitivity and fat and carbohydrate metabolism, contributing to normalizing adipokine and cytokine levels.[50,51] Increased levels of azelaic acid and 7-hydroxyhexadecanedioic acid and decreased levels of quinic, rosmarinic and 3 (3(4)-hydroxyphenyl) propionic acids in response to diet-induced weight loss might be mainly related to changes in dietary patterns, likely accompanied by modifications of the host endogenous metabolism/physiology and the gut microbiota activity. L-urobilin is a byproduct of bilirubin degradation and is upregulated in fecal samples of OB.2. Bilirubin is reduced to urobilinogen in the gut by the action of the gut microbiota,[52] and this last compound is partly reabsorbed and transported by enterohepatic circulation to the hepatocytes, ultimately being excreted by the kidney. Our results are in agreement with a previous study indicating that bilirubin is a marker of hepatic function, and decreased levels of this metabolite have been reported in the serum of obese individuals.[53] In contrast, urobilinoids were reportedly increased in the cecal and liver meta-metabolomes of obese mice.[54] Homovanillic acid is a dopamine catabolic metabolite whose presence has been reported in feces[28] and is the metabolite that exhibited a more drastic reduction in the feces of the OB.2 group in our study. Obesity has been associated with elevated synthesis of catecholamines and with resistance to these compounds.[55] Moreover, reduced activity of monoamine oxidase, an enzyme participating in the degradation of dopamine to homovanillic acid, has also been reported in the adipose tissue of obese subjects.[56] Downregulation of homovanillic acid after moderate weight loss may reflect a decrease in dopamine levels in the gut, which is in agreement with previous reports indicating an association between dietary body weight loss and downregulation of serum L-DOPA and norepinephrine in obese and diabetic subjects.[55]

In short, our fecal metabolomics results indicate that changes in dietary patterns are likely the major driving force contributing

to differential fecal metabolomes of extremely obese individuals before and after diet-induced weight loss. Shifts in dietary habits could promote changes in the metabolism of nutrients by the host, which might be accompanied by modifications of the gut microbiota as well.

2.5. Associations between Changes in the Fecal Metabolome and the Fecal Microbiota

To further explore possible associations between the gut microbiota and fecal metabolomic profiles, we examined potential correlations between concentrations of the 13 tentatively identified fecal metabolites and SCFA with the microbial groups quantified by 16S rRNA gene profiling (at the phylum and family level) in samples from NW, OB, and OB.2 groups. Significant correlations are shown in Figure S3, Supporting Information, and the most relevant are discussed below.

The intestinal tripeptide upregulated in OB individuals was positively associated with Bacteroidaceae and Porphyromonodaceae (Bacteroidetes) families and butyric acid. High protein intake from animal origin typical of a western diet in the OB group, together with a probable impairment of peptide absorption in the small intestine, could favor the proliferation of protein-fermenting bacteria, as is the case in some members of the Bacteroidaceae family. Moreover, it is well-established that in response to alterations in dietary protein components, significant changes in SCFA profiles occur.

N-acetyl-L-DOPA, whose levels were decreased in OB with respect to NW individuals, was negatively associated with the Enterococcaceae family, whereas homovanillic acid, downregulated in OB.2 compared to OB, was positively associated with acetic acid. Recent data have shown that *Enterococcus* species have an amino acid decarboxylase enzyme that decarboxylates L-DOPA to dopamine in the small intestine.[47] It is also known that fecal SCFA levels are influenced by microbial colonic fermentation of undigested carbohydrates/proteins from the diet, and SCFA can modulate the brain's catecholamine system, modifying dopaminergic levels through regulation of tyrosine carboxylase gene expression.[57]

In spite of the associations observed, our study design does not allow establishing causality and requires further animal experimentation to decipher the mechanisms explaining the potential role of the gut–brain axis in the metabolism of tyrosine and catecholamines in the context of extreme obesity and diet-induced weight loss. In this regard, a rodent model of weight loss and recurrent obesity was used by Thaiss and coworkers to demonstrate that the microbiome plays a key role in postdiet weight gain, and flavonoids supplementation can reset the weight-rebound clock.[58] Similarly, a recent study in humans suggested a potential link between gut microbiota plasticity and sustained weight loss.[59]

3. Concluding Remarks

There is increasing evidence suggesting that the gut microbiota should be considered in the pathophysiology of several metabolic diseases associated with low-grade inflammation and in tight association with diet, such as obesity. It is currently unknown whether the altered gut microbiota is a cause or a consequence of human obesity, due to the complex metabolic interactions between the host and its microbial community; however, our data support the usefulness of the fecal microbiota and metabolome analyses to improve our understanding of the interactions among diet and gut metabolism in extreme obesity. We also assessed the impact of weight loss, and this study is, to the best of our knowledge, the first to evaluate the impact of diet-induced weight loss not based on a very low caloric restricted diet in the fecal metabolome and microbiota of extremely obese subjects. The most evident changes identified in the fecal metabolome relating to host health status after weight loss were associated with the metabolism of bilirubin and catecholamines and their association with SCFA and the gut microbiota. However, the above features related to the gut–brain and liver–gut axes deserve further investigation in larger studies in which the diet and other environmental factors, such as exercise or other clinical parameters, are carefully monitored and controlled. In previous reports, changes in the fecal microbiota have been associated with plasma metabolites in obesity and weight loss, but this exploratory study supports the utility of using the fecal metabolome to evaluate the impact of host diets on gut microbiota functionality and the potential use of certain fecal metabolites or members of the gut microbiota as biomarkers to assess the efficacy of weight loss diets in the context of obesity.

4. Experimental Section

Participants, Fecal Sample Collection, and Study Design: Fecal samples were obtained from nine healthy normal-weight adults (NW, 7 males, 2 females; BMI < 25 kg m^{-2}) and nine extremely obese volunteers (OB, 5 males, 4 females; BMI > 40 kg m^{-2}) recruited at the Digestive and Endocrinology Services of the Asturias Central University Hospital (HUCA, Asturias, Spain). The mean age of control adults and obese individuals was 44.00 ± 13.75 and 49.67 ± 7.81, respectively. Four obese women (mean age 46.75 ± 15.44) were selected from the OB group among those who achieved at least a 5% decrease in body weight during the 6–8 months following a diet containing 20 kcal kg^{-1} of body weight (≈1800–2000 Kcal per day) (OB.2: extremely obese subjects after weight loss). As part of routine care, obese patients were seen by a dietician specialist nurse, and the volunteers were instructed to follow a hypocaloric diet containing 15% protein, 30% fat (<10% saturated fat), 55% carbohydrates, and 20–25 g dietary fiber. Lifestyle changes and behavioral strategies to facilitate adherence to the diet were also recommended, including regular physical activity. Inclusion criteria were as follows: no diagnosis of type 2 diabetes, autoimmune diseases, inflammatory bowel disease or other conditions known to affect intestinal function, as well as not being pregnant or having undergone medical treatment with oral corticoids, immunosuppressive agents, monoclonal antibodies, antibiotics or immunotherapy, not having a history of chronic physical/mental disease or gastro-esophageal reflux treated with high doses of proton pump inhibitors or having consumed probiotics or prebiotics during the two previous months. This study was approved by Regional Ethics Committee for Clinical Research (Servicio de Salud del Principado de Asturias n°66/2014) and the Ethical Committee of CSIC (Consejo Superior de Investigaciones Científicas) in compliance with the 1964 Declaration of Helsinki. Informed written consent was obtained from each volunteer prior to enrollment in the study.

Fresh fecal samples were collected in plastic sterile containers and immediately introduced into anaerobic jars (Anaerocult A System, Merck, Darmstadt, Germany) for transportation to the laboratory within 1 h after collection. A 1/10 w/v dilution was made in pre-reduced PBS solution and homogenized in a Lab Blender 400 Stomacher (Seward Medical, London,

UK) for 5 min. Homogenates were centrifuged at 16 000 × *g* at 4 °C for 10 min, and resulting FS were used to monitor interactions with the HT29 cell line, to assess SCFAs and to perform untargeted metabolomics analysis. For SCFA and untargeted metabolomics analyses, FS were stored at −20 °C until use. Another 10 mL of the homogenate were used for microbiota isolation using a Nycodenz (PROGEN Biotechnik GmbH, Heidelberg, Denmark) gradient method (44%, w/v) as previously described.[13] The IM was used to monitor interactions with the HT29 cell line and for microbiota 16S rRNA gene profiling and qPCR quantification of relevant intestinal bacterial groups. Analyses were conducted on NW, OB, and OB.2 samples. Comparisons are presented between NW and OB samples with a focus on changes occurring in extreme obesity and between OB.2 and their OB matched pair samples with a focus on changes occurring in extreme obesity after moderate weight loss.

Monitoring the Interaction of Isolated Microbiotas and Fecal Supernatants with HT29 Intestinal Cells: The behavior of HT29 cell monolayers in response to exposure to IM or FS from the different population groups was assessed using a real-time cell analyzer (RTCA-DP) xCelligence apparatus (ACEA Bioscience Inc., San Diego, CA), as previously described.[14] This system allows monitoring in real time the epithelial cell monolayer structure/integrity by measuring the impedance and detecting changes in this parameter that may be due to changes in the morphology of the cells or in their attachment. The culture conditions and maintenance of the intestinal epithelial cell line HT29 (ECACC 91072201) are detailed in a previous work in which the functional model was developed.[13]

Gut Microbiota Profiling, SCFA, and Untargeted Metabolomics Analyses: Experimental procedures and analyses for microbiota 16S rRNA gene sequencing and qPCR quantification, as well as fecal SCFA profiles and untargeted fecal metabolomics analyses, are described in detail in the Supporting Information.

Statistical Analysis: Statistical analysis of RTCA, 16S rRNA gene profile, qPCR, and SCFA was performed using SPSS v.25 software (SPSS Inc., Chicago, USA) to compare NW and OB subjects and to discriminate the effects of weight loss in the same individual, by assessing differences before (OB) and after (OB.2) losing weight. For all analyses, the Shapiro–Wilk and Levene tests were applied to verify normal distribution of the data. In the analyses of variables showing a normal distribution, a two-tailed Student's *t*-test was conducted. In the remaining cases (variables showing non-normal distribution), U-Mann–Whitney for comparison between NW and OB and Wilcoxon matched pairs tests for comparison between OB and OB.2 were applied. A *p*-value of 0.05 was considered to be significant for the interpretation of results.

For untargeted metabolomics analysis, multivariate statistical analysis was performed using MetaboAnalyst 3.0.[60] Metabolomics data was normalized by adjusting to the sum and scaled using the Pareto algorithm. Normal distribution of the fecal metabolic profiles was verified using the Shapiro Wilk test. A Volcano plot (fold change ratio >1.5 and *p*-value <0.05) was performed constructed to compare NW and OB samples. The same statistical approach but with matched paired comparison was employed for OB and OB.2 samples. Principal component analysis (PCA) was applied to examine variability between fecal metabolomics profiles from NW and OB groups and to identify possible trends in the variables due to weight loss between OB and OB.2. Partial least squares discriminant analysis (PLS-DA) was performed to examine the differences between fecal metabolite profiles in OB and OB.2. VIP >2.5 was taken to identify features significantly differentiating between OB and OB.2, and the fold change ratio was then obtained for each feature. For correlation analysis, univariate associations among tentatively identified fecal metabolites and gut microbiota were tested using Spearman correlation coefficient. Partial correlations with BMI were used to control for potential confounding variables.

Supporting Information

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

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Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

Conflict of interest

The authors declare no conflict of interest.

Author Contributions

C.G.R.-G., M.G., and N.S. conceived and designed the research; C.M.-F. and A.S. recruited volunteers and obtained clinical and anthropometrical data; A.M.N., P.R.-M., L.M., and A.C. conducted research; A.M.N., C.G.R.-G., L.M., M.V., C.L., M.G., and N.S. analyzed the data; C.G.R.-G. and NS wrote the paper; All authors read and approved the final version to be submitted for publication.

Keywords

gut microbiota, in vitro model, fecal metabolomics, extremely obese, diet-induced weight loss

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