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The gut microbiome of Mexican children affected by obesity

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ABSTRACT

Obesity is a metabolic disorder and global health issue. In Mexico 34.4% of children between 5 and 11 years-old are overweight or obese. Here we address this issue studying the gut microbiome in a sample of Mexican children affected by obesity. We performed metagenomic shotgun-sequencing of DNA isolated from fecal samples from a cohort of normal weight and obese Mexican children using Illumina platform with HiSeq 2500. We also examined their metabolic factors and fecal short-chain fatty acids concentration. The results show that a remarkable dysbiosis of bacteria, archaea and viruses was not observed in the obese children group compared to the normal weight group; however, the archaeal community exhibited an increase of unclassified *Methanobrevibacter* spp. in obese children. The bacterial communities of all participants were clustered into three different enterotypes. Most normal weight children have a gut bacterial community dominated by *Ruminococcus* spp. (Enterotype 3), while most obese children had a community dominated by *Prevotella* spp. (Enterotype 2). On the other hand, changes in the gut microbiome were correlated with clinical metadata and could be used to stratify individuals based on their phenotype. The species *Megamonas* spp. were over-represented in obese children, whereas members of the family Oscillospiraceae were depleted in the same individuals and negatively correlated with levels of serum cholesterol. A microbiome comparative metabolic pathway analysis showed that two KEGG pathway modules of glycolysis, Glycolysis I (from Glucose 6-Phosphate), and Glycolysis II (from Fructose 6-Phosphate) were significantly overrepresented in normal weight children. Our results establish specific alterations in the gut microbiome of Mexican children affected of obesity, along with clinical alterations, providing information on the microbiome composition that may be useful for prognosis, diagnosis, and treatment.

1. Introduction

Obesity and overweight are global maladies, and currently approximately 2.8 million people in the world die each year due to its complications [1,2]. Obesity and overweight have a prevalence of ≤10% among school-age children in global regions [3], and in countries like Mexico they have epidemic proportions, where at least 34.4% of children between 5 and 11 years-old are overweight or obese [4]. As multifactorial diseases, the development of obesity and overweight involves dietary and physical activity issues, dysfunctional mitochondria [5], genetic factors like polymorphisms in candidate genes such as FTO, NPC1, ENPP1, NEGR1, GNPDA2, and MC4R [6], which affects gene expression of some of them [7], and

Abbreviations: EggNOG, Evolutionary genealogy of genes: Non-supervised Orthologous Groups; HUMAnN2, Human Microbiome Project (HMP) Unified Metabolic Analysis Network 2; KEGG, Kyoto Encyclopedia of Genes and Genomes; LDA, Linear discrimination analysis; MaAsLin, Multivariate microbial association by the linear model; MetaPhlAn, Metagenomic Phylogenetic Analysis; MGWAS, Microbial Genome-Wide Association Studies; SCFAs, Short-chain fatty acids; T2D, Type 2 diabetes.

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the microbiome.

Microbiota in the human gut is composed of trillions of inhabiting microorganisms, with a remarkable portfolio of microbial genes (microbiome). In the gut, the microbiome may modulate human health through diet-energy extraction in form of short-chain fatty acids (SCFAs) through fermentation of undigested carbohydrate fibers, maintenance of immune system, and anti-pathogenic activity [8,9]. Gut microbiota is mainly composed of bacterial phyla such as Firmicutes (60–65%), Bacteroidetes (20–25%), Proteobacteria (5–10%) and Actinobacteria (3%) [10]. One interesting hypothesis is that obese children harbor a gut microbiota with higher capacity of fermentation of resistant starch. So, the existence of a selected microbiota in obese children is plausible, with higher abundance of fermenting metabolic pathways of soluble polysaccharides. In a recent study of microbial diversity between normal-weight, overweight and obese Mexican children of our group, a significant increase in the abundance of *Blautia* spp., *Coprococcus* spp., *Enterobacteriaceae* in overweight, and significant increase in the abundance of *Faecalibacterium* spp., *Roseburia* spp., *Lachnospiraceae* in obese children was revealed [11].

Microbiome richness is illustrated by the compilation of approximately 6 million complete reference genes in the catalog of the human gut microbiome [12], in addition, genomics approaches to characterize the gut microbial biome using the 16S rDNA gene as phylogenetic marker, and whole genome shotgun sequencing, have provided useful taxonomic and functional profiles. In recent years, understanding of the functional association between the gut microbiota and host physiology has progressed enormously. Recent studies proved the important influence of the gut microbiome in metabolic disorders such as obesity, type 2 diabetes (T2D), and metabolic syndrome [13–16].

Microbial Genome-Wide Association Studies (MGWAS) revealed association of gut microbiome with many complex diseases, not only by taxa abundance but also by their functional metabolic profile [17]. Limited MGWAS have explored diseases such as T2D, colorectal cancer and rheumatoid arthritis [18–20]. There are reports of MGWAS in obese Danish and French adults, exploring the metabolic markers and dietary links with obesity [21,22]. However, to our knowledge, there are not enough published reports of systematic MGWAS about children with obesity.

In this study, we performed a metagenome-wide association study of the gut microbiota to identify disease associated metagenomic markers, highlighting how gut microbiota composition, and their metabolic routes differ between normal weight and obese Mexican children.

2. Methods

2.1. Study participants and specimen collection

Our general work pipeline was as described in Fig. 1. We selected twenty unrelated children between 9 and 11 years-old (ten normal, and ten obese) from an obesity database [11]. Children attended a public primary school located in Greater Mexico City area. Informed consent was signed-out by parents and children in accordance with the Helsinki Declaration revised in 2013. The research protocol was approved by the Local Ethical Committee Board of Health from the Instituto Mexicano del Seguro Social R-2011–1402 1402–10, Mexico City. Fecal samples were taken in conjunction with a health examination and stored immediately at −80 °C. Blood samples were drawn by vein puncture after an overnight fast and sent to a central laboratory to measure biochemical factors. Individuals also completed a questionnaire recording their dietary intake for analysis. Anthropometrical measurements, biochemical tests, and fecal short chain fatty acids (SCFA) were measured as described previously [11].

2.2. Nucleic acid extraction and metagenomic shotgun sequencing

Total DNA was extracted from 250.0 mg of homogenized wet fecal sample (71.5% water content ±7.26 SD) using the MoBio Power Soil DNA Isolation kit (MoBio, Solana Beach, CA, USA) according to the manufacturer's instructions and stored at −80 °C until sequencing. All samples were sequenced using the Illumina platform with HiSeq 2500 instrument, which produced paired-end 2 × 126 read length (Otogenetics Corporation, USA), with ≥40 M reads, and ≥5.2 Gb data per sample (Appendix A, Supplementary Table S6).

2.3. Data processing

FastQC was used to assess the 126-bp paired-end reads for the average quality score per base position [23]. The low-quality regions were trimmed out with Trimmomatic [24]. After trimming, sequence reads were aligned against human genome assembly hg19 obtained from UCSC Genome Browser [25] using Bowtie 2 [26] with default parameters to remove potential human reads. Possible human DNA sequences were identified and discarded.

2.4. Metagenomic shotgun sequences analysis

The gut microbial composition of each sample was profiled using MetaPhlAn2 [27]. The relative abundances of the gut microbial functional pathways from metagenomically-sequenced communities were determined using HUMAnN2 [28] using genes, extracted from the KEGG database [29].

2.5. Bacterial, archaeal, and viral abundance analysis

Differences in the abundance of bacterial and archaeal taxa (family, genus and species) between normal weight and obese children, was made by linear discriminant analysis (LDA) followed by Wilcoxon Mann-Whitney test, coupled to size effect (LEfSe) using default parameters [30]. Viral profiling analysis was made using ViromeScan [31].

2.6. Enterotyping and functional clustering

We calculated the distance for genus-level relative abundance profiles and KEGG pathway relative abundance profiles. Enterotypes of the gut microbiome were assigned using default parameters available in the tutorials (http://enterotyping.embl.de/enterotypes.html) [32]. In brief, primarily relative abundance of all gut microbiome was taken and script *data = read.table("Mexican_child-obese.genus.txt", header = T, row.names = 1, dec = ".", sep = "\|t"); data = data[-1,]* used to load and read the file. Subsequently, we used scripts *data.dist = dist.JSD(data); JSD < - function(x,y) sqrt(0.5 * KLD(x, (x + y)/2) + 0.5 * KLD(y, (x + y)/2)), and KLD < - function(x,y) sum(x * log(x/y))* to calculated the Jensen–Shannon (JS) distance for relative abundance profiles at genus-level. To cluster the abundance profiles of the microbiome, we used the Partitioning Around Medoids (PAM) clustering algorithm using the script *pam(as.dist(x), k, diss = TRUE)*, where "x" is a distance matrix and "k" the number of clusters. Next, to assess the optimal number of clusters, we used the Calinski-Harabasz (CH) index, as in the script *data.cluster = pam.Clustering(data.dist, k = 3)*. These clusters were validated by silhouette coefficient S(i) as in the script *obs.silhouette = mean(silhouette(data.cluster, data.dist)[,3])*, and noise was removed using *data.denoized = noise.removal(data, percent = 0.01)* script. Finally, clusters were visualized by principal

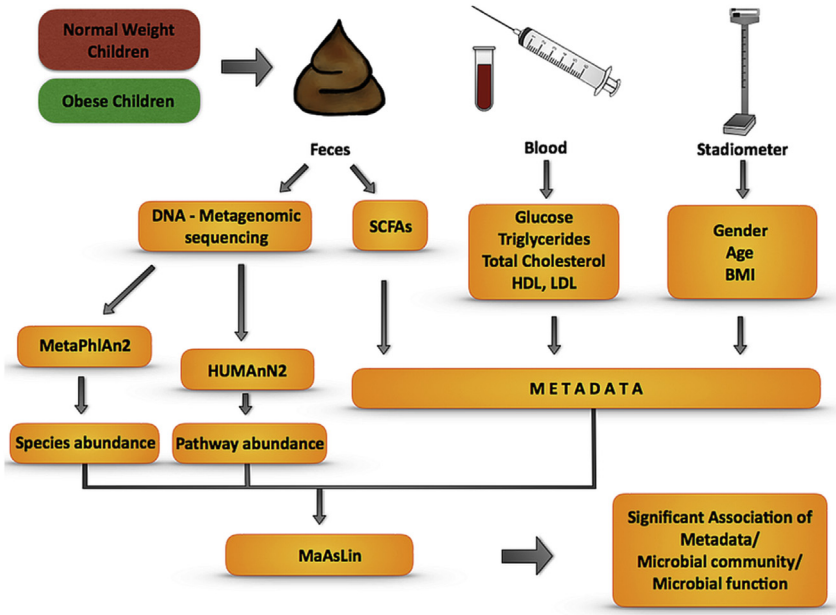

Fig. 1. MGWAS general work pipeline. Fecal and blood samples were collected, and anthropometric data was recorded from all normal weight and obese participant children. Total DNA was extracted from stool and was subjected to metagenomics sequencing; microbial community composition and function were profiled using MetaPhlAn2 [27] and HUMAnN2 [28]. Total Short Chain Fatty acids were also measured from same samples by HPLC [11]. A biochemical profile including glucose, triglycerides, cholesterol and high and low-density lipoproteins was determined in serum. MaAsLin [33] was used to find significant associations among species, pathway abundances, and all metadata.

coordinates analysis (PCoA), using the script *obs.pcoa = dudi.pco(-data.dist, scannf = F, nf = 3)*, and the script *s.class(obs.pcoa$li, fac = as.factor(data.cluster), grid = F)*. Enterotyping was done in "R" environment using biotyper (0.1.2), cluster (v2.07), and ade4 (v1.7) packages.

2.7. Testing for significant associations with the clinical metadata variables

A multivariate association with linear model (MaAsLin) specially adapted to microbiome data, was used to identify significant associations between microbial and phenotypic variables [33]. Selected metadata were used in a general linear model with metadata as predictors and arcsin-square root transformed microbial relative abundances as the responses. In this study, model covariates of interest comprised clinical variables shown in Appendix A, Supplementary Table S1. The anthropometric characteristics were statistically analyzed using chi-square, analysis of variance (ANOVA), and Kruskal–Wallis one-way analysis of variance.

2.8. Metagenome assembly and gene prediction

Metagenomic assembly and gene prediction were performed using the MOCAT2 pipeline [34]. Protein-coding genes were predicted using MetaGeneMark [35].

3. Results

3.1. Obese children showed increased levels of fat metabolites and dyslipidemia

Twenty unrelated children (ten normal weight, and ten obese) aged between 9 and 11 years-old, were selected from an obesity database (Fig. 1; Appendix A, Supplementary Table S1) [11]. The anthropometric assessment indicated no difference in age and height between the two groups (Fig. 2A and C), but a significant difference in weight and BMI percentile was observed in agreement to the phenotype (Fig. 2B and D). In case of biochemical parameters, Mexican obese children showed a significant increase of serum levels of total cholesterol (Fig. 2F), and triglycerides (Fig. 2G); the low-density lipoprotein was also increased (Fig. 2I), however, the difference was not statistically significant. The high-density lipoprotein was significantly decreased in obese, with respect to normal weight children (Fig. 2H). Also, there was not difference for the fasting serum glucose levels between the two groups (Fig. 2E). The short chain fatty acids concentration in feces showed no difference between normal weight and obese children (Fig. 2J–L), although participants for this study were randomly selected from a larger database, where a significant decrease has been reported for propionic and butyric acid in obese children [11].

3.2. Microbiota diversity of normal weight and obese children

A MetaPhlAn analysis of the metagenomic sequencing did not show a global difference in the abundance for the entire bacterial community in obese children compared to the normal weight. For instance, although the relative abundance of *Prevotella copri*, a bacterium belonging to the Bacteroidetes phylum, has been reported to be associated to rheumatoid arthritis in humans, increasing from 3.08% in normal weight to 13.63% in obese children, the difference was not statistically significant ($p = 0.440$). On the other hand, the abundance of *Bacteroides coprocola* another Bacteroidetes associated to T2D decreased from 5.28% in normal weight to 0.54% in obese children, but again the difference was not statistically significant ($p = 0.286$) (Fig. 3A-Bacteria, color code in Appendix A, Supplementary Fig. S1). The archaea community exhibited an increase of unclassified species of *Methanobrevibacter* in obese children (Fig. 3A-Archaea; Appendix A, Supplementary Fig. S1). For the viral community, the analysis of the metagenomics data did not show a significant difference in the abundance in obese children compared to normal weight children. However, the relative abundance of some viruses like the Human

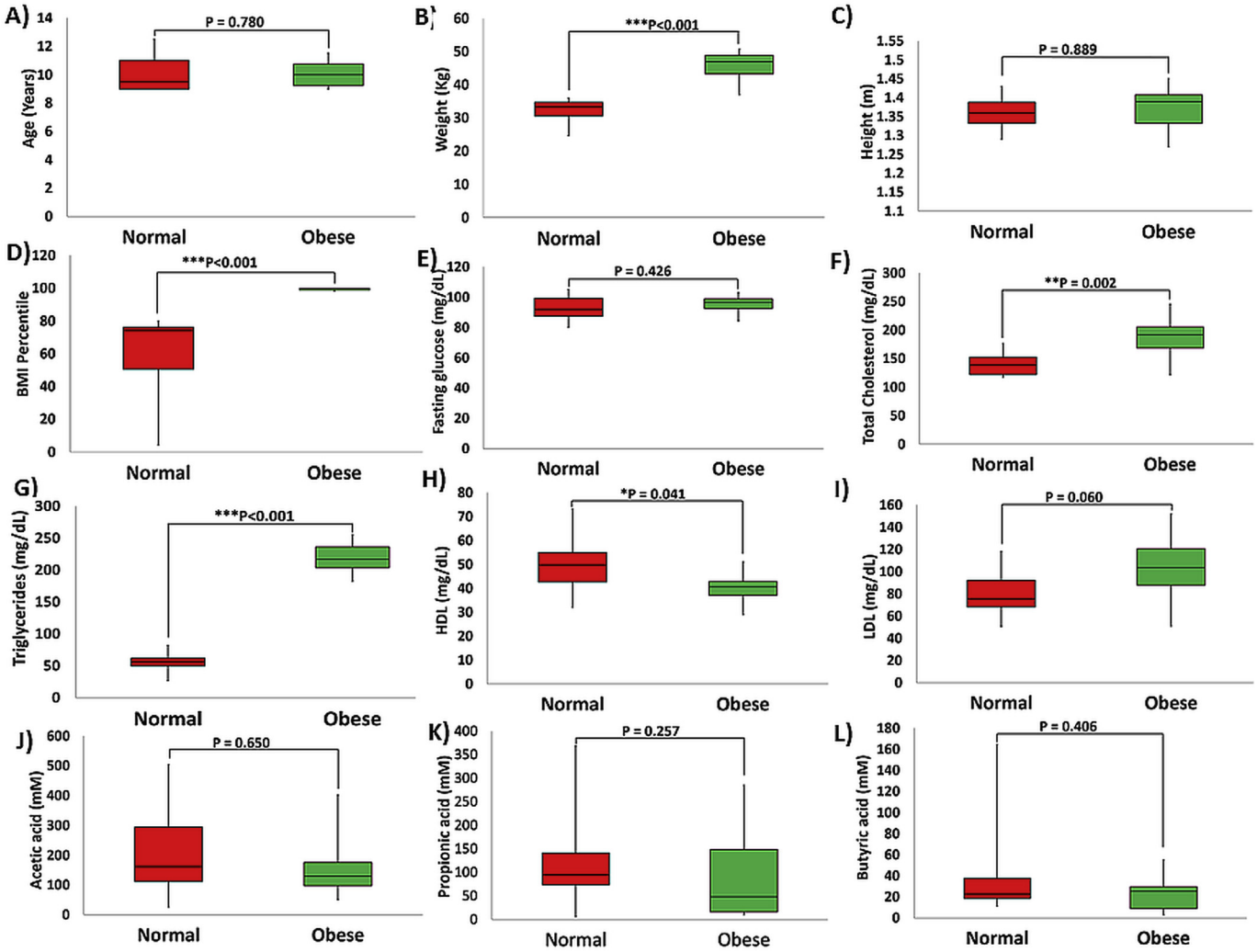

Fig. 2. Metadata statistical significance comparison. A) Age; B) Weight; C) Height; D) body mass index percentile BMI; E) Fasting glucose; F) Total cholesterol; G) Triglycerides; H High-density lipoprotein (HDL); I) Low-density lipoprotein (LDL); J) Acetic acid; K) Propionic Acid; L) Butyric acid. P-Values were calculated according to Chi-square test, ANOVA test for equal variances, and Kruskal–Wallis test for different variances. (*) $P < 0.05$, (**) $P < 0.01$, (***) $P < 0.0001$ are considered statistically significant; Red color indicates normal weight, and Green color indicates obese children. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

herpesvirus 4 (NC_007605.1) increased from 0.08% in normal weight to 1.53% in obese children ($p = 0.017$), while the relative abundance of Torque teno midi virus 1 (NC_009225.1) decreased from 2.21% in normal weight to 0.72% in obese children ($p = 0.282$) (Fig. 3A-Virus; color code in Appendix A, Supplementary Fig. S2). We also explored the abundance of CrAssphage virus reads, which went from 29,748 in normal weight to 78,111 in obese children; however, this difference was not statistically significant ($p = 0.473$) (Fig. 3B). A clustering analysis of the gut microbiome sequencing data revealed the presence of three previously reported enterotypes in our study subjects: Enterotype 1, enriched in *Bacteroides* spp., Enterotype 2 enriched in *Prevotella* spp. with 50% of obese children, and Enterotype 3, enriched in *Ruminoccocus* spp. with 50% of normal weight children (Fig. 3C; Appendix A, Supplementary Table S3).

3.3. Compositional and functional characteristics of normal weight and obese children microbiomes

We performed a MetaPhlAn2 and HUMAnN2 analysis on the metagenomics data (Fig. 1). It was observed that *Megamonas* spp., a Firmicutes bacteria was at least 2-fold more significantly abundant in obese children than in normal weight (Fig. 4A). On the other hand, some members of the phyla Firmicutes, Bacteroidetes and Proteobacteria were at least 2-fold more significantly abundant in normal weight than in obese children. Here, Firmicutes included bacteria such as *Ruminococcus* spp., *Clostridium citroniae*, *Coprococcus comes*, *Streptococcus thermophilus*, *Dorea formicigenerans*, *Oscillibacter* spp., and other members of the Family Oscillospiraceae. The phylum Bacteroidetes included *Bacteroides ovatus*, *B. xylanisolvens*, *B. salyersiae*, and *B. faecis*, while the phylum Proteobacteria included members of the Family Desulfovibrionaceae (Fig. 4A). In addition, to explore the microbiota diversity, we characterized the microbiome of our subjects. Path abundance analysis showed that two KEGG pathway modules of glycolysis, Glycolysis I (from Glucose 6-Phosphate) ($p = 0.013$), and Glycolysis II (from Fructose 6-Phosphate) ($p = 0.016$) were significantly over-represented in normal weight children (Fig. 4B; Appendix A, Supplementary Table S4). The KEGG path coverage analysis of metabolic routes showed almost 4-fold more abundance of three anabolic routes for nucleotide synthesis such as pyrimidine, guanosine in normal weight, and four abundant anabolic routes for

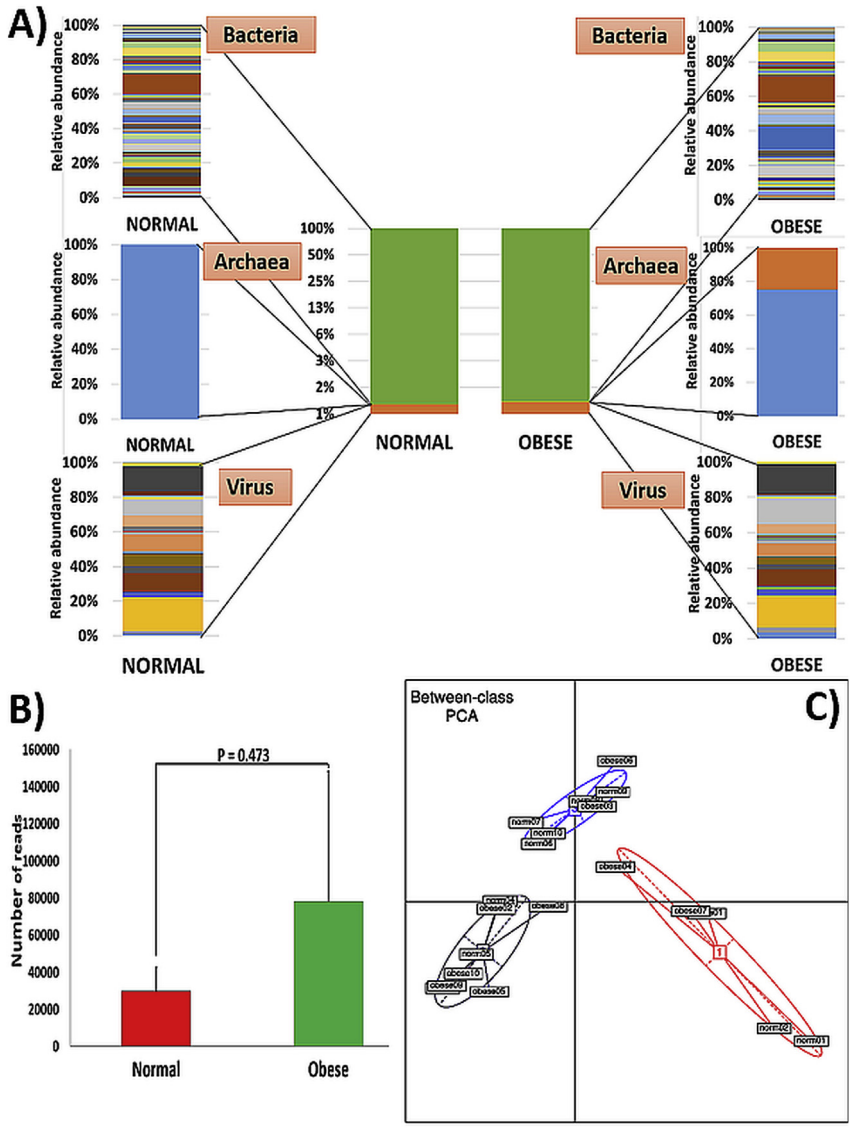

Fig. 3. Characterization of the distal gut microbiome in stool samples from normal weight and obese children. DNA was extracted from each sample and shot-gun metagenomic sequenced as described in the Methods section. A) Relative abundance of bacterial and archaeal (color code for each entry shown in Appendix A, Supplementary Fig. S1); and viral populations (color code shown in Appendix A, Supplementary Fig. S2); B) Comparison of total number of CrAssphage reads. Red color indicates normal weight and green color indicates obese children; C) Bacterial enterotypes were defined as describe in 2.6 Enterotyping and functional clustering section of the Methods section. Samples are colored by enterotype as identified by the partitioning around medoids (PAM) clustering algorithm. Red color is Enterotype 1, violet color is Enterotype 2, and blue color indicates Enterotype 3. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

aminoacids or sugar synthesis in obese children (Fig. 4C; Appendix A, Supplementary Table S4). On regard of KEGG gene families, data analysis showed an increase in the significant abundance of at least 2-fold, of genes such as 4-O-β-D-mannosyl-D-glucose phosphorylase (RXN_12977) in normal weight and catabolic genes such as α-amylase (ALPHA_AMYL_RXN), an enzyme for hydrolysis of (1 → 4)-α-D-glucosidic linkages of polysaccharides in obese children (Fig. 4D; Appendix A, Supplementary Table S4). However, when a Bray-Curtis-Principal coordinate analysis (PCoA) was made for KEGG pathway module abundances, coverage, and gene families using distance matrices, no clustering was observed between the two experimental groups (Appendix A, Supplementary Fig. S3). On the other hand, when metadata and bacterial were probed together, a significant association was found, where high serum cholesterol levels are related to lower abundance of the Oscillospiraceae family in obese children (Fig. 4E).

3.4. Phylogenetic and functional diversity in the normal weight and obese children microbiomes

We performed a clustering analysis using the top 15 abundant bacterial species from the microbiome, the full set of metadata, and the 15 orthologous more relevant gene clusters for our data from the EggNOG database. The graphic heatmap of this analysis showed a hierarchical clustering of the normal weight and obese children with gut microbes from the phylum Bacteroidetes such as *Alistipes putrenidis*, *A. shahii*, *Prevotella copri*, *Parabacteroides merdae*, and seven different bacteroides: *Bacteroides ovatus*, *B. vulgatus*, *B. uniformis*, *B. stercoris*, *B. dorei*, *B. cacae*, and *B. coprocola*. The same occurred with members of the phylum Firmicutes, like *Subdoligranulum* spp., *Eubacterium rectale*, *Faecalibacterium prausnitzii*, and *Ruminococcus bromii* (Fig. 5A). The analysis with orthologous genes derived from metagenomics functional profiling using

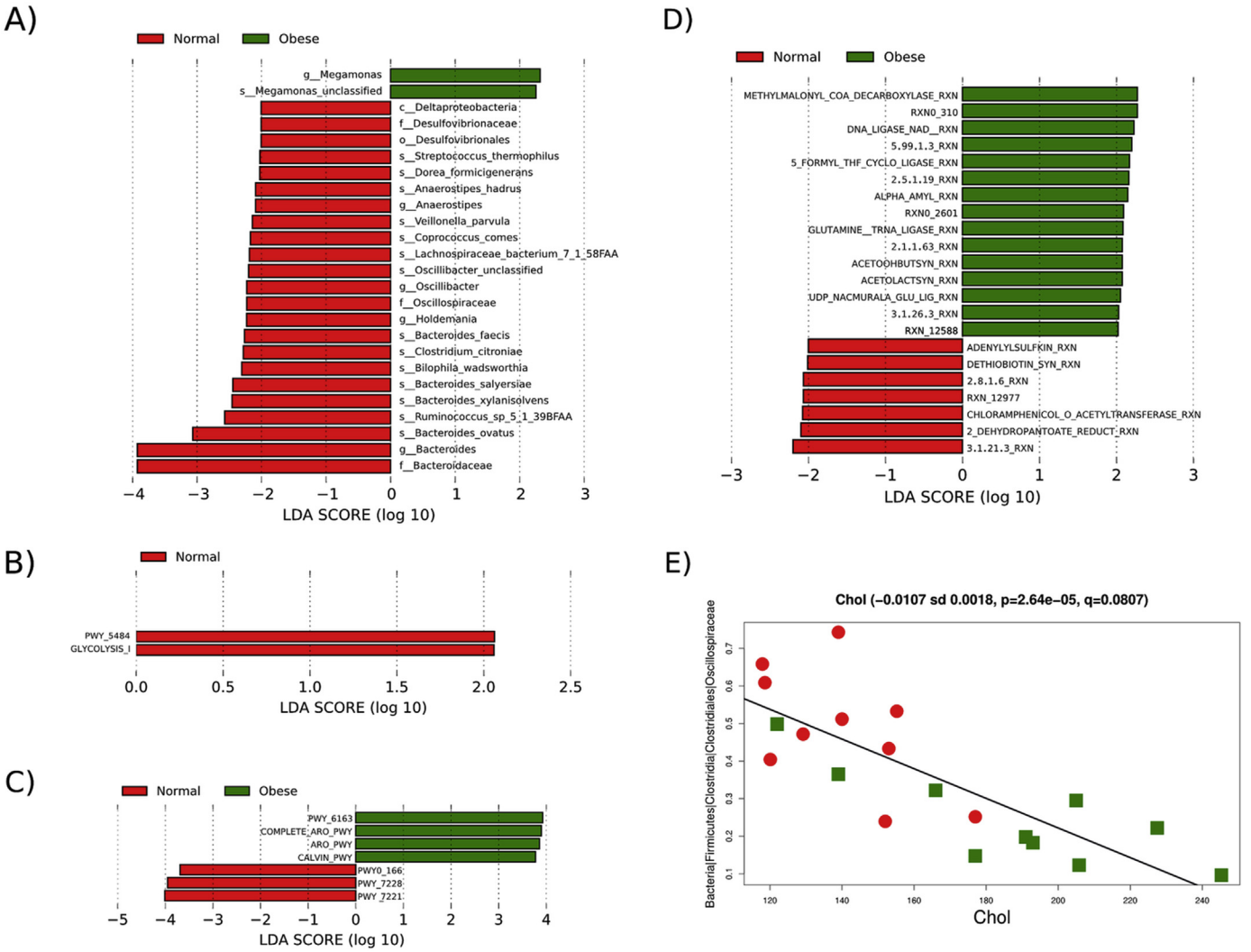

Fig. 4. Significant bacteria, path abundance-coverage, gene families found in the gut microbiome and association with metadata. Figure shows graphs of linear discriminant analysis (LDA) scores for A) Differentially abundant bacterial genera, families or species; B) Differentially abundant KEGG Pathway modules; C) Differentially abundant KEGG pathway coverage; D) Differentially abundant KEGG gene families. Negative and positive LDA scores indicate overrepresented data in normal weight (red) and obese (green) children. Features with LDA scores ≥ 2 are presented. Full description of path abundance, coverage, and gene families is shown in Appendix A, Supplementary Table S4; E) Significant association of cholesterol level with Oscillospiraceae family abundance. Lines represent linear model fit after transform to accommodate compositional, non-normally distributed data and cholesterol level. Red color circles indicate normal weight children; green color squares indicate obese children. Nominal *p*-values and FDR corrected *q*-values are assigned by MaAsLin [33]. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

EggNOG database entries (Fig. 5B; Appendix A, Supplementary Table S5) showed four main clustering of metabolic modules, one including cell wall, membrane, and envelope biogenesis, as well as amino acid and lipid transport and metabolism. A second clustering included energy production and conversion, and signal transduction mechanisms; while a third clustering grouped nucleotide and carbohydrate transport and metabolism, DNA transcription, ribosomal structure and biogenesis, and energy production and conversion orthologous gene clusters. The fourth clustering included replication, recombination, and repair, ribosome structure and biogenesis, and the enzyme pyruvate phosphate dikinase, from the carbohydrate transport and metabolism group (Fig. 5B). Finally, the analysis using the clinical metadata, showed three clustering, the first grouped triglycerides, the percentile of body mass index, and weight; the second grouped glucose, acetate, cholesterol, and low-density lipoprotein; while the third grouped butyrate, high density lipoprotein, height and propionate (Fig. 5C).

4. Discussion

Overweight and obesity are global diseases where according to the Organization for Economic Co-operation and Development, at least one in three adults and one in five children suffer of it among its 35 members [36]. In countries such as Mexico, these two conditions have increased at an alarming rate in the last years, where at least one in three children and adolescents between 5 and 11 years-old are affected [37]. Recent work from our group on gut bacteria in overweight and obesity, has shown its association with the level of SCFAs production in Mexican children [11]. Overweight and obesity usually starts in childhood or adolescence and involves genetic factors in the human genome as well as in the human microbiome.

Microbial Genome Wide Association Studies (MGWAS) has proved the important role of the microbiome in the development of diseases such as type 2 diabetes [18], colorectal cancer [19], and rheumatoid arthritis in adults [20], which has revealed enriched microbial genes, strains and functions in each study group. In this

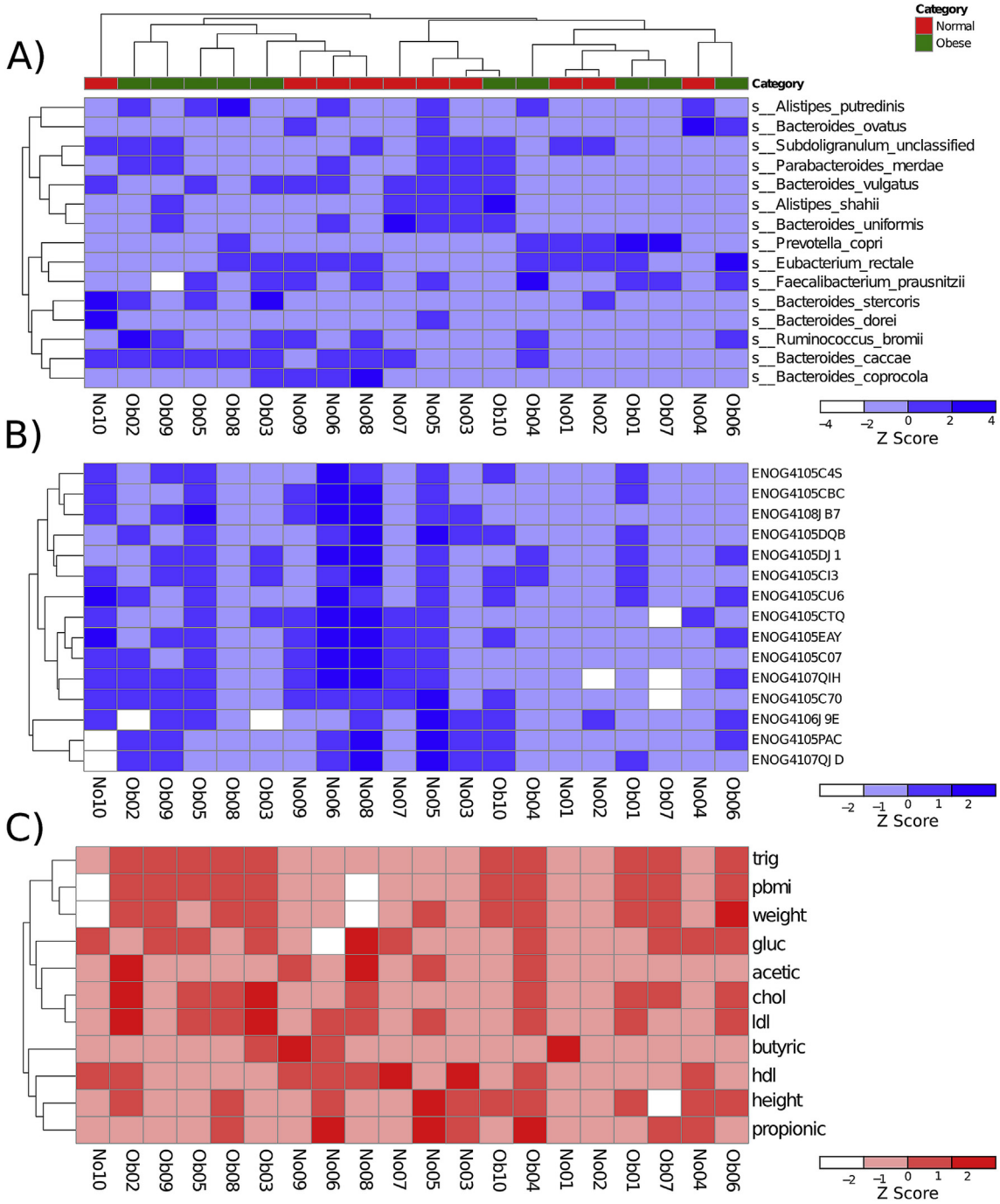

Fig. 5. Heatmap and hierarchical clustering of taxonomic and functional profiles of gut microbiomes. A) Columns, the top 15 bacterial abundance profiles for every individual clustered the two categories (normal-weight, obese children) in groups. Rows, the bacterial abundance in each individual, clustered the members of the top 15 bacteria; B) Columns, the sample clustering retains ordering from A), Rows, clustering of metabolic modules derived from metagenomic functional profiling using EggNOG database, (Appendix A, Supplementary Table S5); C) Columns, the sample clustering retains ordering from A), Rows, clustering of clinical metadata profile. trig, triglycerides; pbmi, percentile of body mass index; gluc, glucose; acetic, acetate; chol, cholesterol; ldl, low density lipoprotein; butyric, butyrate; hdl, high density lipoprotein; propionic, from data shown in Fig. 2, and Appendix A, Supplementary Table S1. Red color indicates normal weight children. Green color indicates obese children. Z Scores in blue or red color indicates how many standard deviations a value is from the mean. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

work, we made a comparative study of the microbiome in normal weight and obese Mexican children from a study cohort on obesity.

Our results show that obese children in the sample have increased weight and BMI percentile, besides to increased triglycerides and cholesterol levels (dyslipidemia) (Fig. 2G and F, Appendix A, Supplementary Table S1). The microbial diversity characterization based on the shotgun metagenomic analysis showed that in general, the abundance of bacterial, archaeal, and viral communities was comparable between normal weight and obese children. However, on regard of viruses, there is a significant 19-fold increase of the Human herpesvirus 4 (HHV-4) in feces of obese children (Fig. 3A, color code in Appendix A, Supplementary Fig. S2). There is one report of an association of a different Human herpesvirus 1 with obesity in US population between 20 and

49 years-old [38], thus ours is the first report of an association of HHV-4 with obesity in children. We also observed an increase in the number of reads for the CrAssphage virus in obese children; this virus infects bacterial members of the phylum Bacteroidetes (e.g. *Prevotella intermedia*, *Bacteroides* spp.), and it is thought to be associated with obesity [39] (Fig. 3B).

Regarding archaea, there was a decrease in the abundance of the human gut methanogenic archaeon *Methanobrevibacter smithii* in obese children of this study (Fig. 3A-Archaea, color code in Appendix A, Supplementary Fig. S1), this result agrees with a report in French adults [40]. However, there was an increase in the relative abundance of a different unclassified species of *Methanobrevibacter* spp. in the same children (orange color in Fig. 3A-Archaea). Members of this genus have a productive saccharolytic activity allowing digestion of polysaccharides in the gut [41] and are reported to be responsible for diet-related increased body weight in a murine model [42]. Obesity also involves low-grade chronic inflammation [43], and a strain of *Methanobrevibacter smithii* is reported capable of inducing an inflammatory cytokine response by activation and release of pro-inflammatory cytokines in monocyte-derived human dendritic cells contributing to obesity [44]. In a report of a study made in US population adults 18-40 years-old, there was a positive association of *Methanobrevibacter* spp. with a diet high in carbohydrates [45], with greater body mass index, and body fat [46]. Moreover, the same, *M. smithii* colonization was positively associated with an increased risk of overweight in 6–10 years-old children in Netherlands [47].

When the bacterial community was analyzed, the normal-weight and the obese children were stratified based on three different bacterial enterotypes [32,48]. We observed that most normal weight children have a gut bacterial community dominated by *Ruminococcus* spp (Enterotype 3), while most obese children had a community dominated by *Prevotella* spp. (Enterotype 2) (Fig. 3C, Appendix A, Supplementary Table S3). These two enterotypes accounted for 75% of the studied children. Stratification of microbial communities in enterotypes has been reported for tumor-associated and non-tumor-associated microbiota in colorectal cancer [49], and as a dietary-associated cardiometabolic risk factor [50]. In our study, we observed that the relative abundance of *Prevotella copri* increased in obese children; in this manner, we think that a particular *Prevotella* spp. dominating the gut microbiota (Enterotype 2) is associated with obesity in Mexican children 9 to 11 years-old.

On the other hand, based on LEfSe analysis, we observed that in normal weight, several members of the phyla Firmicutes, Bacteroidetes and Proteobacteria were at least 2-fold more abundant in normal-weight than in obese children (Fig. 4A), being the ratio Firmicutes/Bacteroidetes in agreement with previous reports for obesity [51].

Regarding the phylum Proteobacteria, in our study, the MetaPhlAn2 analysis showed that members of the Family Desulfovibrionaceae, e.g. *Bilophila wadsworthia* were 2-fold more abundant in normal-weight than in obese children (Fig. 4A). Members of this family are strict anaerobic sulfate-reducing bacteria, and a decrease in its abundance is reported associated to obesity (Table 1).

Among the phylum Bacteroidetes, our study showed that members of the Family *Bacteroidaceae*, such as *Bacteroides faecis*, *B. ovatus*, *B. xylanisolvens*, and *B. salyersiae* were 2- to 4-fold more abundant in normal-weight than in obese children (Fig. 4A). The functional relationship between the host and members of the phylum Bacteroidetes is complex, due mostly to its genomic diversity, plus a functional metabolic versatility, which allows members of this phylum to adapt quickly to rapid environment changes in the gut [52]. High abundance of *Bacteroides* spp. is reported associated to normal weight, with the important role of maintaining an ecological balance of the concurrent microbiota in the gut, due to its saccharolytic activity producing acetate, propionate and succinate (Table 1).

We identified several Firmicutes which are more abundant among normal weight than obese children: *Streptococcus thermophilus*, *Dorea formicigenerans*, *Anaerostipes hadrus*, *Veillonella parvula, Coprococcus comes*, *Lachnospiraceae* bacterium 7 1 58 FAA, *Oscillibacter* spp., *Holdemania* spp., *Clostridium citroniae*, and *Ruminococcus* sp. 5 1 39BFAA (Fig. 4A). There is reported evidence that some of these bacteria play a major role in the prevention of obesity in humans through metabolite production or immunological modulation. The abundance of *S. thermophilus* increases adipocyte lipolysis in aP2-agouti transgenic obesity model mice, which is susceptible to diet-induced obesity, and exhibit a human pattern of expression of obesity-related genes [53] (Table 1). In another report, a strain of the genus *Anaerostipes* spp. was shown to increase butyrate content in the gut of healthy mice models; butyrate is an energy source for intestinal epithelium, stimulates the production of regulatory T cells, inhibits inflammation, and regulates gene expression (Table 1). A decrease in butyrate-producing bacteria is associated with several inflammatory diseases like obesity [54]. By this, we have previously reported a decline in butyrate production by the gut microbiota in Mexican obese children under 11 years-old from the same study cohort [11]. *Veillonella parvula* is an opportunistic pathogen, and members of this genus *Veillonella* spp. negatively correlated with short-chain triglycerides (Table 1). In another report, *V. parvula* has potential immunomodulatory properties inducing cytokine responses like interleukine-6 (IL-6) production in dendritic cells. Furthermore, in the same report, a combination of *Veillonella* spp. with *Streptococcus* spp. augmented IL-8, IL-6, IL-10, and TNF-α response [55]. It is of interest that IL-8, and TNF-α, are reported elevated in human obesity [56], cytokine IL-6 has anti-inflammatory effect and a compensatory role in obesity by increasing islet glucagon-like peptide-1 (GLP-1) production [57], and IL-10 participates in the prevention of systemic low-grade inflammation caused by obesity [58]. In our study, *S. thermophilus* exhibited a comparable 2-fold more abundance in normal-weight children (Fig. 4A), so we believe a pairwise abundance of *V. parvula* and *S. thermophilus* is associated to normal-weight in Mexican children where they synergistically regulate the production of IL-6 to contribute to the reduction of inflammation. The abundance of *C. comes*, a bacterium producer of butyric, acetic, formic, and propionic acids [59], is also higher in normal-weight children (Fig. 4A), the abundance of this bacteria decreases along with BMI, serum triglycerides, cholesterol and LDL-cholesterol after Roux-N-Y gastric-bypass surgery in German adults (Table 1).

In normal weight children, bacteria belonging to the family Oscillospiraceae such as unclassified *Oscillibacter* spp. were significantly more abundant than in obese children (Fig. 4A). *Oscillibacter valericigenes* (Oscillaspiraceae) is a well-known producer of valeric acid (Table 1); we especially found an important significant negative association ($SD = 0.0018$, $p = 2.64e\text{-}25$, $q = 0.0807$) of this family with serum cholesterol levels, and BMI among children of our study (Fig. 4E). This result agrees with a role of inhibition of hepatic cholesterol synthesis produced by valeric acid derivatives in rats [60]. Another Firmicutes, *Holdemania* spp. was 2-fold more abundant in normal weight children; this bacterium is an acetic- and lactic acid producer, and members of this genus are reported associated to leanness in a study made in Japanese men (Table 1). *Clostridium citroniae* is a Firmicutes 2-fold more abundant in normal weight children in our study. A strain of this bacterium was reported as more abundant in lean than obese adults in Japanese population; however, it appears to be an opportunistic pathogen associated to colitis in adults (Table 1). *Ruminococcus* sp. 5 1

Table 1
Gut bacteria with significant increase or decrease among children of this study.

Phylum Proteobacteria			
bacteria	this work	other reports	reference
Family Desulfovibrionaceae, *Bilophila wadsworthia*	2-fold more abundant in normal weight children than obese children	Family Desulfovibrionaceae, a decrease in its abundance has been associated to overweight and obesity in a study made in Swedish preschool children.	[74]
		B. wadsworthia significantly more abundant in lean than obese Japanese adult population.	[75]
		Bilophila spp. a high sugar diet increases the abundance in rat model.	[76]
Phylum Bacteroidetes			
bacteria	this work	other reports	reference
Family Bacteroidaceae, Genera *Bacteroides faecis*, *B. ovatus*, *B. xylanisolvens*, and *B. salyersiae*.	2 to 4-fold more abundant in normal-weight than obese children	Increase in Bacteroidetes reported due to alimentary intervention reducing the BMI in Spanish adolescents	[77]
		B. acidifaciens members of the Family Bacteroidaceae, its increase prevented obesity and improved insulin sensitivity in mice model	[78]
		B. faecis negatively associated with markers for dyslipidemia in Danish adult women.	[79]
		B. ovatus maintains diversity of gut microbiota by efficient hydrolysis of soluble fiber like starch producing oligosaccharides and dextrins, which are available to other non-saccharolytic bacteria.	[80]
		B. xylanisolvens in the human gut degrades xylans to acetate, propionate and succinate.	[81]
		B. salyersiae has been isolated from human feces.	[82]
Phylum Firmicutes			
bacteria	this work	other reports	reference
Streptococcus thermophilus	2-fold more abundant in normal-weight than in obese children	*S. thermophilus* a probiotic associated to reduction of body weight, fat accumulation, fatty acid synthase activity in adipocytes.	[53]
Dorea formicigenerans		*D. formicigenerans*, a serine protease producer, whose abundance increases after bowel cleansing in English adult people.	[83]
		D. formicigenerans abundance decreases after antibiotic treatment in adult Japanese people suffering of ulcerative colitis.	[84]
Anaerostipes hadrus		*A. hadrus*, a strain of this genus has shown to increase butyrate content in the gut of healthy mice models.	[85]
Veillonella parvula		*V. parvula* usually considered part of the oral microbiota, or an opportunistic human pathogen.	[86]
		Veillonella spp. negatively correlated with short-chain triglycerides in serum and stool, in a study made in preschool Finnish and Estonian children.	[87]
Coprococcus comes		*C. comes*, its abundance decreases along BMI, serum triglycerides, cholesterol and LDL-cholesterol in German adults affected of type 2 diabetes/obesity after Roux-en-Y gastric-bypass surgery.	[88]
Lachnospiraceae bacterium 7 1 58 FAA		Family Lachnospiraceae, associates with lower long-term weight gain in women of Caucasian ancestry.	[89]
Oscillibacter sp. unclassified		*O. valericigenes* well-known producer of valeric acid.	[90]
Holdemania spp.		*H. filiformis* acetic and lactic acid producer.	[91]
		Holdemania spp. associated to leanness in Japanese men.	[92]
Clostridium citroniae		*C. citroniae* more abundant in lean than obese adults in Japanese population.	[75]
		C. citroniae strain is an opportunistic pathogen associated to colitis in adults.	[93]
Ruminococcus sp. 5 1 39BFAA		*Ruminococcus* spp. positively correlates with short-chain triglycerides concentration in serum and stool in preschool Finnish and Estonian children.	[87]
Megamonas sp. unclassified	More than 2-fold more abundant in obese than in normal-weight children.	*Megamonas* spp. more abundant in Taiwanese obese adults than in normal weight.	[94]
		Megamonas spp. abundance significantly reduced in Mexican children with Type1 diabetes onset with respect to healthy control.	[63]
		Megamonas spp. more abundant in normal and pre-diabetes Chinese subjects than in recently diagnosed Type 2 diabetes adult individuals.	[64]

39BFAA, this Firmicutes was 2-fold more abundant in normal weight children in our study (Fig. 4A). This strain belongs to a genus where its members are reported as pathogens or probiotics [61]; surprisingly *Ruminococcus* spp. positively correlates with short-chain triglycerides concentration in serum and stool in a study made in preschool Finnish and Estonian children (Table 1).

On the other hand, we found an unclassified gram-negative Firmicutes *Megamonas* spp. more than 2-fold significantly over-represented in obese children than in normal weight children (Fig. 4A). Members of the genus *Megamonas* spp. are active propionic, and acetate producing, saccharolytic bacteria [62]. Similar results about the abundance of this bacterium were observed in Taiwanese obese adults (Table 1). However, *Megamonas* spp. abundance was significantly reduced in Mexican children with Type 1 Diabetes onset with respect to healthy control [63], while in a Type 2 Diabetes (T2D) study in Chinese adults, the genus *Megamonas* was more abundant in normal and pre-diabetes subjects than in recently diagnosed T2D individuals [64] (Table 1). All children in our study exhibited normal fasting glucose levels (Fig. 2E), but serum cholesterol levels were increased in children affected with obesity (Fig. 2F). Members of the genus *Megamonas* are reported to promote isoprenoid cholesterol biosynthesis through propionic acid mediated pyruvate pathway and alanine [65,66], which may explain its abundance in obesity in our study. Furthermore, in a different report, a decrease in the abundance of *Megamonas* spp. was observed during oral treatment with Berberine, an

anticholesteremic drug in African and Chinese adults [67], which adds to its role in cholesterol biosynthesis.

The HUMAnN2 analyses (Fig. 1), showed that the KEGG pathway modules Glycolysis_I (from Glucose 6-Phosphate), and Glycolysis II (pathway-5484, from Fructose 6-Phosphate) were 2-fold significantly overrepresented in the gut microbiota of normal weight children (Fig. 4B; Appendix A, Supplementary Table S4). More glycolytic activity in the colon would increase short chain fatty acids production, which are absorbed by the host. An increase in valeric acid for instance, would decrease acetyl CoA in the host, leading to normal lipid levels [66]. On the other hand, an increase in butyric acid in the host, leads to an increase in anti-inflammatory interleukins [68] (Fig. 6). This result agrees with the depletion of glycolysis observed in a study of the microbiome of obese American twins [69].

Microbiome path coverage revealed a 4-fold overrepresentation of three anabolic routes for nucleotide synthesis such as pyrimidine and guanosine in normal weight, and four abundant anabolic routes for aminoacids or sugar synthesis in obese children (Fig. 4C, Appendix A, Supplementary Table S4); however, we do not find an explanation for this observation. Microbiome gene family abundance revealed that α-amyl RXN (or α-Amylase (EC 3.2.1.1)) was 2-fold significantly more abundant in obese children (Fig. 4D; Appendix A, Supplementary Table S4). This enzyme carries out the hydrolysis of α-1,4 glycosidic bonds of resistant starches reaching the colon. Similar results were observed in American children with non-alcoholic fatty liver disease [70]. Furthermore, obese microbiome has a higher capacity to extract more energy in the form of acetic and propionic acids; *Megamonas* spp. carries bacterial α-amylase, which might lead to dyslipidemia through acetyl-CoA synthesis [65] (Fig. 6).

Clustering analysis using the more relevant 15 orthologous gene clusters for our data from the EggNOG database (Fig. 5B; Appendix A, Supplementary Table S5), showed that carbohydrate transport and metabolism is the most abundant functional group among Mexican gut microbiome, which is similar to another human gut microbiome study in Chinese and Danish adults [12]. In addition, phosphohexokinase (ENOG4105CTQ) orthologue of carbohydrate transport and metabolism, was significantly more abundant ($p = 0.044$) in normal weight children than obese children, which promotes utilization of glucose to regulate the energy in both glycolysis I and II [71] (Fig. 4B). Likewise, methylmalonyl-CoA decarboxylase (ENOG4108JB7) orthologue of lipid transport and metabolism, was more abundant in normal weight than obese children ($p = 0.056$) (Fig. 5B; Appendix A, Supplementary Table S5). Methylmalonyl-CoA decarboxylase plays a vital role in propionic acid production in *Megamonas* spp., which is involved in cholesterol synthesis through Acetyl CoA [65].

We think these enzymatic activities allow efficient catabolic metabolism of undigested polysaccharides from the diet that reach the colon in children and are metabolized by the gut microbiota. The fermentation products are short-chain fatty acids which have immunomodulatory and anti-inflammatory properties [68]. Resistant starch is a dietary fiber that may contribute to reduce fat accumulation, enhance insulin sensitivity, regulate blood glucose level and lipid metabolism (Fig. 6), making it a promising dietary fiber for the prevention or treatment of obesity and its related diseases [72]. In another report, dietary resistant starch and chitosan showed anti-obesity effects in a murine model [73]. So, in this manner, the gut microbiota of normal gut children would carry among other gene function, the capacity to ferment resistant polysaccharides efficiently in the gut.

5. Conclusions

The gut microbiome of normal and obese children studied in this work, was quite similar in their microbial communities or their protein assemblies (Fig. 5A; Fig. 5B). However, individual members

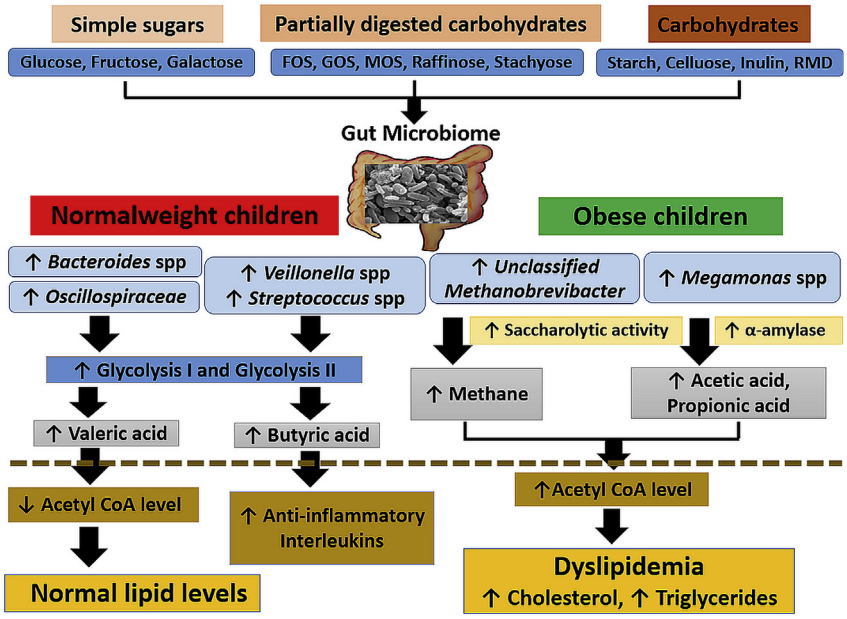

Fig. 6. A model of the gut microbiome role in metabolism of normal-weight and obese Mexican children. In our model, when simple sugars and partially digested carbohydrates reach the colon they have a different fermentation fate. In the case of normal weight children bacteria such as *Bacteroides* spp, *Veillonella* spp., *Streptococcus* spp., and members of the family *Oscillospiraceae* are more abundant (Fig. 4A), exhibiting more activity of Glycolysis I and II path abundances (Fig. 4B, Appendix A, Supplementary Table S4), and more valeric acid and butyric acid synthesis. These metabolites decrease acetyl CoA level leading to normal lipid levels by inhibition of triglyceride, and cholesterol synthesis in the host. In the children suffering of obesity, and increase in unclassified *Methanobrevibacter* (Fig. 3A), and *Megamonas* spp. (Fig. 4A) produces more methane and acetic and propionic acids respectively. This metabolite increases acetyl CoA level leading to dyslipidemia by increasing the levels of triglycerides, and cholesterol in the host. Note. FOS—-fructooligosaccharides; GOS—galactooligosaccharides; MOS—mannan-oligosaccharides, RMD, resistant maltodextrin.

of the gut microbial communities, which are significantly different between normal and obese children, affect the synthesis of some metabolites via specific routes, promoting obesity in addition to other genetic and environmental factors to which the children are exposed. We conclude that the study of MGWAS of normal weight and obese children adds useful information to understand obesity among children.

Declarations

Ethics approval and consent to participate

Informed consent was signed-out by parents and children in accordance with the Helsinki Declaration revised in 2013. All participants were informed of the scope of this study and provided their written informed consent. The research protocol was approved by the Local Ethical Committee Board of Health from the Instituto Mexicano del Seguro Social R-2011–1402 1402–10, Mexico City.

Consent for publication

Written informed consent was obtained from all parents and children.

Availability of data and materials

Raw sequence data reported in this study have been submitted to the National Center for Biotechnology Information BioProject Archive under accession no. PRJNA385215 and link is https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA385215, https://www.ncbi.nlm.nih.gov/sra?linkname=bioproject_sra_all&from_uid=385215.

Declarations of interest

None.

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Author contributions

Author contributions: JGM, SM, KN performed literature search, conceived and designed the study. SM, MLPZ, and OML collected data and conducted the experiments. SM, OML conceived developed and implemented the computational methods. JGM, CHV, LDA, SM, OML, participated in study design and interpretation. JGM, SM, KN wrote the manuscript with critical review input from all the authors.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.anaerobe.2018.10.009.

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