CENTRO DE INVESTIGACIÓN Y DE ESTUDIOS AVANZADOS DEL INSTITUTO POLITÉCNICO NACIONAL

UNIDAD ZACATENCO

DEPARTAMENTO DE FARMACOLOGÍA

"La microbiota intestinal y los marcadores de disfunción endotelial en niños y adolescentes mexicanos con obesidad"

TESIS

Que presenta

KHEMLAL NIRMALKAR

Para obtener el grado de

DOCTOR EN CIENCIAS

EN LA ESPECIALIDAD DE

FARMACOLOGÍA

Directores de Tesis

Dr. Carlos Hoyo Vadillo

Dr. Jaime García Mena

Ciudad de México **Junio, 2019**

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THESIS

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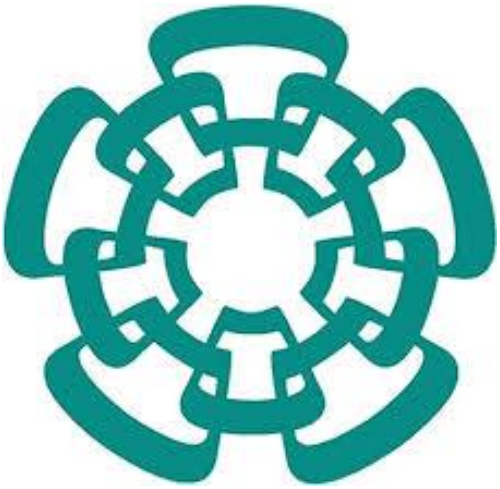
Mexico City **June, 2019**

This work has been done under the guidance of Dr. Jaime García Mena at Lab-0, Laboratorio de Referencia y Apoyo para la Caracterización de Genomas, Transcriptomas y Microbiomas, Departamento de Genética y Biología Molecular, and Dr. Carlos Hoyo Vadillo, at Lab-10, Departamento de Farmacología, Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional (Cinvestav), Unidad Zacatenco, CDMX, Mexico.



This work was financed by funding organizations CONACyT-163235 INFR-2011-01, and FONSEC SS/IMSS/ISSSTE-CONACYT-233361 and Instituto Nacional de Perinatología 212250-3310-11402-01-14. We thank Lic. Flor María Galván Rodríguez, Biol. Alberto Piña Escobedo, and Mr. Rodrigo García Gutiérrez for technical assistance. Ms. Viridiana Rosas Ocegueda for administrative assistance.

Acknowledgments

I would like to deeply thank Consejo Nacional de Ciencia y Tecnología (CONACyT), Mexico for financial support during four years of my doctorate thesis work with CONACyT scholarship number (Becario) - 589896, and CVU 707138.

Foremost, I would like to express my deepest gratitude to my thesis supervisors Dr. Jaime García Mena and Dr. Carlos Hoyo Vadillo for their continuous support, encouragement, motivation, patience, and immense knowledge. Dr. Jaime García Mena, I have been extremely lucky to have you as a supervisor who cared so much about my work, finding funds for the work, providing a great platform, and who responded to my questions, and queries so promptly during all this thesis work. I really learnt a lot during the work, thanks for explaining me about Mexican culture and social life here. Thank you so much Dr. García for your kind support academically as well as personal during the ups and down all these years. I could not have had a better supervisor. I hope, I will have your support in future as well.

Dr. Carlos Hoyo Vadillo, it has been a great privilege to have you as a mentor and thank you for giving me the opportunity to be a student of doctorate in the Departamento de Farmacología, Cinvestav. Dr. Hoyo, I would like to thank for your guidance, caring, patience, and providing me an excellent atmosphere, resources and freedom for doing research, always willing to help and giving me your best suggestions.

My sincere gratitude to Dra. María Luisa Pizano Zárate for recruiting the study subjects for this work, doing clinical studies, and for providing funds. Thanks Dra. Pizano for your great and useful suggestion during this study. It's been my great pleasure to work with you. I would like to also thank your team including Cristina García-González, Rosa María Morales-Hernández, Dr. Jorge Arturo Nuñez-Hernández, Dra. María del Socorro Romero-Figueroa for making recruitment, collecting samples, biochemical analysis and helping me with great support and suggestions. Thank you so much all of you for making this work possible. I would like to thank all children and their parents for giving permission and providing samples for this study. I deeply indebted to all the participants of this study.

I express deepest appreciation to my thesis committee members including Dra. Nora Ruiz Ordaz, Dra. Claudia Pérez-Cruz, Dr. Francisco Javier Camacho, Dr. Ranier Gutiérrez Mendoza for their suggestions, constructive comments, questions, and guidance that helped me a lot to improve my project. It has been a deeply enriching, educational and rewarding process, and for that always I will be grateful.

I am very thankful to Dr. Selvasankar Murugesan for his special contribution to this work, he has been a great mentor, companion and friend, being patience with me, teaching me basics of gut microbiota world. Thank you so much for the tips of Spanish language, Mexican culture, and making me comfortable around this city. I do not know, when I started feeling that you are like my elder brother and as a guardian, you always take me out from any trouble I got whether it is professional or personal. I thank you also for introducing Olivia and her family. My sincere gratitude to them for their warm welcome and making feel home in their home. I cannot define your contribution in words. Thank you so much for everything Selva.

My special thanks to Flor Maria Galvan Rodriguez for her extreme support and for the invaluable help. Thanks for technical assistance during work and teaching me the difference between many confusing Spanish words. Many thanks to Beatriz Cecilia Alcantara Castro for the great academic assistant of my doctorate. I would like to offer my sincere thanks to Alberto Piña Escobedo for his advice and expertise, especially for sequencing of this work; also, José Rodrigo García Gutiérrez for technical assistance and Ms. Viridiana Rosas Ocegueda for administrative assistance.

I am thankful to Dra. Igrid García González for her tips for statistical analysis. Dra. Igrid always treat me like her son, thank you so much for your immense support and love. I also thank Carolina Miranda Brito for her encouragement, explaining me Mexican culture and giving me opportunity to taste many foods like special jaggery bread.

I thank my fellow lab mates and friends Fernandote, Fernandito, Loan, Otoniel, Daniela, Marcos, Karina, Kari, Daniel, Alejandra, Cintia, Emmanuel, Arlyn. It’s been my pleasure to work with you all, thanks for helping me directly-indirectly for experiments, warm support, guiding and tips around the places, and teaching me Mexican culture and Spanish. It’s been always a friendly environment and more comfortable worked together with all of you guys.

My special gratitude to Dr. Pragya Kulkarni, Dr. Savitri Sharma, and Professor Pankaj Jain for their guidance, vision and support for making possible my doctorate journey here. More importantly I would like to thank whole heartedly to my

dear friends Sandip, Umesh, Dushyant, Tarendra, Ajay, Thirupati, Shaisav, Noopur, Rhisita, Nagveni for their great friendship. I thank Chappu for her moral support, love and friendship. You taught me many things, inspired me, always supportive and you show me another side of the world. Tumhe pata hai, kya kimat me mere life me tumhari. Shiv mere dost, thanks for your dosti, friendship and care, and supporting me financially. Neeshu and Joice, my special thanks to you guys for providing me tasty Indian food and making me happy here in Mexico. I also thank Tauqeer, for her great support and friendship, and for guiding me to come Mexico. Ye dosti hum nahi bhulenge, nahi chodenge. Gaurav, Samantha, Ishwar, Gayatri, Karthik, Digpal, Rakesh Dada, Jitu, Ranjith, John, Chandan, Prashant and their family, I thank you all guys for your friendship, and being my strength. I would like to thank all people including friends, colleagues, relatives who helped me, motivate me directly-indirectly. Thank you so much.

Finally, I wish to avail myself this opportunity to express my deepest gratitude, and love to my family; parents Mr. Kaushal Ram Nirmalkar, Mrs. Radha Nirmalkar, brother Mr. Devanand Nirmalkar, sister Mrs. Lalita Nirmalkar, and my Mama-Mami for their immense support, love, inspiration, and encouragement. My doctorate could not be possible without you all. This thesis is dedicated to you guys; words alone cannot express what I owe you all. Thank you so much god for giving me such wonderful family and for being my strength.

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Abbreviations

16S rDNA	-	16S ribosomal Deoxyribonucleic Acid
ANOVA	-	Analysis of variance
BMI	-	Body Mass Index
CRP	-	C-reactive Protein
CDC	-	Centre for Disease Control and Prevention
DNA	-	Deoxyribonucleic Acid
ELISA	-	Enzyme-linked immunosorbent assay
EDF	-	Endothelial Dysfunction
FDR	-	False Discovery Rate
HOMA-IR	-	Homeostatic Model Assessment for Insulin Resistance
HPLC	-	High Performance Liquid Chromatography
ICAM	-	Intercellular Adhesion Molecule
NIH	-	National Institute of Health
OECD	-	Organization for Economic Co-operation and Development
OTUs	-	Operational Taxonomy Units
PCR	-	Polymerase Chain Reaction
QIIME	-	Quantitative Insights Into Microbial Ecology
SCFAs	-	Short Chain Fatty Acids
TNF	-	Tumor Necrosis Factor
VCAM	-	Vascular Cell Adhesion Molecule
WHO	-	World Health Organization

Resumen

La obesidad es una enfermedad metabólica que se caracteriza por una inflamación de bajo grado que se acompaña de dislipidemia y una regulación a la alza de moléculas bioactivas, creando una predisposición a la disfunción endotelial y al síndrome metabólico. En este trabajo se estudió la asociación entre la diversidad de la microbiota intestinal y los marcadores de disfunción endotelial (DFE) en niños y adolescentes obesos de origen mexicano. Se examinaron los datos clínicos, incluidos los factores metabólicos y los marcadores de DFE en muestras de sangre. La diversidad bacteriana intestinal se caracterizó mediante la secuenciación de alto rendimiento de las bibliotecas de ADNr V3-16S. Se encontró que los niveles de triglicéridos, de insulina, la evaluación del modelo de homeostasis resistente a la insulina (HOMA-IR), la leptina, la proteína C reactiva (PCR) y la molécula de adhesión intercelular 1 marcador DFE (ICAM-1) fueron significativamente más altos en niños y adolescentes obeso en comparación con sujetos normopeso. El análisis multivariado mostró asociaciones positivas estadísticamente significativas entre la molécula de adhesión celular vascular 1 (VCAM-1) y Veillonellaceae, y entre ICAM-1 y *Ruminococcus*, en niños obesos. En adolescentes obesos, hubo una asociación positiva estadísticamente significativa entre el colesterol total y *Ruminococcus*, y entre la ICAM-1 y *Bacteroides*. El análisis de LEfSe mostró que el género *Lactobacillus* y la familia Coriobacteriaceae se enriquecieron en niños, y los géneros *Collinsella* y *Prevotella* se enriquecieron en adolescentes obesos. Los niños y adolescentes obesos tuvieron niveles más altos de resistencia a la insulina y síndrome metabólico. Estos resultados sugieren que los niños y adolescentes mexicanos con obesidad presentan niveles elevados de PCR y una reducción de la adiponectina, lo que provoca una mayor expresión de los marcadores de DFE, afectando la función endotelial y asociándose con cambios en la microbiota intestinal.

Abstract

Obesity is a metabolic disease characterized by low-grade inflammation and accompanied by dyslipidemia and up-regulation of other bioactive molecules, creating a predisposition to endothelial dysfunction and metabolic syndrome. We studied the association between gut microbiota diversity and endothelial dysfunction (EDF) markers in obese Mexican children and adolescents. We examined clinical data including metabolic factors and EDF markers in blood samples. Gut bacterial diversity was characterized by high-throughput sequencing of V3-16S rDNA libraries. Triglycerides, insulin, homeostasis model assessment-insulin resistant (HOMA-IR), leptin, C-reactive protein (CRP), and EDF marker intercellular adhesion molecule 1 (ICAM-1) were significantly higher in obese children and adolescents. Multivariate analysis showed statistically significant positive associations between vascular cell adhesion molecule 1 (VCAM-1) and Veillonellaceae, and between ICAM-1 and *Ruminococcus* in obese children. In obese adolescents, there was a statistically significant positive association between total cholesterol and *Ruminococcus*, and between ICAM-1 and *Bacteroides*. LEfSe analysis showed that the genus *Lactobacillus* and family Coriobacteriaceae were enriched in children, and genera *Collinsella* and *Prevotella* were enriched in obese adolescents. Obese children and adolescents had higher levels of insulin resistance and metabolic syndrome. These results suggest that obese Mexican children and adolescents had increased levels of CRP and a reduction of adiponectin, which causes higher expression of EDF markers, affecting endothelial function and associating with changes in the gut microbiota.

1. Introduction

1.1 Obesity

Obesity is a metabolic disorder and a serious global health issue. The World Health Organization (WHO) have defined obesity as excessive or abnormal body fat accumulation that drives a risk to health and is measured using the Body Mass Index (BMI), with a BMI >30 classified as obese for adults (WHO, 2008). In the United States, criteria for obesity in children are based on the Centers for Disease Control and Prevention (CDC) BMI-for-age growth charts. At risk for obesity is defined as having a BMI >95th percentiles of the BMI-for-age growth charts.

Obesity is a process that usually starts in childhood or adolescence and leads by an imbalance between energy intake and expenditure. It can induce complications of physical, social and emotional well-being to a child. Some of those physical complications are high cholesterol level, high blood pressure, asthma, sleep disorders and further it can cause chronic diseases, such as Type 2 Diabetes and Metabolic syndrome (Kannel *et al*., 1996; Carey *et al*., 1997; Wild et al., 2006). Obese children and adolescents are more likely to have risk factors for cardiovascular disease, such as high cholesterol or high blood pressure. In a population-based sample of 5 to 17-year-olds, 70% of obese youth had at least one risk factor for cardiovascular disease (Freedman *et al*., 2007).

1.2 Prevalence of obesity

Most of the world's population are suffering of obesity. In 2016, more than 650 million adults were obese, whereas children and adolescents (aged between 5 and 19 years) who were obese exceeded 340 million [WHO Obesity and Overweight, 2018]. The global prevalence of obesity has nearly doubled in last three decades. In 2015, 32.4% of adults in Mexico were reported as obese [Obesity Update 2017], being second only to the United States. In 2016, ENSANUT reported (Figure 1) that 15.3% of Mexican children (aged between 5 and 11 years), and 13.9% adolescents (aged between 12 and 19 years) were reported as being obese [ENSANUT, 2016]. Mexico is now just behind the United States experiencing the worst epidemic of adolescent obesity in the world (Holub *et al*., 2013).

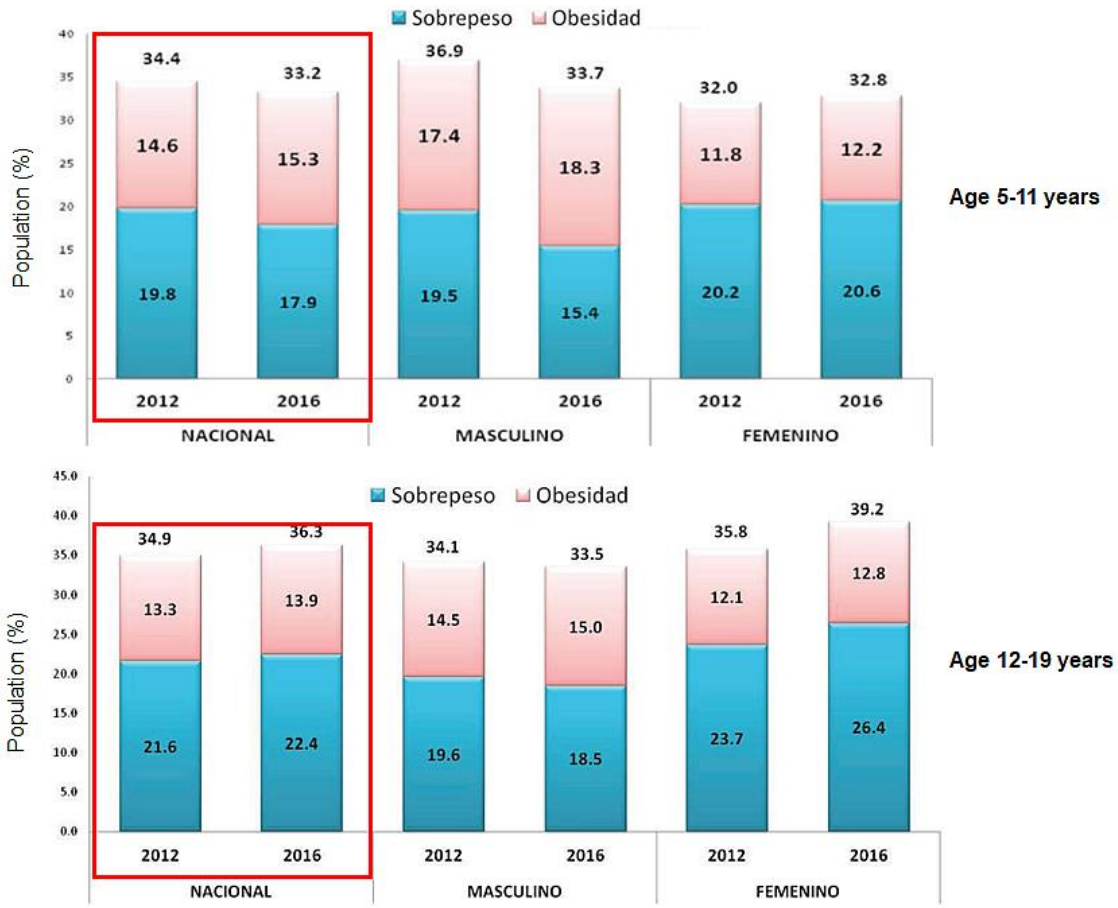


Figure 1. Obesity status in Mexican Children and adolescents**.** Figure shows the national comparative prevalence of overweight and obesity in Mexican population between 5-11 years-old children and 12-19 years-old adolescents, ENSANUT 2016, Mexico.

1.3 The human gut microbiota

Human gastrointestinal microbiota or gut microbiota is a complex community of microorganisms that live in the digestive tracts of humans. It is estimated that 100 trillion microorganisms reside in our gut [Ley *et al*., 2006]. Gut microbiota is composed of bacteria, archaea, viruses, and eukaryotic microbes. Among them, bacteria are the major proportion, and comprise Firmicutes and Bacteroidetes as the most abundant phyla members, and Actinobacteria, Verrucomicrobia and Proteobacteria being the least abundant [Marchesi *et al*., 2016]. Gut microbiota has a remarkable potential to influence our physiology, both in health and in disease. Healthy gut microbiota provides several health benefits to the host such as protection from pathogens, production of nutrients and vitamins, host metabolism,

and immune modulation [Jandhyala *et al*., 2015]. Diet is the essential link between gut microbiota composition and metabolism [Jandhyala *et al*., 2015]. These significant characteristics of the gut microbiota drive the focus of research to study functional aspects of microbial diversity in host health.

The gut microbiota harvests energy from dietary fiber through fermentation, producing short-chain fatty acids (SCFAs) such as acetate, propionate, and butyrate, and influencing host energy metabolism [Turnbaugh *et al*., 2006; Murugesan *et al*., 2018]. The molar ratio of SCFAs acetate, propionate, and butyrate is 60:20:20 in the colon, and this ratio varies from the caecum to the descending colon [Cummings *et al*., 1987]. Gut bacteria such as *Akkermansia muciniphila*, *Bacteroides* spp., *Bifidobacterium* spp., *Blautia* spp. are acetic acid producers [Louis *et al.*, 2010; Rey *et al.*, 2010], while *Bacteroides* spp., *Dialister* spp., *Coprococcus catus*, *Roseburia inulinivorans* are propionic acid producers [Scott *et al*., 2006]. On the other hand, *Anaerostipes* spp., *Coprococcus catus*, *Eubacterium rectale*, *Faecalibacterium prausnitzii*, *Roseburia* spp. are butyric acid-producing bacteria [Duncan *et al*., 2002].

1.4 The gut microbiota and obesity

Obesity makes many physiological changes in human body and influences the gut microbiota. Primarily it has been reported in genetically obese mice that the gut microbiota is associated with obesity [Ley *et al*., 2006], and is mainly dominated by two bacterial phyla; Firmicutes and Bacteroidetes, of which Firmicutes is more abundant in obese individuals (Figure 2) [Ley *et al*., 2006]. They next demonstrated that colonization of obese *ob/ob* mice with a cecum-derived microbiota from conventional mice produces a 60% increase in body fat mass within 2 weeks. The increase in body fat was accompanied by insulin resistance, adipocyte hypertrophy, and increased levels of circulating leptin and glucose. In addition, obese *ob/ob* mice were reported to show higher amounts of SCFAs in the caecum and less in their feces in comparison to their lean littermates and increases the capacity for energy harvest [Ley *et al*., 2006; Turnbaugh *et al*., 2006]. Moreover, it has been reported that alterations in the composition and metabolic capacity of gut microbiota in

obesity promotes the adiposity and influence the metabolic processes in peripheral organs, such as the control of satiety in the brain; the release of hormones from the gut and the synthesis, storage or metabolism of lipids in the adipose tissue, liver and muscle (Figure 2). Microbial molecules also increase intestinal permeability, leading to systemic inflammation and insulin resistance [Tremaroli & Bäckhed, 2012].

Similarly, it has been also found that the gut microbiota is associated with obesity in Mexican children; phyla Firmicutes was more abundant, genera like *Faecalibacterium*, *Blautia*, *Coprococcus*, *Roseburia* were dominant in overweight or obese Mexican children compared to control subjects [Murugesan *et al*., 2015]. On the other hand, gut microbiota has been also associated with obesity plus metabolic syndrome in Mexican women; genera *Coprococcus*, *Faecalibacterium*, *Megamonas*, *Ruminococcus* were more dominant than control group [Chávez-Carbajal *et al*., 2019]. The first Microbial Genome-Wide Association Studies (MGWAS) of obese Mexican children revealed an archaeal genus *Methanobrevibacter* spp. and a bacterial genus *Megamonas* spp. is associated with obesity and dominated by Enterotype 2 bacteria *Prevotella* spp. in Mexican children [Maya-Lucas *et al*., 2019].

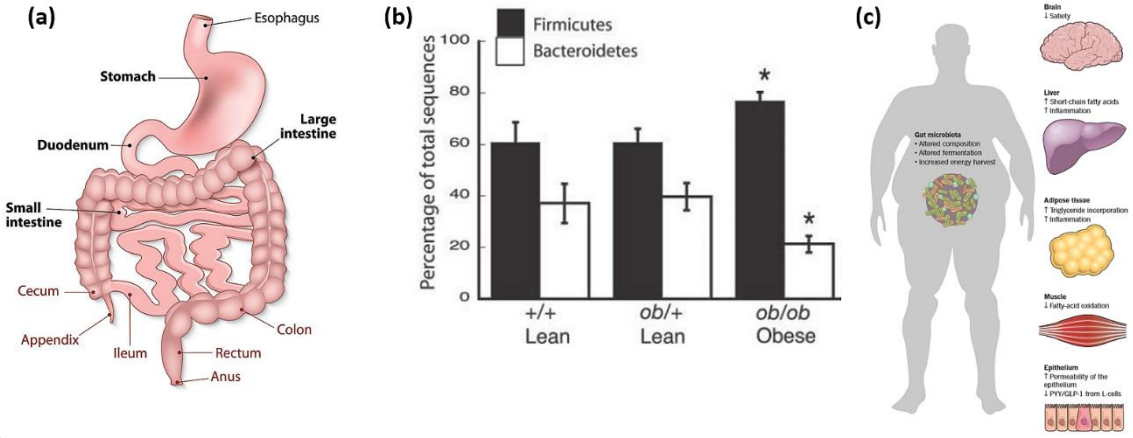


Figure 2. Overview of gut microbiota and obesity. Figure (a) shows the anatomy of human gastrointestinal tract; (b) shows the comparative study of gut microbiota between genetically obese *ob/ob* mice and lean mice; (c) explains the obesity alters the gut microbiota, increases the energy harvest in the body and causes inflammation. Adapted from Michigan University health care webpage; Ley *et al*., 2006; Tremaroli & Bäckhed, 2012.

1.5 The gut microbiota and atherosclerosis

Atherosclerosis is an inflammatory disease, caused by an accumulation of cholesterol in particular of low-density lipoprotein (LDL) cholesterol, and inflammation reaction by immune cells like macrophages and dendritic cells, which promote the formation of atherosclerotic plaques into artery wall and it may lead to myocardial infarction and stroke [Ross, 1999]. It has been reported that obesity is one of the causing factors for atherosclerosis [Yamaoka-Tojo, 2017]. Interestingly, it has been found that gut microbiota is also associated with atherosclerosis. Shotgun sequencing of the gut metagenome revealed that the genus *Collinsella* was enriched in patients with atherosclerosis, whereas *Roseburia* and *Eubacterium* were enriched in healthy Swedish controls. Furthermore, they found that the patients were enriched in genes encoding peptidoglycan synthesis and reduced in serum levels of beta-carotene [Karlsson *et al*., 2012]. In addition, gut and plaque study of atherosclerosis suffering Swedish adults, showed higher abundance of *Chryseomonas*, *Veillonella*, and *Streptococcus, Ruminococcus, Bacteroides* than controls (Koren *et al*., 2011). Recent findings have revealed that the microbial metabolism of dietary choline to betaine and trimethylamine, which can be further metabolized in the liver to trimethylamine N-oxide, strongly correlates with atherosclerosis and cardiovascular disease (Tang *et al*., 2013). These observations suggest that it may be possible to develop strategies to prevent atherosclerotic events based on the gut microbiota.

1.6 The gut microbiota and endothelial dysfunction (EDF)

Endothelial dysfunction (EDF) is an impairment of vasodilatation/vasoconstriction, or diminished availability of nitric oxide (NO) [Hadi *et al*., 2005]. EDF leads to the up-regulation of Reactive Oxygen Species (ROS), C-reactive protein (CRP), and stimulates the secretion of primary proinflammatory cytokines, such as interleukin (IL-1) and tumor necrosis factor-alpha (TNF-α) [Hadi *et al*., 2005; Gomes *et al*., 2010]. These cytokines enhance the expression of adhesion molecules such as intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), and E-, L-, and P-selectins in the endothelial cells [Gomes *et al*., 2010], with blood thickening and formation of small plaques

(Figure 3). Subsequently, EDF may cause atherosclerosis and other cardiovascular diseases [Hadi *et al*., 2005].

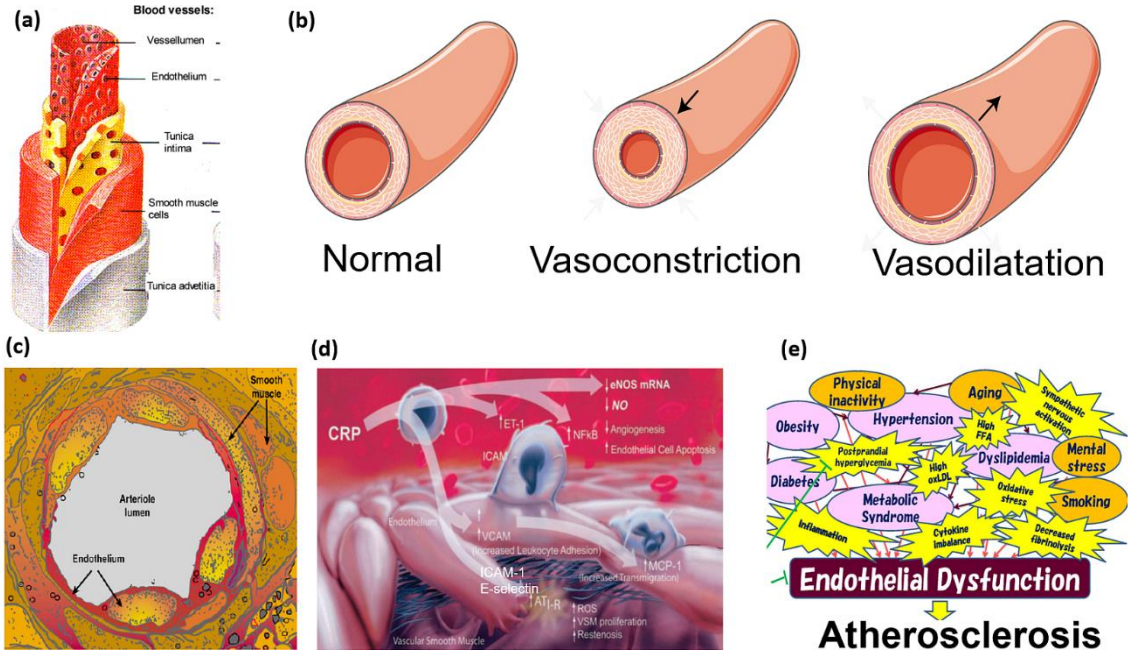


Figure 3. Outline of endothelial dysfunction (EDF) and their causing factors. Figure (a) explains the anatomy of blood vessels, showing endothelium layer, tunica intima *etc*.; figure (b) shows the normal, vasoconstriction and vasodilatation process of blood vessel; (c) shows the inflammation in endothelium layer; (d) explain the role of inflammatory marker and EDF markers in developing of EDF. In the state of inflammation C-reactive protein (CRP) up-regulates, and stimulates the secretion of primary proinflammatory cytokines, subsequently leads the expression of Intercellular adhesion molecule 1 (ICAM-1), Vascular cell adhesion molecule 1 (VCAM-1), and cause immune reaction with macrophages and dendritic cells, causes cell apoptosis with blood thickening and formation of small plaques, which can lead atherosclerosis; (e) shows the causing factors of EDF and atherosclerosis. This Figure 3 adapted from Lerman *et al*., 1992; Szmitko *et al*., 2003; Davignon & Ganz, 2004; Yamaoka-Tojo, 2017.

EDF can be diagnosed by the gold standard method of angiography with acetylcholine injection [Ludmer *et al*., 1986], the Flow Mediated Dilation (non-invasive) method (FMD) [Korkmaz & Onalan, 2008], or by measurement of EDF markers such as VCAM-1, ICAM-1, and E-selectin [López-García *et al*., 2004; Eikemo *et al*., 2004]. In Mexico, around 29.8% of children (aged between 3 and 17 years) have EDF [Madrigal *et al*., 2011]. EDF is also an early marker for atherosclerosis [Davignon & Ganz, 2004]; atherosclerosis was observed in 53% of autopsies in Mexico during 2005–2007 [Rodríguez-Saldaña *et al*., 2014].

Obesity was reported to be associated with endothelial dysfunction [Meyers & Gokce, 2007], and atherosclerosis is also associated with endothelial dysfunction [Schächinger & Zeiher, 2000]. In addition, human gut microbiota is associated with obesity [Ley *et al*., 2006] and some specific members of the gut microbiota found in the feces of atherosclerosis patients are also found in their plaques [Koren *et al*. 2011; Karlsson *et al*., 2012].

These reports suggest that the gut microbiota may be associated with EDF or EDF markers. Diet is an important factor modulating microbial diversity. It was also reported that high fat diet is associated with obesity, whereas fiber-rich diet can reduce the risk of obesity [Du & Feskens, 2010]. As mentioned above, obesity and EDF prevalence are 15.3% and 29.8%, respectively, in Mexican children, and 5–17-year-old obese children have a higher risk of cardiovascular disease [Freedman *et al*., 2007].

To the best of our knowledge, there is no published report about the association between EDF markers and gut microbiota in any population. Therefore, we aimed to investigate whether there is an association between EDF markers and the intestinal microbiota in obese Mexican children and adolescents [Nirmalkar *et al.*, 2017]. We summarized our aim as a model in Figure 4.

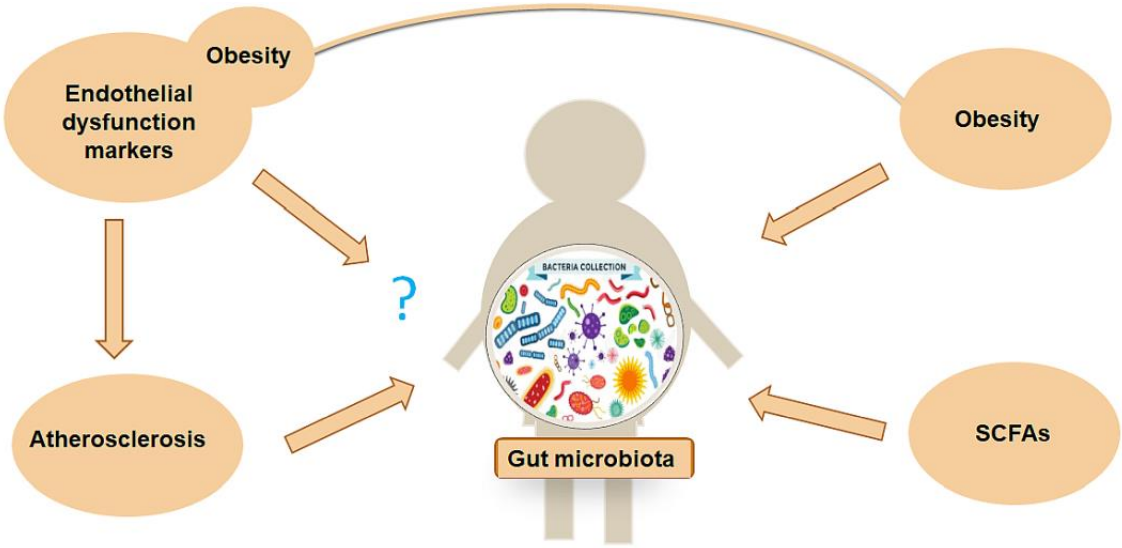


Figure 4. A model of association between gut microbiota and endothelial dysfunction markers with obesity.

2. Rationale

Endothelial dysfunction is a predisposing factor for atherosclerosis and for cardiovascular diseases. To our knowledge, there is no study about the association between EDF markers and gut microbiota in any population. We believe that understanding gut microbiota changes associated to endothelial dysfunction markers in obese subjects may reveal possible connections with potential use for medical therapies. Therefore, we address this issue in Mexican obese children and adolescents, characterizing their gut microbiota profile along with EDF markers. This study can make a path for the futurist clinician to treat the endothelial dysfunction patient, also it will make a platform for researchers to investigate about the different factors for vascular and heart diseases.

3. Hypothesis

Obese Mexican children and adolescents will show higher relative abundance of Firmicutes in their gut microbiota and less concentration of SCFAs in in fecal samples, and higher levels of endothelial dysfunction (EDF) markers in blood with respect to normal weight Mexican children and adolescents.

4. Objective

The general objective of this work was to investigate the association between gut microbiota composition and endothelial dysfunction markers in obese Mexican children and adolescents. This general objective is accomplished through the following specific objectives:

4.1 Specific objective 1.

To select study subjects based on their anthropometric data, divide them into children and adolescent's category with normal weight or obesity. Characterize the clinical parameters including metabolic factors, adipokines and inflammatory markers from blood of all individuals.

4.2 Specific objective 2.

To characterize and quantify the concentration of SCFAs using High Performance Liquid Chromatography (HPLC) from fecal samples.

4.3 Specific objective 3.

To extract the genomic DNA of bacteria from fecal samples and prepare 16S rDNA libraries for high throughput sequencing. Determine the presence EDF markers in blood samples.

4.4 Specific objective 4.

To analyze the sequencing data, describe the gut microbiota diversity and find the potential association of microbiota with clinical parameters including with EDF markers in children and adolescents with obesity.

5. Subjects

In this work, association of gut microbiota with endothelial dysfunction markers is studied in obese Mexican children and adolescents. Children and adolescents categorized into two group, normal weight and obese of both sexes and 172 in total number for each type of samples, blood and feces were collected separately, from Instituto Nacional de Perinatología, CDMX, Mexico.

6. Materials and methods

This work has been divided into the following phases (Figure 5)

6.1 Phase 1. Selection of study subjects, clinical studies and SCFAs analysis

Activity 1.1 Selection of Mexican children and adolescents

A total of 172 individuals was selected, including children (n = 111) between the age of 6 and 11 years, and adolescents (n = 61) between the age of 12 and 18 years. These individuals were divided into two groups: Children; normal weight (n = 49) and obese individuals (n = 62), and Adolescents; normal weight (n = 27) and obese individuals (n = 34).

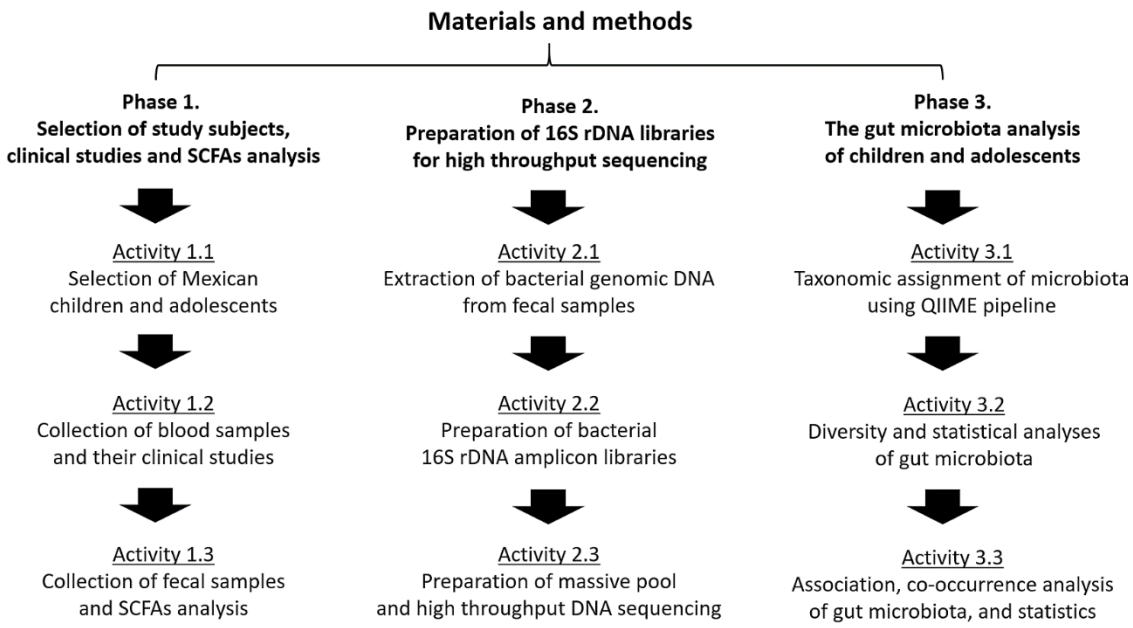


Figure 5. Flow-chart of the work of this thesis.

Subjects were selected from three different Mexican public schools: Escuela Juan Fernández Albarrán, Centro Escolar Lázaro Cárdenas, Secundaria Técnica Tierra y Libertad, and one hospital (220 IMSS, Instituto Mexicano del Seguro Social), located in the city of Toluca, Mexico. All individuals were interviewed and screened by a certified pediatrician for inclusion and exclusion of participants. All selected participants were healthy with no gastrointestinal diseases or probiotics use in the previous 3 months. The exclusion criteria were: Chronic diseases, smoking, pregnancy, allergies, thyroid disease, eating disorders, consumption of any supplement, atherosclerotic cardiovascular disease, and administration of oral antibiotics in the previous 3 months. Informed consent was obtained from all participants and their parents in accordance with the Helsinki Declaration revised in 2013. The protocol was approved by the Research and Ethical Committee Boards of the Instituto Nacional de Perinatología, 212250-3310-11402-01-14, Mexico City (see Appendix A5).

Systolic blood pressure (SBP), diastolic blood pressure (DBP), weight, height, and waist circumference (WC) were measured. Body mass index (BMI) and BMI percentile were calculated and classified based on the World Health

Organization (WHO) norms and calculated as weight (kg)/height2 (m2) [de Onis *et al*., 2007]. According to this, individuals were classified into two groups: Normal weight (BMI < 85th percentile) and obese individuals (BMI ≥ 95th percentile).

Diversity in the diet intake of all participants was assessed using a 7-day dietary recall survey applied by certified dietitians. Diet intake was divided into seven food groups as follows: (1) Starchy staples, (2) legumes, (3) dairy, (4) meat, (5) vitamin-A rich fruits and vegetables, (6) other fruits and vegetables or fruit juices, and (7) foods made with oil, fat, or butter. Food groups that were consumed ≥3 days by each participant in the previous week received a score of 1, and those food groups that were consumed <3 days by each participant in the past week were scored 0. A final score was calculated for each participant adding the values of all the consumed food groups. Thus, a score of 7 was the maximum possible value as previously described [Murugesan *et al*., 2015].

Activity 1.2 Collection of blood samples and their clinical studies

Blood samples were collected from each participant after 12 h of fasting in a vacutainer (BD, Mississauga, Canada) rapid serum tube. Fasting glucose, total cholesterol, high-density lipoprotein (HDL), and triglycerides (enzymatic colorimetric; Diasys, Holzheim, Germany) were analyzed using an automatic analyzer (LORY 2000, Diasys; Diagnostic Systems GmbH, Holzheim, Germany). Friedewald formula was used to calculate low-density lipoprotein (LDL) [Friedewald *et al*., 1972]. C-reactive protein (CRP), insulin, and interleukins were measured by chemiluminescence (Immulite 1000; Siemens Health Care Diagnostic, (Malvern, PA, USA). Leptin and adiponectin concentrations were quantified with the Enzyme-Linked ImmunoSorbent Assay (ELISA), sandwich type (R&D Systems, Minneapolis, MN, USA) [Perichart-Perera *et al*., 2017]. Homeostasis model assessment-insulin resistant (HOMA-IR) was calculated using the formula: Fasting insulin (mU/mL) × fasting glucose (mmol/L)/22.5 for all participants [Aradillas-García *et al*., 2012; Matthews *et al*., 1985]. The cut-off value was 2.89 ± 0.7 [Aradillas-García *et al*., 2012]. A participant was classified as having metabolic syndrome (MetS), if they had at least 3 out of 5 criteria, including WC ≥ 75th percentile; triglycerides ≥ 100 mg/dL;

HDL < 50 mg/dL for children, or <45 mg/dL for adolescents; glucose ≥ 100 mg/dL, and blood pressure (SBP/DBP) > 90th percentile for the age and sex categories. These criteria were considered for Mexican children and adolescents based on previous reports [Burguete-García *et al*., 2014; NCEP report 1992; Weiss *et al*., 2004; Magge *et al*., 2017].

The concentration of EDF markers including sVCAM-1 (soluble vascular cell adhesion molecule, Cat. #DVC00), sICAM-1 (soluble Intercellular adhesion molecule, Cat. #DCD540), and E-selectin (Cat. #DSLE00) were measured in serum of all individuals [Eikemo *et al*., 2004], using quantitative immunoassay technique kit (R&D Systems, Minneapolis, MN, USA).

Activity 1.3 Collection of fecal samples and SCFAs analysis

Fecal samples from both children and adolescents were collected aseptically in a sterile stool container at home in the morning. Once received, samples were immediately transported to the laboratory in cold boxes with ice-gel-packs previously cooled at −70 °C. Samples were aliquoted in multiple tubes and instantly stored at −70 °C.

SCFAs were measured in 100 mg of dehydrated fecal samples using PerkinElmer-Flexar (Waltham, MA, USA) high performance liquid chromatography (HPLC) equipment as previously reported [Murugesan *et al*., 2015]. Mobile phase consisted of two solutions; 80% of (A) 20mM NaH_2PO4 (Sigma-Aldrich Cat. #S8282, St. Louis, MO, USA) pH 2.2 adjusted with phosphoric acid (J.T. Baker, State of Mexico, Xalostoc, Mexico, Cat. #0260-05), and 20% of (B) Acetonitrile (J.T. Baker, Cat. #9012-03, State of Mexico, Xalostoc, Mexico), using a 1.0 mL/min flow rate in a 15 cm C-18 column [de Baere *et al*., 2013]. All chromatographic data were processed using Chromera (v4.1.2.6410, PerkinElmer, Waltham, MA, USA)—HPLC Flexar Software (PerkinElmer, Waltham, MA, USA).

6.2 Phase 2. Preparation of 16S rDNA libraries for high throughput sequencing

Activity 2.1 Extraction of bacterial genomic DNA from fecal samples

DNA was extracted from 100 mg of fecal sample of all individuals using stool kit method (Favorgen Biotech Corp., Ping-Tung, Taiwan; Favor prep stool kit, Cat. #FASTI001-1). DNA concentration was measured by NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA) and quality evaluated by 0.5% agarose gel electrophoresis.

Activity 2.2 Preparation of bacterial 16S rDNA amplicon libraries

For each fecal DNA a polymerase chain reaction (PCR) was performed and obtained an amplicon of approximately 281 bp containing the V3 polymorphic region of the bacterial 16S rDNA (Figure 6). V3 region was amplified using a sense V3-341F primer containing a 12 bp Golay barcode [Murugesan *et al*., 2015], an A–adapter for massive sequencing in Ion Torrent PGM (Life Technologies), and an antisense V3-518R containing Truncated P1 (TrP1)–adapters [Murugesan *et al*., 2015]. The PCR thermocycler program was 5 min at 95° C; 25-cycles of 15 s at 94° C; 15 s at 62° C; and 15 s at 72° C; followed by 10 min at 72° C. Amplification was carried out using GeneAmp PCR System 2700 Thermocycler (Applied Biosystems).

Activity 2.3 Preparation of massive pool and high throughput DNA sequencing

For high throughput sequencing, equivalent amounts (10 µg each) of amplicons, were mixed in groups of 50 individuals, regardless of their normal weight or obese phenotype of children and adolescents. Each massive pool of 16S rDNA V3 libraries were fractionated by electrophoresis in 2% agarose gel, cut and purified using Wizard SV Gen PCR Clean-Up System (Promega). The DNA concentration of each library was measured by Nanodrop (Thermo Scientific). From the concentration and the average size of each amplicon library, the amount of DNA fragments per microliter was calculated using Agilent Bioanalyzer 2100, and libraries for each run were diluted to 22 pM prior to clonal amplification. Emulsion PCR was carried out using the Ion OneTouchTM 200 Template Kit v2 DL (Life Technologies) according to the manufacturer's instructions. Amplicon enrichment with ion spheres was done using Ion One Touch ES kit. The sequencing was made using Ion 318 v2 Chips and Ion Torrent PGM sequencing system (Figure 6). After sequencing, reads

were filtered by the PGM software to remove low quality and polyclonal sequences. During this process sequences matching the 3'- adapter were automatically trimmed and filtered [Murugesan *et al*., 2015]. Sequences were submitted to National Center for Biotechnology Information (NCBI) BioProject database with accession number PRJNA433269 and can be accessed through the following link: https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA433269.

6.3 Phase 3. The gut microbiota analysis of children and adolescents

Activity 3.1 Taxonomical assignment of gut microbiota using QIIME pipeline

After high throughput sequencing, Ion torrent PGM software Torrent Suite v4.0.2 was used to demultiplex the sequenced data based on their barcodes for each phenotypic category including normal weight and obese children and adolescents. Poor quality reads were eliminated from the datasets such as quality score <20, containing homopolymers >6, length <200 nt, and containing errors in primers and barcodes. Filtered data were exported as FASTQ format files. Demultiplexed sequencing data were analyzed using Quantitative Insights Into Microbial Ecology (QIIME) software v1.9.0 pipeline. FASTQ files were converted into FASTA files, and open reference Operational Taxonomic Units (OTUs) were picked at 97% similarity level against the Greengenes database v13.8. Subsequently, gut microbiota population were calculated in the form of relative abundance for all children and adolescents with respect to their phenotypic category.

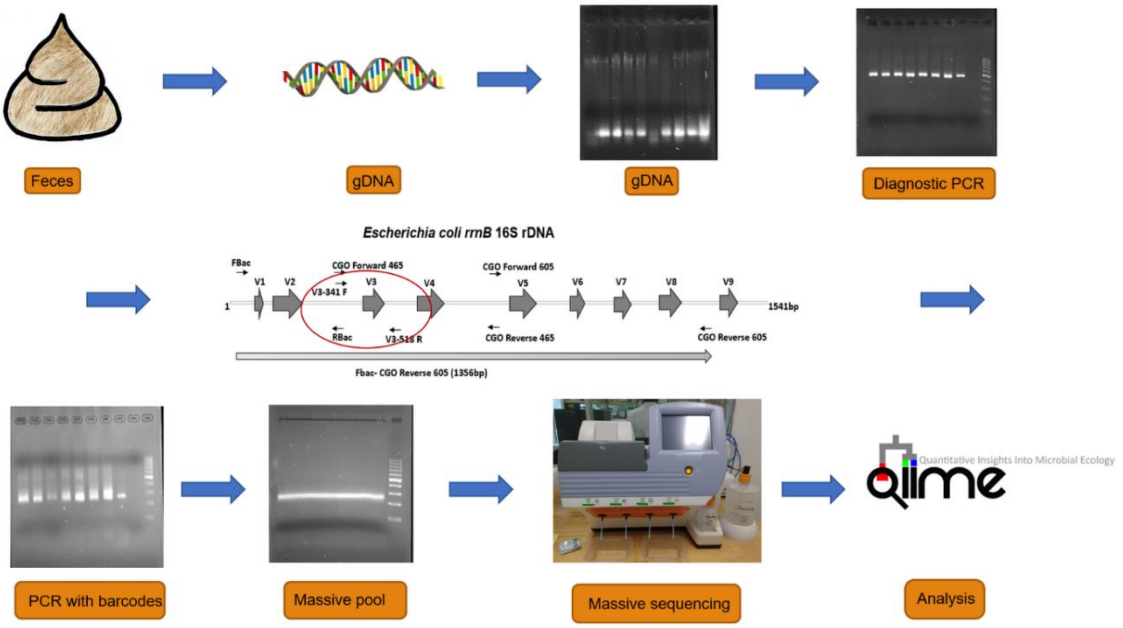


Figure 6. Outline of bacterial V3-16S rDNA high throughput sequencing using Ion Torrent PGM sequencer.

Activity 3.2 Diversity and statistical analyses of gut microbiota

To evaluate the alpha diversity of gut microbial communities, we calculated Shannon, Simpson, and Chao1 indexes and observed species using phyloseq (vegan (v2.2.1), and ggplot2 packages) in the R environment (v3.3.3.). To assess the beta-diversity, dissimilarity index was calculated by UniFrac distance metric, and visualized by principal coordinate analysis as previously described [Murugesan *et al*., 2015]. Core microbiota analysis was performed using QIIME pipeline (v1.9.0). Linear discriminant analysis (LDA) effect size (LEfSe, v1.0) was used to elucidate significantly different relative abundances of bacterial taxa, associated with both children and adolescents. These analyses are presented in a bar plot and the parameters set with default *p*-value, α = 0.05, and an LDA score of 2.0 with LEfSe [Segata *et al*., 2011].

Activity 3.3 Association, co-occurrence analysis of gut microbiota, and statistics

Multivariate association with linear models (MaAsLin, v0.0.4) was performed to investigate the associations between taxa abundances and clinical metadata using default parameters in R. These analyses were used to explore associations

between figures reporting *p*- and *q*-values. The false discovery rate (FDR) (*q*-value) was calculated using the Benjamini-Hochberg method to avoid the inclusion of false positives [Benjamini & Hochberg, 1995]. *p*-values less than 0.05 and *q*-values less than 0.25 were considered significant [Morgan *et al*., 2012].

Co-occurrence analysis was performed using *otu_table.biom* files in CoNet (Co-occurrence Network Inference) plugin tool [Faust & Raes, 2016], and generated co-occurrence networks were visualized and analyzed in Cytoscape (v3.6.1) software. To avoid false positive results, corrections were made using the Benjamini-Hochberg method (*q*-value). *p*- and *q*-values < 0.05 were considered statistically significant, and correlations analysis (Pearson/Spearman) were sorted for statistically significant ($p < 0.05$) and $R > 0.8$.

The clinical characteristics of all individuals including anthropometric parameters, metabolic factors, EDF markers, SCFAs analysis, and other characteristics were statistically calculated using one-way analysis of variance (ANOVA), and the Mann–Whitney U test. Clinical data are expressed in means ± standard error. Sequencing data were analyzed using Quantitative Insights Into Microbial Ecology (QIIME) pipeline (v1.9.0). Operational taxonomic units (OTUs) picking was run against the Greengenes (v13.8) database. Images were plotted using ggplot2 and RcolorBrewer (v1.1-2) packages. To correct the *p*-values, multiple testing correction [Benjamini & Hochberg, 1995] was performed using the *p.adjust* function in R to avoid the inclusion of false positives, including alpha-diversity and bacterial relative abundance. Gut bacterial diversity (alpha-diversity) was assessed with phyloseq, and vegan (v2.2-1) packages in R (v3.3.3).

7. Results

7.1. Obese children and adolescents have different clinical characteristics than normal weight

We studied a total of 172 individuals divided into two groups: 111 children (6–11 years-old) and 61 adolescents (12–18 years-old). Obese children and adolescents weighed significantly more (both, $p < 0.001$), had higher BMI

percentiles (both, $p < 0.001$), larger WC (both, $p < 0.001$), and higher WC percentiles (both, $p < 0.001$) with respect to normal-weight children and adolescents. In addition, SBP (children, $p = 0.010$; adolescents, $p < 0.001$), and DBP (both, $p = 0.048$) were significantly higher in obese individuals. However, their percentiles were not statistically significant, except the SBP percentile of adolescents (Tables 1 and 2).

Obese children and adolescents had significantly higher triglycerides (both, $p < 0.001$) and leptin (both, $p < 0.001$) levels and lower HDL-cholesterol only in obese children ($p < 0.001$), whereas total cholesterol was higher only in obese adolescents ($p = 0.041$). Obese children and adolescents had significantly higher CRP (children, $p < 0.001$; adolescents, $p = 0.022$) and insulin (both, $p < 0.001$), but similar fasting glucose levels (children, $p = 0.223$; adolescents, $p = 0.345$). Only obese children had higher TNF-α ($p = 0.006$) but lower levels of adiponectin ($p = 0.019$). The measurement of EDF markers in the participants showed an increase in E-selectin (both, $p < 0.001$), and ICAM-1 was significantly increased only in obese adolescents ($p < 0.001$). Only the short-chain fatty acid propionic acid was significantly lower among obese children ($p = 0.027$). The 7-day recall dietary profile did not show any significant difference between normal weight and obese individuals (Table 3).

Children and adolescents were classified as having metabolic syndrome (MetS), as they fulfilled three out of five criteria including: WC ≥ 75th percentile, triglycerides ≥ 100 mg/dL, and HDL < 50 mg/dL for children or HDL < 45 mg/dL for adolescents (Tables 1 and 2). Overall, 15 out of 34 obese children (44.1%) and 30 out of 62 obese adolescents (48.4%) were affected by MetS.

Table 1. Clinical characteristics of 6 – 11 years old children.

Characteristics	Normal weight	Obesity	*P*-value
Number (F/M)	49 (30/19)	62 (27/35)	Nd
Age (years)	9.14 ±0.22	9.50 ±0.18	0.146 $^{\mu}$
Age range (years)	7 to 11	6 to 11	Nd
Anthropometric			
Weight (kg)	31.49 ±1.02	48.54 ±1.37	<0.001 $^{\mu}$
Height (m)	1.35 ±0.01	1.39 ±0.01	0.094 $^{\Omega}$
BMI (kg/m^2)	16.89 ±0.21	24.90 ±0.42	<0.001 $^{\mu}$
BMI pc	57.44 ±2.71	98.78 ±0.18	<0.001 $^{\mu}$
BMI pc scale	<85	>95	Nd
WC (cm)	59.53 ±0.68	80.55 ±1.29	<0.001 $^{\mu}$
WC pc	59.46 ±3.78	97.47 ±0.72	<0.001 $^{\mu}$
Blood pressure			
SBP (mm Hg)	88.42 ±3.64	100.61 ±1.78	0.010 $^{\mu}$
SBP pc	33.20 ±3.69	38.96 ±3.53	0.312 $^{\mu}$
DBP (mm Hg)	59.44 ±2.66	65.56 ±1.42	0.048 $^{\mu}$
DBP pc	56.56 ±3.87	64.54 ±2.86	0.123 $^{\mu}$
Metabolic factors			
Fasting glucose (mg/dL)	89.04 ±0.99	91.32 ±1.33	0.223 $^{\mu}$
Triglycerides (mg/dL)	84.64 ±5.30	119.73 ±7.74	<0.001 $^{\mu}$
Total cholesterol (mg/dL)	162.88 ±3.45	166.62 ±2.70	0.651 $^{\mu}$
HDL (mg/dL)	53.25 ±1.57	46.34 ±2.16	<0.001 $^{\mu}$
LDL (mg/dL)	92.70 ±2.82	96.34 ±2.67	0.394 $^{\mu}$
Insulin (µIU/mL)	6.49 ±0.61 $^{\circ}$	13.53 ±1.42	<0.001 $^{\mu}$
Leptin (ng/mL)	6.71 ±0.69 $^{¥}$	25.11 ±1.98	<0.001 ^{b}
HOMA-IR	1.44 ±0.14	3.12 ±0.33	<0.001 $^{\mu}$
HOMA-β (%)	91.89 ±8.86	148.49 ±34.61	<0.001 $^{\mu}$
Adipokines			
CRP (mg/L)	1.03 ±0.21 *	4.05 ±0.87 **	<0.001 $^{\mu}$
IL-1β (pg/mL)	2.40 ±0.10 $^{\alpha}$	2.58 ±1.55 $^{\alpha\alpha}$	0.879 $^{\Omega}$
IL-6 (pg/mL)	2.83 ±0.36 $^{\beta}$	3.03 ±0.23 $^{\beta\beta}$	0.192 $^{\Omega}$
TNF-α (pg/mL)	12.45 ±0.73 $^{€}$	16.32 ±1.70 $^{€€}$	0.006 $^{\Omega}$
Adiponectin (µg/mL)	13.86 ±0.81 $^{£}$	11.24 ±0.61	0.019 $^{\Omega}$
Endothelial dysfunction markers			
VCAM-1 (ng/mL)	761.73 ±31.60	789.37 ±34.93	0.771 $^{\Omega}$
ICAM-1 (ng/mL)	205.36 ±8.62	246.81 ±19.16	0.137 $^{\Omega}$
E-selectin (ng/mL)	58.09 ±4.53	175.88 ±71.52	<0.001 $^{\Omega}$
Fecal SCFAs			
Acetic acid (mM/100mg)	237.84 ±20.19	221.80 ±17.14	0.489 $^{\Omega}$
Propionic acid (mM/100mg)	14.57 ±1.91	11.68 ±2.07	0.027 $^{\Omega}$
Butyric acid (mM/100mg)	13.81 ±1.44	12.18 ±1.08	0.289 $^{\Omega}$

The results appear like mean ± standard error. *P* -values were calculated according to: µ Mann-Whitney U test for unequal variances, Ω One-way ANOVA for equal variance. *P* <0.05 is considered statistically significance. Abbreviations are F – female, M – male, BMI - body mass index, WC – waist circumference, SBP – systolic blood pressure, DBP – diastolic blood pressure, HDL - high-density lipoprotein, LDL - low-density lipoprotein, HOMA-IR – homeostasis model assessment-insulin resistant, , HOMA-β - homeostasis model assessment-beta cell function, CRP - C-reactive protein, IL – interleukin, TNF - tumor necrosis factor, VCAM - vascular adhesion molecule, ICAM - intercellular adhesion molecule, SCFAs – short chain fatty acids, pc - percentile, Nd - not determined. Different symbols show the number of participants for the data: $^{\circ}$48, $^{¥}$48, *35, $^{\alpha}$25, $^{\beta}$25, $^{€}$25, $^{£}$48, out of 49 for Normal weight; or **56, $^{\alpha\alpha}$41, $^{\beta\beta}$41, $^{€€}$42, out of 62 for Obesity. WC pc was adjusted according to sex and age.

Table 2. Clinical characteristics 12 - 18 years old adolescents.

Characteristics	Normal weight	Obesity	*P*-value
Number (F/M)	27 (12/15)	34 (18/16)	Nd
Age (years)	13.00 ±0.28	13.61± 0.28	0.254 $^{\mu}$
Age range (years)	12 to 16	12 to 18	Nd
Anthropometric			
Weight (kg)	44.83 ±1.54	69.43± 2.46	<0.001 $^{\Omega}$
Height (m)	1.52 ±0.01	1.55± 0.01	0.224 $^{\Omega}$
BMI (kg/m^2)	19.25 ±0.42	28.78± 0.83	<0.001 $^{\Omega}$
BMI pc	52.60 ±4.34	98.01 ± 0.36	<0.001 $^{\mu}$
BMI pc scale	<85	>95	Nd
WC	68.91 ±1.27	88.68 ±1.57	<0.001 $^{\mu}$
WC pc	65.59 ±5.53	97.93 ±0.76	<0.001 $^{\mu}$
Blood pressure			
SBP (mm Hg)	93.84 ±2.22	104.48 ±1.73	<0.001 $^{\mu}$
SBP pc	20.49 ±3.90	36.51 ±4.73	0.021 $^{\mu}$
DBP (mm Hg)	63.12 ±1.41	68.30 ±1.77	0.048 $^{\mu}$
DBP pc	51.20 ±3.89	62.52 ±4.50	0.074 $^{\mu}$
Metabolic factors			
Fasting glucose (mg/dL)	91.45 ±2.07	92.89 ±1.68	0.345 $^{\mu}$
Triglycerides (mg/dL)	91.14 ±5.67	136.94 ±8.33	<0.001 $^{\mu}$
Total cholesterol (mg/dL)	161.98 ±2.64	176.93 ±5.20	0.041 $^{\mu}$
HDL (mg/dL)	48.61 ±1.84	43.86 ±1.70	0.069 $^{\Omega}$
LDL (mg/dL)	95.13 ±2.35	105.68 ±4.30	0.089 $^{\mu}$
Insulin (µIU/mL)	8.64 ±0.67	17.69 ±1.51	<0.001 $^{\mu}$
Leptin (ng/mL)	14.73 ±4.19	34.13 ±4.61	<0.001 $^{\mu}$
HOMA-IR	1.93 ±0.14	4.07 ±0.36	<0.001 $^{\mu}$
HOMA-β (%)	126.69 ±14.67	256.49 ±38.70	<0.001 $^{\mu}$
Adipokines			
CRP (mg/L)	2.27 ±0.76 *	3.15 ±0.80	0.022 $^{\mu}$
IL-1β (pg/mL)	2.40 ±0.20 $^{\circ}$	2.83 ±0.44 $^{\circ\circ}$	0.926 $^{\mu}$
IL-6 (pg/mL)	2.74 ±0.25 $^{\alpha}$	3.26 ±0.51 $^{\alpha\alpha}$	0.824 $^{\mu}$
TNF-α (pg/mL)	13.50 ±1.23 $^{\beta}$	11.12 ±0.86 $^{\beta\beta}$	0.092 $^{\mu}$
Adiponectin (µg/mL)	12.50 ±1.08	10.49 ±0.86	0.105 $^{\mu}$
Endothelial dysfunction markers			
VCAM-1 (ng/mL)	799.69 ±47.67	792.23 ±60.90	0.127 $^{\mu}$
ICAM-1 (ng/mL)	182.10 ±17.05	263.11 ±17.40	<0.001 $^{\mu}$
E-selectin (ng/mL)	44.33 ±3.35	103.12 ±19.53	<0.001 $^{\mu}$
Fecal SCFAs			
Acetic acid (mM/100mg)	245.10 ±28.42	238.76 ±17.76	0.576 $^{\mu}$
Propionic acid (mM/100mg)	14.79 ±3.25	8.50 ±1.56	0.379 $^{\mu}$
Butyric acid (mM/100mg)	13.60 ±1.82	16.65 ±3.71	0.596 $^{\mu}$

The results appear like mean ± standard error. *P*-values were calculated according to: µ Mann-Whitney U test for unequal variances, Ω One-way ANOVA for equal variance. *P* <0.05 is considered statistically significance. Abbreviations are F – female, M – male, BMI - body mass index, WC – waist circumference, SBP – systolic blood pressure, DBP – diastolic blood pressure, HDL - high-density lipoprotein, LDL - low-density lipoprotein, HOMA-IR – homeostasis model assessment-insulin resistant, HOMA-β - homeostasis model assessment-beta cell function, CRP - C-reactive protein, IL – interleukin, TNF - tumor necrosis factor, VCAM - vascular adhesion molecule, ICAM - intercellular adhesion molecule, SCFAs – short chain fatty acids, pc - percentile, Nd - not determined. In some study all individual could not participate due to unavailability of samples. Different symbols show the number of participants for the data: *26, $^{\circ}$22, $^{\alpha}$22, $^{\beta}$22, out of 27 for Normal weight; or $^{\circ\circ}$30, $^{\alpha\alpha}$30, $^{\beta\beta}$30 out of 34 for Obesity. WC pc was adjusted according to sex and age.

Table 3. Dietary diversity of the studied children and adolescents by phenotypic classification.

Group	Mean diversity score (range 0–7)	% with low diversity	% with middle diversity	% with high diversity
		0–2 food groups	3–4 food groups	5–7 food groups
Children (105)				
Normal weight (46)	6.87	26.71	31.68	41.61
Obesity (59)	6.75	21.18	32.45	42.37
Adolescents (57)				
Normal weight (25)	6.88	26.29	34.86	38.86
Obesity (32)	6.53	32.59	25.00	42.41

Food groups: (1) starchy staples; (2) legumes; (3) dairy; (4) meat; (5) vitamin A-rich fruits and vegetables; (6) other fruits and vegetables or fruit juices; and (7) foods made with oil, fat, or butter. 6 children and 4 adolescents could not participate for dietary diversity study.

7.2. Obese individuals have a trend to present higher abundance of Firmicutes and lower abundance of Bacteroidetes

To evaluate gut microbial composition, we performed high-throughput DNA sequencing of V3-16S rDNA libraries using fecal DNA from all children and adolescents. We processed 13,095,175 total reads for children and 5,819,206 for adolescents. The average number of reads was 118,389 for normal weight children and 117,647 for obese children; in the case of adolescents, the average number of reads was 102,184 for normal weight and 90,007 for obese subjects.

The alpha-diversity analysis showed slightly higher diversity in both obese children and adolescents in comparison to normal weight participants; however, differences were not statistically significant (Figure 7 and Table 4). We also calculated the beta-diversity to assess the distance matrix between normal weight and obesity in both children (Figure 7c) and adolescents (Figure 7d). The results of unweighted UniFrac analyses was plotted by principal coordinates analysis (PCoA) and hierarchical clustering. Clustering was not observed.

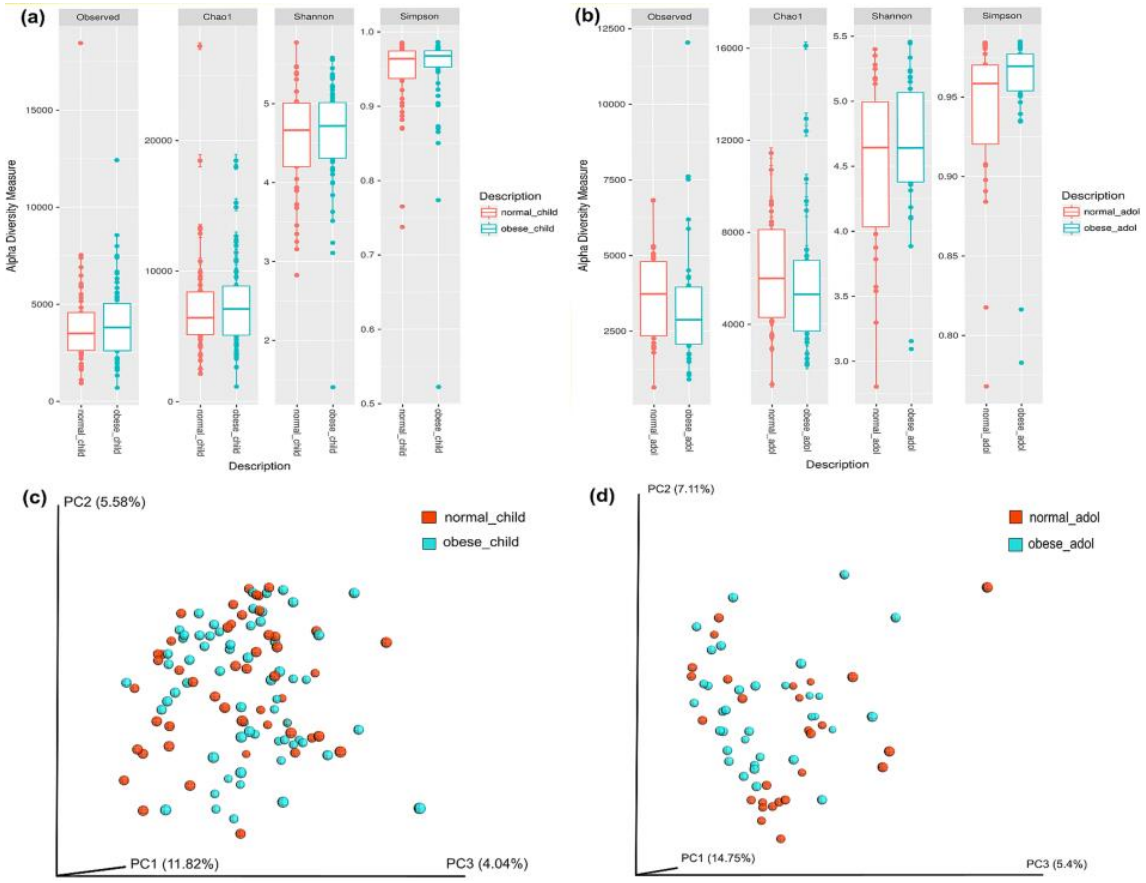


Figure 7. Characterization of diversity of the gut microbiota. Alpha-diversity in children (a), and adolescents (b). beta-diversity in children (c) and adolescents (d).

Table 4. Diversity indexes for children and adolescents.

Diversity index				
Children	Normal weight*	Obesity*	*p*-value	*q*-value
Observed	4,015.55	4,007.79	0.6995	1.0
Chao1	7,424.22	7,636.18	0.4472	1.0
Shannon	4.53	4.61	0.4946	1.0
Simpson	0.94	0.95	0.4615	1.0
Adolescents	Normal weight*	Obesity*	*p* value	*q* value
Observed	3,569.93	3,412.79	0.3876	1.0
Chao1	6,209.36	5,912.67	0.3876	1.0
Shannon	4.48	4.62	0.4904	1.0
Simpson	0.94	0.95	0.0827	1.0

p-values were calculated by Mann-Whitney test for unequal variances. *p*-values corrected by Benjamini-Hochberg, 1995 method and generated FDR value (*q*-value). $p < 0.05$ and $q < 0.05$ are considered statistically significant. Where * shows mean values

Next, we evaluated the composition of the gut microbiota at the phylum level. Obese children had higher relative abundance of Firmicutes and Actinobacteria and decreased Bacteroidetes with respect to the normal weight (Figure 8a); however, these differences were not statistically significant (Table 5). Similar results were obtained for Actinobacteria in obese adolescents; however, after FDR correction, it was not significant (Figure 8b and Table 5).

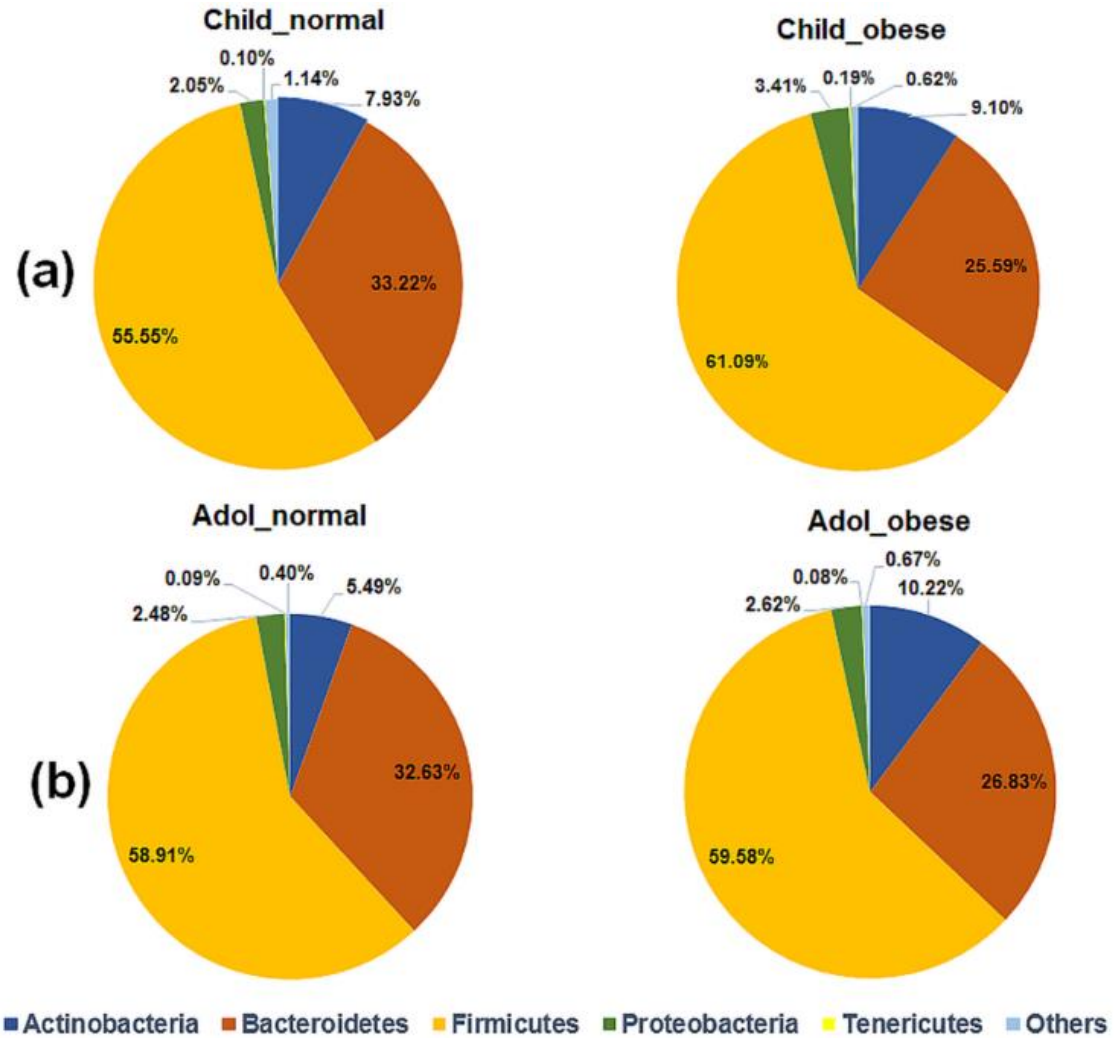


Figure 8. Relative abundance of gut bacterial phyla in children (a) and adolescents (b). Pie charts shows the gut microbial abundance for each phenotypic group which is indicated on top. The relative abundance of each phylum is shown as percentage (%) beside the charts. Actinobacteria, Bacteroidetes, Firmicutes, Proteobacteria, Tenericutes, and "Others" phyla are indicated by different colors, described at the bottom of the figure. Tags on top of each chart are normal weight children, obese children, normal weight adolescents and obese adolescents. Addition information in Table 5.

Table 5. Significant level of bacterial phylum in children.

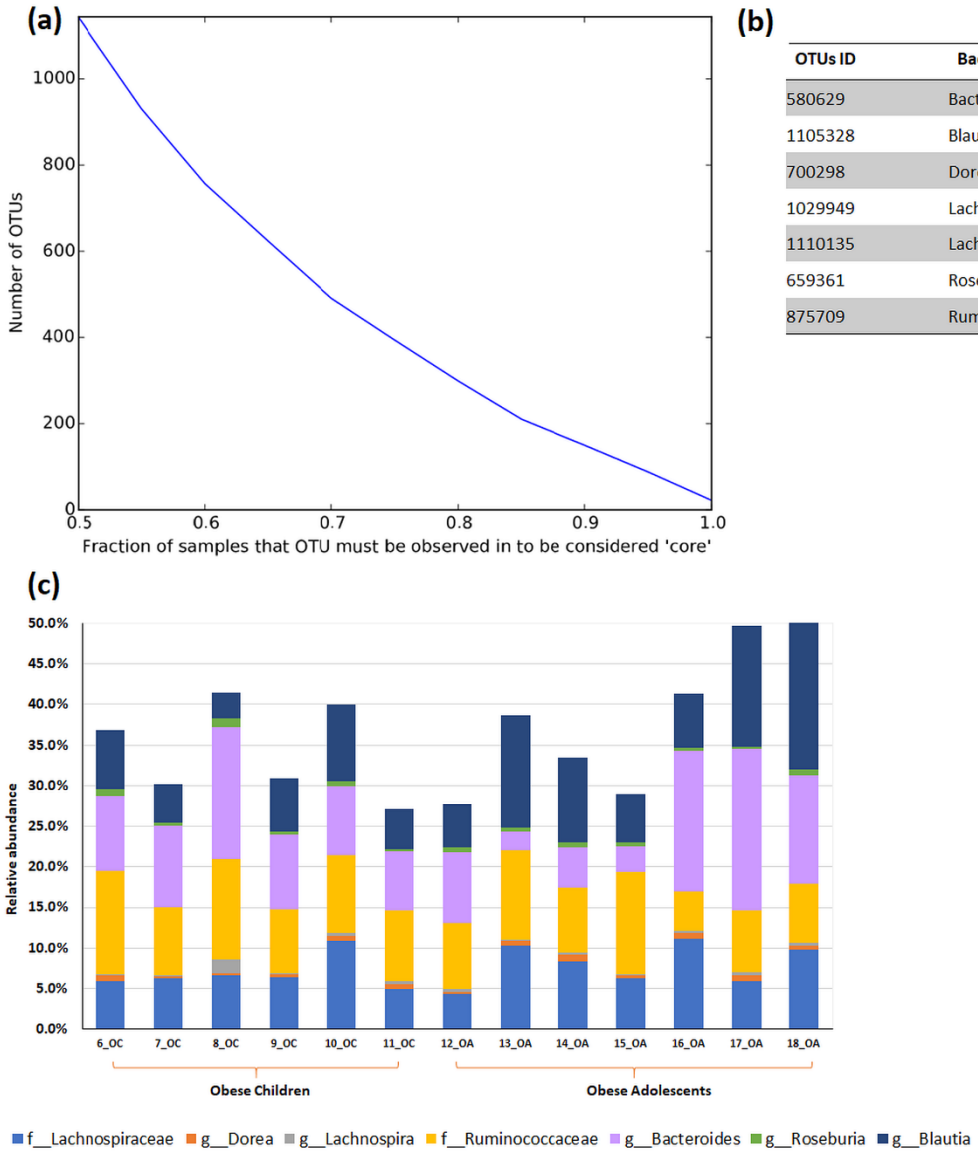
Taxa	Normal weight*	Obesity*	*p*-value	*q*-value
Children				
p_Actinobacteria	7.93%	9.10%	0.1314	0.6906
p_Bacteroidetes	33.22%	25.59%	0.0493	0.6906
p_Firmicutes	55.55%	61.09%	0.0543	0.6906
p_Proteobacteria	2.05%	3.41%	0.2444	0.6906
p_Tenericutes	0.10%	0.19%	0.5328	0.7991
Others	1.00%	0.67%	0.7170	1.0000
Adolescents				
p_Actinobacteria	4.34%	10.04%	0.0025	0.0740
p_Bacteroidetes	32.63%	26.83%	0.3270	0.9771
p_Firmicutes	58.91%	59.58%	0.9248	0.9850
p_Proteobacteria	2.13%	2.06%	0.4723	0.9836
p_Tenericutes	0.09%	0.04%	0.6454	0.9836
Others	0.40%	0.67%	0.7930	1.0000

p-values corrected by Benjamini-Hochberg, 1995 method and generated FDR value (*q*-value). $p < 0.05$ and $q < 0.05$ are considered statistically significant. The name of a higher taxon level was added before its taxon abbreviation "p", phylum. * shows mean values.

Subsequently we analyze the core microbiota in children and adolescents, which showed that in all obese children and adolescents from the age of 6-18 years-old carries some common gut microbiota like genera *Roseburia*, *Blautia*, *Bacteroides*, *Dorea*, *Lachnospira*, family Lachnospiraceae, and Ruminococcaceae (Figure 9).

7.3. Distinct gut microbiota between normal weight and obese children and adolescents

We next used LEfSe analysis to identify bacteria where the relative abundance was significantly increased or decreased in each phenotypic category. Obese children had increased relative abundance of members of the phylum Firmicutes compared to control subjects, e.g., family Peptostreptococcaceae (p = 0.036), and the genus *Lactobacillus* (p = 0.040), were three-fold higher than normal weight children. The genera *Clostridium* (p = 0.025) and *SMB53* (p = 0.018) were also at least two-fold higher. There was at least a three-fold increase in members of the order Bacteroidales (p = 0.019) phylum Bacteroidetes, and members of the family Coriobacteriaceae (p = 0.019) from phylum Actinobacteria. Members of the phylum Proteobacteria, like the genera *Succinivibrio* (p = 0.019), were three-fold higher, whereas the genera Candidatus *Portiera* (p = 0.042) and *Dickeya* (p = 0.042) were at least two-fold higher.



OTUs ID	Bacteria
580629	Bacteroides
1105328	Blautia
700298	Dorea
1029949	Lachnospira
1110135	Lachnospiraceae
659361	Roseburia
875709	Ruminococcaceae

Figure 9. Core gut microbiota of obese Mexican children and adolescents. (a) shows the relation between number of OTUs present in the samples and percentage of the samples. X-axis shows the percentage of the sample from 0.5 (means 50%) to 1.0 (means 100%), Y-axis shows the number of OTUs present in the samples; (b) shows the core bacteria and their OTUs number presents in 100% of the samples; (c) shows the bar diagram of core microbiota presents in all obese individuals. X-axis shows the phenotypic category with age in years from 6_OC to 18_OA and Y-axis shows the relative abundance (%) of microbiota. 6 to 18 are age in years; OC - obese children; OA - obese adolescents.

There was at least a two-fold increased relative abundance of members of the family Elusimicrobiaceae (p = 0.005), phylum Elusimicrobia. In normal weight children, bacteria from the phylum Actinobacteria, order Solirubrobacterales (p = 0.010), the family Conexibacteraceae (p = 0.022), and the genus *Nocardioides* (p = 0.022), and similarly the genus. *Acholeplasma* (p = 0.049) of the phylum Tenericutes, were at least two-fold more abundant (Figure 10a and Table 6).

In obese adolescents the phylum Firmicutes, e.g., genus *Blautia* (p = 0.043) were at least four-fold higher than control subjects; in addition, the genus *Coproccous* (p = 0.020) was three-fold higher and the families Mogibacteriaceae (p = 0.002), Leuconostocaceae (p = 0.016), Gemellaceae (p = 0.004), as well as the genera *Lactococcus* (p < 0.001), and *Gemella* (p = 0.005) were at least two-fold higher than control group.

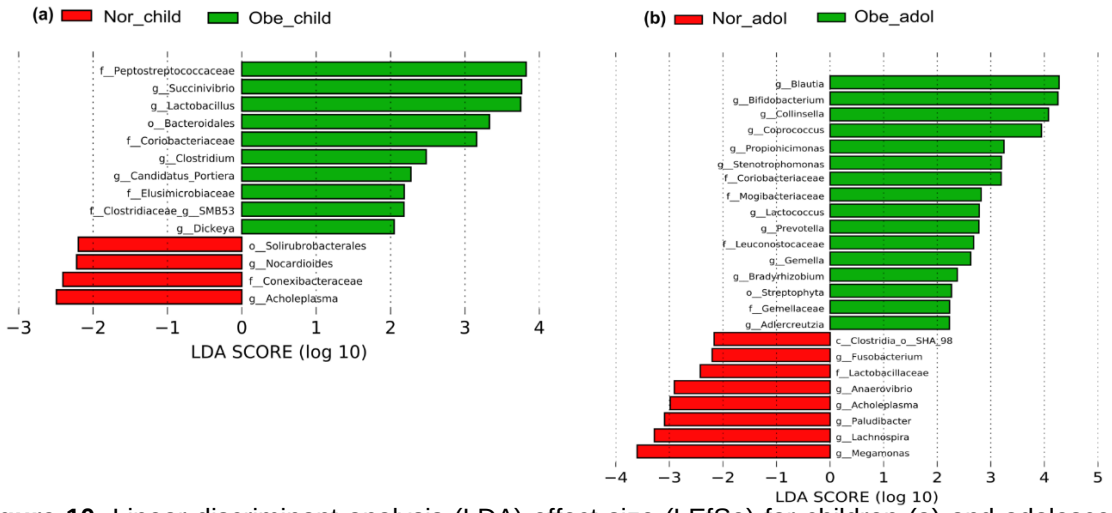


Figure 10. Linear discriminant analysis (LDA) effect size (LEfSe) for children (a) and adolescents (b). Horizontal bars represent the effect size for each taxon. The length of the bar represents the log10 transformed LDA score, indicated by vertical dotted lines. Normal weight children and adolescents are indicated by red, and obesity by green. The threshold on the logarithmic LDA score for discriminative features was set to 2.0. The taxon of bacteria with statistically significant change (p < 0.05) in the relative abundance is written alongside the horizontal lines. The name of the taxon level is abbreviated as p—phylum; c—class; o—order; f—family, and g—genus. Tags above the graphics are normal weight children, obese children, normal weight adolescents, and obese adolescents. Data were processed as described in Materials and Methods section. Statistically significant values are in additional data Table 6 and 7.

Table 6. Linear discriminant analysis (LDA) effect size (LEfSe) analysis for children.

Taxa	Group	LDA score	*p*-value
g_*Lactobacillus*	Obe_child	3.7495	0.0402
g_*Succinivibrio*	Obe_child	3.7613	0.0194
g_*Clostridium*	Obe_child	2.4784	0.0256
f_Elusimicrobiaceae	Obe_child	2.1828	0.0053
f_Coriobacteriaceae	Obe_child	3.1561	0.0194
f_Peptostreptococcaceae	Obe_child	3.8233	0.0363
g_*SMB53*	Obe_child	2.1799	0.0180
g_*Dickeya*	Obe_child	2.0485	0.0429
o_Bacteroidales	Obe_child	3.3284	0.0196
g_Candidatus *portiera*	Obe_child	2.2739	0.0429
g_*Acholeplasma*	Nor_child	2.4934	0.0493
g_*Nocardioides*	Nor_child	2.2210	0.0226
o_Solirubrobacterales	Nor_child	2.1983	0.0104
f_Conexibacteraceae	Nor_child	2.4045	0.0226

The threshold on the logarithmic LDA score for discriminative features was set to 2.0. The name of a higher taxon level was added before its taxon abbreviation. “p”, phylum; “c”, class; “o”, order; “f”, family; “g”, genus. “Obe_child”, children affected of obesity; “Nor_child”, normal weight children; “LDA” Linear discriminant analysis. $p < 0.05$ are considered statistically significant.

Regarding the phylum Bacteroidetes, the abundance of genus *Prevotella* ($p = 0.030$) was at least two-fold higher. Two genera of the phylum Proteobacteria were higher: *Stenotrophomonas* ($p = 0.013$) was three-fold and *Bradyrhizobium* ($p = 0.049$) was at least two-fold higher. Members of the phylum Actinobacteria, like the genera *Bifidobacterium* ($p = 0.021$) and *Collinsella* ($p = 0.001$), were at least four-fold higher; the family Coriobacteriaceae ($p = 0.003$) and the genus *Propiocinimonas* ($p = 0.039$) were also at least three-fold higher, and finally the genus *Adlercreutzia* ($p = 0.004$) increased by at least two-fold. We detected at least a two-fold increase in the phylum Cyanobacteria, order Streptophyta ($p = 0.030$). Conversely, in normal weight adolescents, genera from the phylum Firmicutes, like *Lachnospira* ($p = 0.031$) and *Megamonas* ($p = 0.036$), were at least three-fold higher, whereas the order SHA-98 ($p = 0.024$), the family Lactobacillaceae ($p = 0.002$), and the genus *Anaerovibrio* ($p = 0.048$) were at least two-fold higher. There was at least a three-fold increase in genus *Paludibacter* ($p = 0.040$) of the phylum Bacteroidetes, and two additional phyla, Tenericutes and Fusobacteria, had members whose abundances increased at least two-fold: genus *Acholeplasma* ($p = 0.048$) and genus *Fusobacterium* ($p = 0.032$), respectively (Figure 10b and Table 7).

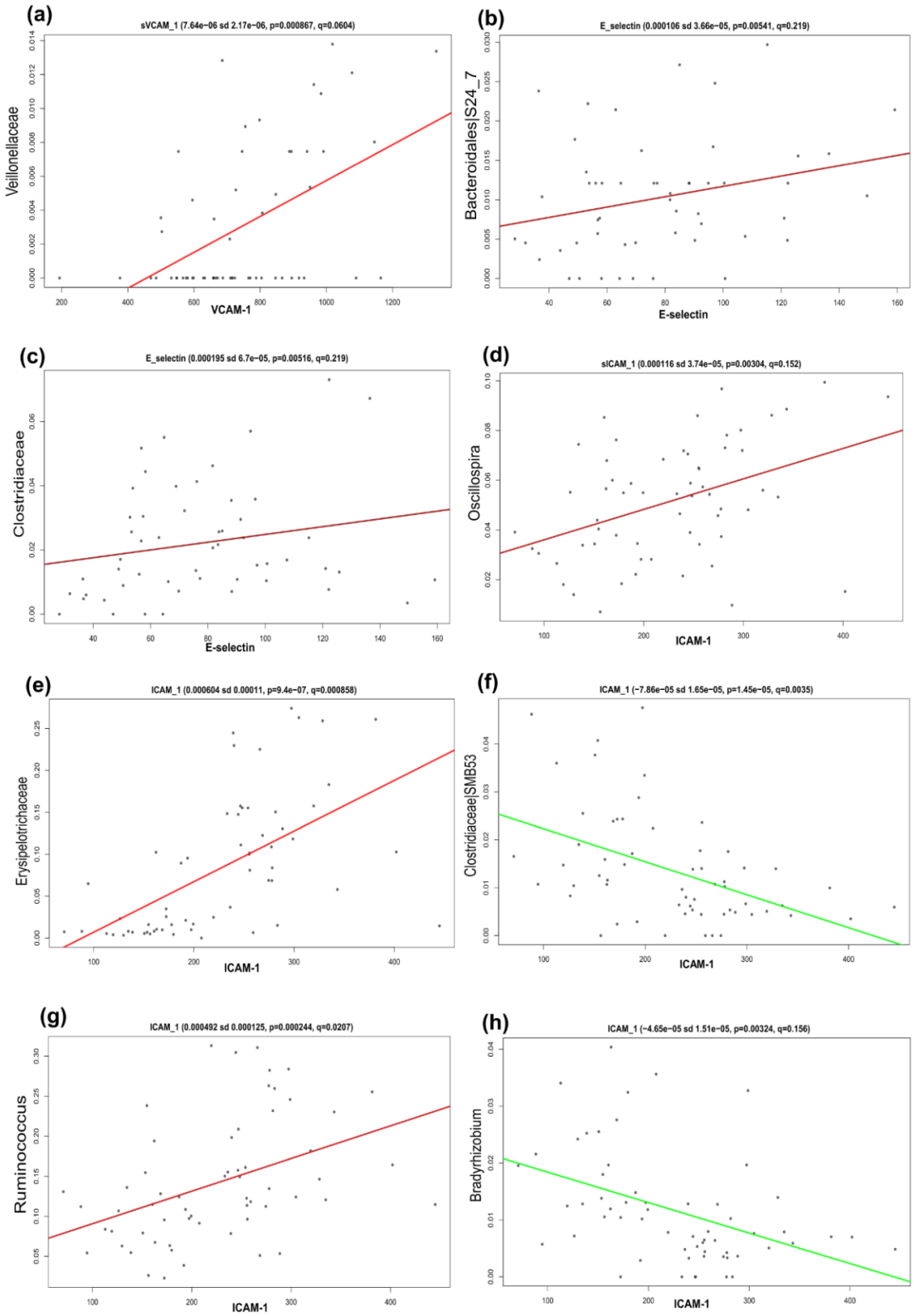
Table 7. Linear discriminant analysis (LDA) effect size (LEfSe) analysis for adolescents.

Taxa	Group	LDA score	*p*-value
g_*Adlercreutzia*	Obe_adol	2.2280	0.0041
g_*Bradyrhizobium*	Obe_adol	2.3751	0.0499
g_*Bifidobacterium*	Obe_adol	4.2532	0.0210
f_Leuconostocaceae	Obe_adol	2.6803	0.0160
g_*Blautia*	Obe_adol	4.2739	0.0436
g_*Collinsella*	Obe_adol	4.0767	0.0018
f_Coriobacteriaceae	Obe_adol	3.1937	0.0035
g_*Lactococcus*	Obe_adol	2.7848	<0.001
g_*Coprococcus*	Obe_adol	3.9461	0.0202
o_Streptophyta	Obe_adol	2.2671	0.0303
g_*Prevotella*	Obe_adol	2.7781	0.0309
f_Mogibacteriaceae	Obe_adol	2.8203	0.0024
f_Gemellaceae	Obe_adol	2.2309	0.0040
g_*Propionicimonas*	Obe_adol	3.2475	0.0394
g_*Gemella*	Obe_adol	2.6242	0.0058
g_*Stenotrophomonas*	Obe_adol	3.1971	0.0132
g_*Paludibacter*	Nor_adol	3.0903	0.0403
g_*Acholeplasma*	Nor_adol	2.9836	0.0481
g_*Megamonas*	Nor_adol	3.6010	0.0361
g_*Lachnospira*	Nor_adol	3.2791	0.0316
g_*Anaerovibrio*	Nor_adol	2.9063	0.0481
o_SHA_98	Nor_adol	2.1641	0.0249
g_*Fusobacterium*	Nor_adol	2.2006	0.0321
f_Lactobacillaceae	Nor_adol	2.4231	0.0024

The threshold on the logarithmic LDA score for discriminative features was set to 2.0. The name of a higher taxon level was added before its taxon abbreviation. “p”, phylum; “c”, class; “o”, order; “f”, family; “g”, genus. “Obe_adol”, adolescent affected of obesity; “Nor_adol”, normal weight adolescent; “LDA” Linear discriminant analysis. $p < 0.05$ are considered statistically significant.

7.4. Gut microbiota is associated with EDF and dyslipidemia markers in obese individuals

The association between clinical metadata (Tables 1 and 2) and the relative abundance of gut microbiota was explored via MaAsLin for both children and adolescents. The results showed a positive association in obese children between VCAM-1 and Veillonellaceae ($p < 0.001$, $q = 0.060$), E-selectin and family S24-7 ($p = 0.005$, $q = 0.219$), and between ICAM-1, and *Oscillospira* ($p = 0.003$, $q = 0.152$) (Figure 11). A positive association was also found in obese children between ICAM-1 and *Ruminococcus* ($p < 0.001$, $q = 0.020$). On the other hand, there was a negative association between ICAM-1 and SMB53 ($p < 0.001$, $q = 0.003$) in the same individuals. In contrast, obese adolescents had a positive association between total cholesterol and *Ruminococcus* ($p = 0.004$, $q = 0.193$), and between ICAM-1 and *Bacteroides* ($p = 0.0001$, $q = 0.102$). Finally, there was a negative association between LDL and *Parvimonas* ($p = 0.0012$, $q = 0.146$) (Figure 12).


(a)
sVCAM_1 (7.64e−06 sd 2.17e−06, p=0.000867, q=0.0604)
Veillonellaceae
VCAM-1
(b)
E_selectin (0.000106 sd 3.66e−05, p=0.00541, q=0.219)
Bacteroidales|S24_7
E-selectin
(c)
E_selectin (0.000195 sd 6.7e−05, p=0.00516, q=0.219)
Clostridiaceae
E-selectin
(d)
sICAM_1 (0.000116 sd 3.74e−05, p=0.00304, q=0.152)
Oscillospira
ICAM-1
(e)
ICAM_1 (0.000604 sd 0.00011, p=9.4e−07, q=0.000858)
Erysipelotrichaceae
ICAM-1
(f)
ICAM_1 (−7.86e−05 sd 1.65e−05, p=1.45e−05, q=0.0035)
Clostridiaceae|SMB53
ICAM-1
(g)
ICAM_1 (0.000492 sd 0.000125, p=0.000244, q=0.0207)
Ruminococcus
ICAM-1
(h)
ICAM_1 (−4.65e−05 sd 1.51e−05, p=0.00324, q=0.156)
Bradyrhizobium
ICAM-1

[….*Legend from previous figure*]

Figure 11. Multivariate linear associations of clinical metadata and bacterial relative abundance in obese children. Scatter plots show the significant associations of VCAM-1 with Veillonellaceae (a), E-selectin with S24-7 (family Bacteroidales) (b), E-selectin with Clostridiaceae (c), ICAM-1 with *Oscillospira* (d), ICAM-1 with Erysipelotrichaceae (e), ICAM-1 with *SBM53* (f), ICAM-1 with *Ruminococcus* (g), and ICAM-1 with *Bradyrhizobium* (h), as described in Materials and methods section and Table 8. y-axes show the relative abundance of gut microbiota; x-axes show the clinical metadata. Numerical data on top of each graphic are Coefficient (positive coefficient shows positive association, and negative coefficient shows negative association between metadata and gut microbiota), sd—standard deviation; *p*-values; e—times 10 is raised to the power of, and FDR corrected *q*-values which are assigned by MaAsLin (v0.0.4). VCAM-1-vascular cell adhesion molecule-1, and ICAM-1-intercellular adhesion molecule-1.

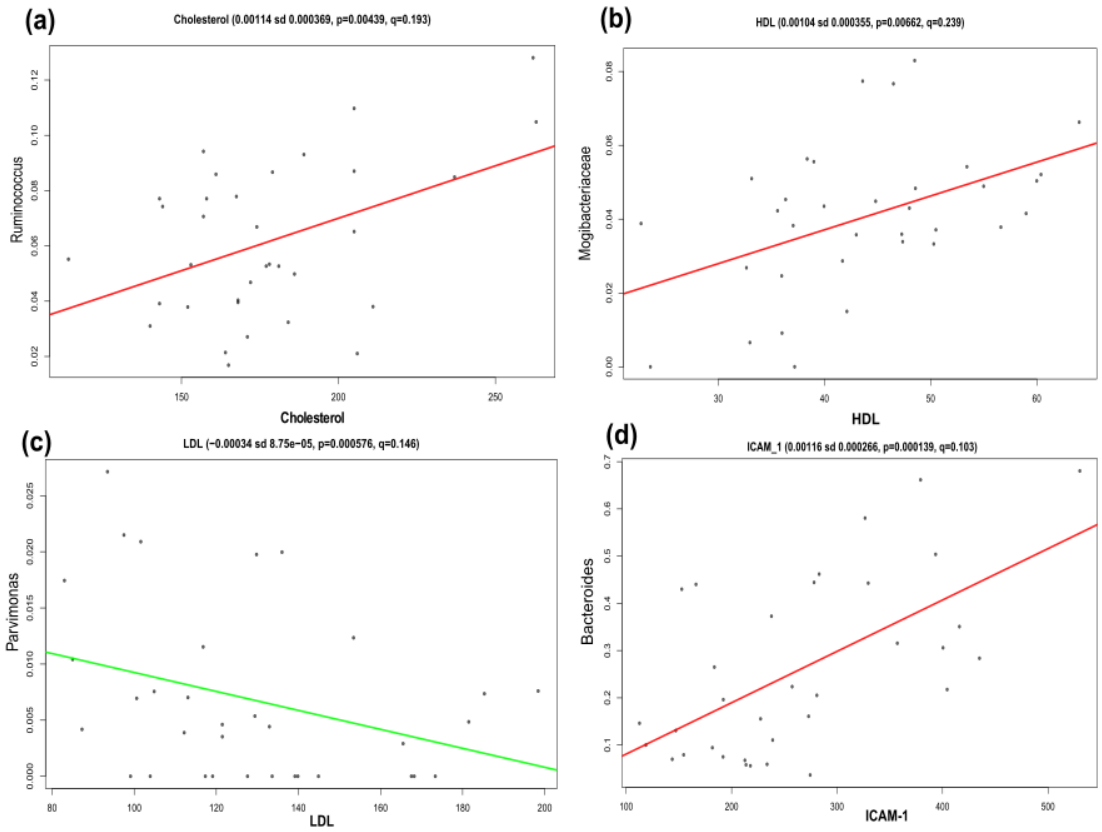


Figure 12. Multivariate linear associations of clinical metadata and bacterial relative abundance in obese adolescents. Scatter plot explains the significant association of Cholesterol with *Ruminococcus* (a), HDL with Mogibacteriaceae (b), LDL with *Parvimonas* (c), and ICAM-1 with *Bacteroides* (d), as described in Materials and Methods section and Table 9. *p*-values and FDR corrected *q*-values are assigned by MaAsLin (v0.0.4). y-axes show the relative abundance of gut microbiota; x-axes show the clinical metadata. Numerical data on top of each graphic are Coefficient (positive coefficient shows positive association, and negative coefficient shows negative association between metadata and gut microbiota), sd—standard deviation; e—times 10 is raised to the power of ; p-values, and FDR corrected q-values which are assigned by MaAsLin (v0.0.4). HDL—high-density lipoprotein; LDL—low-density lipoprotein, and ICAM-1—intercellular adhesion molecule-1.

Table 8. Taxonomic composition of gut microbiota with different metadata in children with obesity.

Variable	Feature	Coefficient	*p*-value	*q*-value
VCAM-1	f_Veillonellaceae	<0.00010	<0.001	0.060
E-selectin	f_Clostridiaceae	0.00019	0.005	0.219
E-selectin	f_S24-7	0.00010	0.005	0.219
ICAM-1	g_*Oscillospira*	0.00012	0.003	0.152
ICAM-1	f_Erysipelotrichaceae	0.00060	<0.001	<0.001
ICAM-1	g_*SMB53*	-0.00007	<0.001	0.003
ICAM-1	g_*Ruminococcus*	0.00040	<0.001	0.020
ICAM-1	g_*Bradyrhizobium*	-0.00004	0.003	0.156

A positive coefficient means gut microbial abundance increases with respective variable, while a negative coefficient means gut microbial abundance decreases with respective variable. For readability the kingdom label is not present. “ICAM-1”, intercellular adhesion molecule-1; “VCAM-1”, vascular cell adhesion molecule. The name of a higher taxon level was added before its taxon abbreviation. “p”, phylum; “c”, class; “o”, order; “f”. family; “g”, genus.

Table 9. Taxonomic composition of gut microbiota with different metadata in adolescents with obesity.

Variable	Feature	Coefficient	*p*-value	*q*-value
Cholesterol	g_*Ruminococcus*	0.00114	0.0040	0.192
HDL	f_Mogibacteriaceae	0.00103	0.0060	0.239
LDL	g_*Parvimonas*	-0.00033	0.0012	0.146
ICAM-1	g_*Bacteroides*	0.00110	0.0001	0.102

A positive coefficient means gut microbial abundance increases with respective variable, while a negative coefficient means gut microbial abundance decreases with respective variable. For readability the kingdom label is not present. “HDL”, high-density lipoprotein,” LDL”, low-density protein, “ICAM-1”, intercellular adhesion molecule-1. The name of a higher taxon level was added before its taxon abbreviation. “p”, phylum; “c”, class; “o”, order; “f”. family; “g”, genus.

7.5. Gut microbial interactions in obese children and adolescents

To investigate the interactions between gut microbiota, we performed a co-occurrence analysis. This analysis showed an interesting network including 35 statistically significant bacterial copresence (positive) and mutual exclusion (negative) interactions (Figure 13a and Table 10) in obese children. Whereas in obese adolescents, it showed 29 statistically significant bacterial copresence (positive) and mutual exclusion (negative) interactions (Figure 13b and Table 10). In contrast, we did not find comparable large complex networks of gut microbiota in normal weight children (Appendix, Figure A1 and Table A1) or adolescents (Appendix, Figure A2 and Table A2).

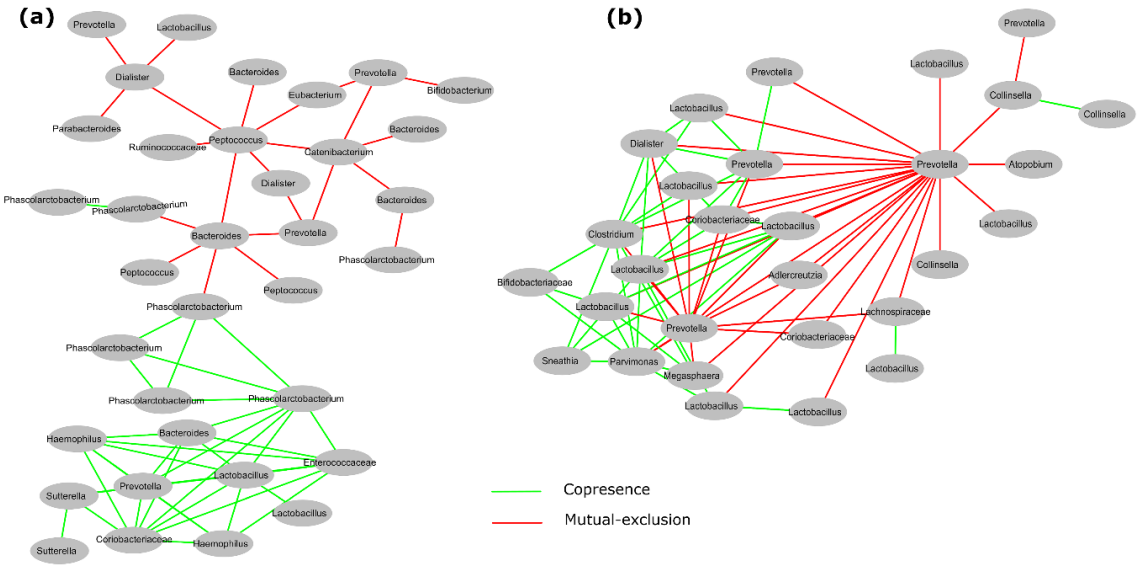


Figure 13. Significant co-occurrence analysis between gut microbiota in obese Mexican children (a) and adolescents (b). This graphic shows selected interactions between different bacterial communities; copresence (positive, green lines) and mutual exclusion (negative, red lines). This analysis was performed using *otu_table.biom* file in CoNet plugin tool and generated co-occurrence networks were visualized by Cytoscape (v3.6.1) software as described in Material and Methods. Each node indicates a microbial clade (bacterial taxon) belonging to a unique OTUs number. Edges (lines) connecting two nodes, represent significant correlations ($p < 0.05$; $q < 0.05$; $R > 0.8$). In this graphics some bacteria names are shown more than one time e.g., *Lactobacillus*, *Prevotella*, etc., however they belong to different OTUs number. Bacteria names and corresponding OTUs number are shown in Table 10. A complete set of networks in Appendix, Figure A3 and A4, and their corresponding OTU number are shown in Appendix, Table A3 and A4.

Table 10. OTUs ID of gut bacteria.

Obese Children (Figure 13a)			Obese Adolescents (Figure 13b)		
No.	**OTUs ID**	**Bacteria**	**No.**	**OTUs ID**	**Bacteria**
1	335	*Phascolarctobacterium*	1	1184	*Collinsella*
2	4007	*Peptococcus*	2	7367	*Prevotella*
3	6529	*Lactobacillus*	3	9022	*Lactobacillus*
4	7389	*Haemophilus*	4	9328	*Collinsella*
5	9453	*Prevotella*	5	10278	*Lactobacillus*
6	11321	*Phascolarctobacterium*	6	10560	*Collinsella*
7	12345	*Phascolarctobacterium*	7	12479	*Lactobacillus*
8	13188	*Prevotella*	8	12723	Lachnospiraceae
9	19296	*Bacteroides*	9	12974	*Lactobacillus*
10	19314	*Parabacteroides*	10	29566	*Sneathia*
11	21736	*Phascolarctobacterium*	11	128300	*Lactobacillus*
12	22231	*Haemophilus*	12	130468	*Lactobacillus*
13	24722	*Bacteroides*	13	130864	*Lactobacillus*
14	41229	*Sutterella*	14	133372	*Parvimonas*
15	157424	*Phascolarctobacterium*	15	137183	Bifidobacteriaceae
16	179261	*Sutterella*	16	225846	*Dialister*
17	183603	*Bacteroides*	17	236308	*Lactobacillus*
18	196604	*Catenibacterium*	18	272516	*Adlercreutzia*
19	215331	*Peptococcus*	19	292921	*Prevotella*
20	235591	*Lactobacillus*	20	354905	*Lactobacillus*
21	269937	*Prevotella*	21	383885	*Lactobacillus*
22	293883	*Phascolarctobacterium*	22	469663	*Atopobium*
23	309133	Enterococcaceae	23	566154	Coriobacteriaceae
24	339685	*Peptococcus*	24	568118	*Prevotella*
25	365496	*Bacteroides*	25	663885	*Prevotella*
26	370086	Ruminococcaceae	26	840914	*Prevotella*
27	403701	*Dialister*	27	851726	*Megasphaera*
28	524371	*Prevotella*	28	858535	Coriobacteriaceae
29	524884	*Eubacterium*	29	986513	*Clostridium*
30	583746	*Dialister*			
31	587753	Coriobacteriaceae			
32	639310	*Bifidobacterium*			
33	716286	*Lactobacillus*			
34	850218	*Phascolarctobacterium*			
35	4226929	*Bacteroides*			

8. Discussion

Obesity is a metabolic disease characterized by low grade chronic inflammation, usually accompanied by dyslipidemia and up-regulation of other bioactive molecules such as CRP and TNF-α [Murugesan *et al*., 2015; Gomes *et al*., 2010]. In this work, we studied a sample of Mexican children and adolescents characterizing clinical aspects, EDF markers, and their association with the gut microbial diversity.

Obese children and adolescents had higher BMI percentiles, waist circumference above the 95th percentiles, more hypertriglyceridemia and hypercholesterolemia, and reduced HDL levels (Tables 1 and 2). In addition, leptin and the percentage of active β-cells were increased in obese children and adolescents. Furthermore, obese children and adolescents presented with greater levels of insulin resistance, as reflected by the elevated glucose, insulin and HOMA-IR values (Tables 1 and 2). Moreover, these obese children and adolescents had higher blood pressure and metabolic syndrome at this early age. This is the first report that describes the presence of metabolic syndrome in this very young Mexican population.

Among obese children and adolescents there were significant differences for the adipokines levels, specifically CRP was increased, while adiponectin was decreased in both groups. Surprisingly, TNF-α was increased in obese children, but not in obese adolescents. For the EDF markers, obese children had significantly elevated levels of E-selectin, though ICAM-1 and VCAM-1 were not significantly increased. In obese adolescents, E-selectin and ICAM-1 were significantly elevated, whereas VCAM-1 was slightly decreased (Tables 1 and 2). Increased levels of CRP along with reduced levels of adiponectin increased the expression of EDF markers in obese individuals by impairing endothelium-dependent vasodilatation and nitric acid production [Gomes *et al*., 2010; Rojas *et al*., 2014]. These adipokines are also associated with insulin resistance, dyslipidemia, atherosclerosis, endothelial dysfunction, and cardiovascular diseases [Rojas *et al*., 2014; Yadav *et al*., 2013; Ohashi *et al*., 2014]. Thus, it seems that EDF markers increased with age or get worst along the time span with obesity. We hypothesize that obesity in obese

children and adolescents increases the risk for the development of metabolic diseases. It has been also reported that hypertriglyceridemia is associated with atherosclerosis and is also predisposition for the development of cardiovascular disease [Dron *et al*., 2017].

Gut microbiota and its microbiome are involved in atherosclerosis and obesity in humans [Murugesan *et al*., 2015; Karlsson *et al*., 2012]. The characterization of gut bacterial diversity by high-throughput DNA sequencing of V3-16S rDNA libraries showed higher relative abundance of Firmicutes and lower relative abundance of Bacteroidetes in obese children and adolescents (Figure 8), as has been similarly reported in mice [Ley *et al*., 2006], and American [Turnbaugh *et al*., 2006] and Japanese human guts [Kasai *et al*., 2015]. However, changes were not statistically significant for our data (Table 5). Core microbiota analysis revealed that genera *Blautia*, *Roseburia* and family Lachnospiraceae were present in all obese Mexican children and adolescents (Figure 9). It has been already mentioned that these gut bacteria were abundant in Mexican overweight and obese children [Murugesan *et al*., 2015].

To explore the differences in the relative abundance of bacterial taxa, we performed a LEfSe analysis that showed that obese children and adolescents have significant changes in the abundance of various distinct gut bacteria with respect to normal weight (Figure 10 and Table 6 and 7).For the phylum Actinobacteria, obese children and adolescents showed an increase in the abundance of members of the family Coriobacteriaceae (Table 11).

In the feces from mice/hamsters, genera *Eggerthella* and *Enterorhabdus* (family Coriobacteriaceae) were reported to be positively correlated with intrahepatic levels of triglycerides and non-HDL plasma concentrations, suggesting gut barrier and metabolic dysfunction [Clavel *et al*., 2014] and chronic inflammation [Wurdemann *et al*., 2009]. It may be that gut members of the Coriobacteriaceae family are involved with the high levels of triglycerides and cholesterol in obese children. In obese adolescents, the genus *Collinsella* was dominant.

Table 11. Selected gut bacteria with significant changes in abundance according to linear discriminant analysis (LDA) effect size (LEfSe) analysis

Taxa	This Work	Other Reports	Reference
Phylum Actinobacteria			
Family Coriobacteriaceae	3-fold more abundant in obese children and adolescents than normal weight	Higher abundance in human and mouse gut. Involved in bile acid metabolism and linked to gut barrier and metabolic dysfunctions	[Clavel *et al*., 2014]
		Isolated from the gut of Crohn's disease suffering adult patient from Germany	[Wurdemann *et al*., 2009]
Collinsella	4-fold more abundant in obese adolescents than normal weight	More than 3-fold enriched in Swedish adult patients with symptomatic atherosclerosis	[Karlsson *et al*., 2012]
		Abundant in American rheumatoid arthritis patients, strongly correlated with production of pro-inflammatory molecules and alters the gut permeability	[Chen *et al*., 2016]
Phylum Bacteroidetes			
Order Bacteroidales	3-fold more abundant in obese children than normal weight	Higher abundance in high-fat diet (HFD) fed Sprague-Dawley rats compared with low fat diet (LFD) fed rats (LFD vs. HFD, $p < 0.01$)	[de La Serre *et al*., 2010]
		Reported in intestinal biopsies of American children and adults with Inflammatory Bowel Disease	[Zitomersky *et al*., 2013]
Prevotella	2-fold more abundant in obese adolescents than normal weight	Higher abundance of *Prevotella* in American adult rheumatoid arthritis patients. Induces inflammatory reactions by stimulating epithelial cells and production of interleukins	[Larsen *et al*., 2017]
Phylum Firmicutes			
Lactobacillus	3-fold more abundant in obese children than normal weight	Different *Lactobacillus* species are associated with weight gain in farm animals	[Million *et al*., 2012]
		Higher abundance in obese French adults than normal weight	[Armougom *et al*., 2009]
Blautia	4-fold more abundant in obese adolescents than normal weight	Found higher abundance of *Blautia* in overweight and obese Mexican children	[Murugesan *et al*., 2015]
Coprococcus	3-fold more abundant in obese adolescents than normal weight	Reported higher abundance of *Coprococcus* in overweight and obese Mexican children	[Murugesan *et al*., 2015]

This bacterium has been found in plaque and feces of symptomatic atherosclerosis patients [Karlsson *et al*., 2012] and in American rheumatoid arthritis patients [Chen *et al*., 2016], which explains that *Collinsella* can be also associated with EDF or EDF markers.

For the phylum Bacteroidetes, members of the order Bacteroidales were more abundant in obese children (Figure 10), similar to what is reported for Sprague-Dawley rats fed a high-fat diet in comparison to rats fed a low-fat diet [de La Serre *et al*., 2010], and in American children and adults affected by inflammatory bowel disease (Table 11) [Zitomersky *et al*., 2013]. Additionally, *Prevotella* showed higher abundance in obese adolescents, as observed in American rheumatoid arthritis adult patients. It is suggested that this bacterium can stimulate the epithelial cells to produce IL-8, IL-6, and CCL20, which can promote mucosal Th17 immune responses and neutrophil recruitment and mediate inflammatory reactions [Larsen *et al*., 2017].

For the phylum Firmicutes, *Lactobacillus* was more abundant in obese children. *Lactobacillus* species were reported to be associated with weight gain in farm animals [Million *et al*., 2012] and French obese adults [Armougom *et al*., 2009] (Table 11). *Lactobacillus* species are commonly used as probiotics; it is possible that species with increased abundance in obesity are strains with additional genes in the core genome supplied by its pangenome [Kant *et al*., 2011]. *Coprococcus* and *Blautia* showed higher abundances only in obese adolescents. An increase in the abundance of these two bacteria has been reported in overweight and obese Mexican children (Table 11) [Murugesan *et al*., 2015].

We were interested in the association of EDF markers (VCAM-1, ICAM-1, and E-selectin) and gut microbiota diversity. For this, we performed a multivariate analysis (MaAsLin), which showed a positive association between some bacteria and clinical data, for example, between the family Veillonellaceae and VCAM-1 (Figure 11a), and between *Ruminococcus* and ICAM-1 (Figure 11g) in obese children (Table 8). For obese adolescents, there was a positive association between *Ruminococcus* and cholesterol (Figure 12a), and between Bacteroides and ICAM-1 (Figure 12d and Table 9). In addition, there was a positive association of E-selectin

with the S24-7 family of the order Bacteroidales (Figure 11b and Table 8). The order Bacteroidales was increased in obese children in this work (Figure 10). Adherent Bacteroidales were reported to trigger an inflammatory reaction in individuals with inflammatory bowel disease [Zitomersky *et al*., 2013]. Based on the data mentioned above, we propose that these bacteria stimulate the endothelium to produce more EDF markers, which subsequently affect the endothelial function in these studied obese subjects.

We looked for interactions among members of the gut microbiota using co-occurrence analysis. We observed that microbial interaction is different in normal weight children and adolescents, and obese children and adolescents (Figures 13). For obese children, we found that *Lactobacillus* showed co-presence (positive interaction) with many other bacteria (Figure 13a). LEfSe analysis showed higher abundance of this bacteria in obese children (Figure 10a). It is possible that *Lactobacillus* may have mutualistic relationships, such as syntrophic interactions with other bacteria. Similarly, in obese adolescents, we found that *Prevotella* showed mutual exclusion (negative interaction) with other gut bacteria, especially with *Collinsella* (Figure 13b). *Prevotella* and *Collinsella* were highly abundant in obese adolescents according to LEfSe analysis (Figure 10b). These negative interactions reflect the trade-off or competition between gut bacteria taxa in the gastrointestinal (GI) tract. Interestingly, one *Prevotella* (OTU568118) in obese adolescents (Figure 13b) showed negative interaction with many other gut bacteria including *Lactobacilllus*, *Collinsella, Atopobium* and other *Prevotella* (OTU663885). It has been mentioned that this *Prevotella* with OTU ID number OTU568118 is belong to a species called *Prevotella copri* [Liu *et al*., 2019]. Further it revealed that this specific bacteria *P. copri* is associated to coronary artery disease in Chinese patients [Liu *et al*., 2019] and to Rheumatoid arthritis in American patients [Scher *et al*., 2013; Chen *et al*., 2016]. We did not find this kind of interaction for normal weight children (Appendix, Figure A1 and Table A1) or adolescents (Appendix, Figure A2 and Table A2). Co-occurrence analysis revealed that members of the gut microbiota, especially more abundant bacteria, create large significant networks with other microbiota members in obese children and adolescents. Furthermore, it suggests

that these interactions may help a particular group of microbiota to develop ecological dominance and obtain more space and food, and maintain convenient host-microbe interactions inside the gut.

Endothelial dysfunction is an early predisposing factor for atherosclerosis [Davignon & Ganz, 2004], and it has been reported that gut bacteria, including the family Veillonellaceae, genera *Ruminococcus*, and Bacteroides, were present in the feces and plaque of adult patients with atherosclerosis [Koren *et al*., 2011], and these bacteria were associated with EDF markers in our obese subjects. The genus *Collinsella*, which was highly abundant in obese adolescents in our study (Figure 10), has also been found in feces and plaque of Swedish adult patients with symptomatic atherosclerosis [Karlsson *et al*., 2012], and in American rheumatoid arthritis patients (Table 11) [Chen *et al*., 2016]. All this evidence supports that members of the gut microbiota, especially the family Veillonellaceae and genera *Ruminococcus* and Bacteroides, are associated with EDF markers in our studied obese subjects and contributing to EDF.

miRNAs have been reported as potential biomarkers for endothelial dysfunction in obese children [Khalyfa *et al*., 2016; Karolina *et al*., 2014]. Vascular microRNA-204 (miR-204) expression is remotely regulated by the microbiome and impairs endothelial function by targeting the Sirtuin1 lysine deacetylase (Sirt1) in mice [Vikram *et al*., 2016]. Since we observed that some gut microbiota like genera *Ruminococcus*, *Bacteroides*, and family Veillonellaceae were associated with EDF markers in our obese participants, we think that miRNAs may be related to EDF or EDF markers, and their expression would be regulated by gut microbiota in our obese children and adolescents.

To improve endothelial function, dietary fiber, antioxidant-containing food/vegetables [Widlansky *et al*., 2003], or supplementation with inulin or Inulin Like Fructans (ITF) as a prebiotic could be important therapeutic solutions. It has been reported in a mice model that ITF can help improve endothelial function by increasing nitric oxide (NO) synthase and reducing oxidative stress [Catry *et al*., 2018]. ITF also improves gut health by increasing NO-producing bacteria and increasing *Akkermansia muciniphila* abundance, which may help reduce the level of

EDF markers in patients. In addition, there are many potential pharmacological interventions available, like angiotensin-converting enzyme (ACE)-inhibitors, angiotensin-receptor blocker, calcium channel blockers (CCB), and certain β-blockers, in particular the NO-group (containing molecule nebivolol), which might reverse endothelial dysfunction [Widlansky *et al*., 2003; Flammer *et al*., 2012]. Limitation on eating high-fat food or consumption of the Western diet is also very important.

With regard to fecal SCFA, its low concentration in obese children and adolescents (Tables 1 and 2) may be explained by higher mucosal absorption, as has been suggested in other studies [Murugesan *et al*., 2015; Turnbaugh *et al*., 2006]. We were not able to measure the SCFAs in plasma to confirm the higher absorption due to insufficient blood samples. A strength of our work is that we demonstrated significant changes in gut microbial composition of obese Mexican children and adolescents. Some specific members of the gut microbiota were positively associated with EDF markers in the same individuals affected by obesity. Indeed, this is an emerging field of interest with regards to obesity and pathophysiology and may be helpful for future intervention studies. Our study is not without limitations, including the small sample size, and homogenous cohort. Future studies should include adults, and diverse race/ethnic groups where the prevalence of EDF, atherosclerosis or cardiovascular disease may be higher. Finally, dietary interventions using high-fiber containing foods might be useful for improving endothelial function through the modification of the gut microbiota.

9. Conclusions

We find an association between featured gut microbiota and endothelial dysfunction markers in obese Mexican children and adolescents. These obese individuals had increased levels of CRP and a reduction of adiponectin, which causes higher expression of EDF markers, affecting endothelial function and associating with changes in the gut microbiota. They also suffer of possible leptin-resistance, insulin-resistance, and metabolic syndrome at this early age.

10. Perspective

Given that an early onset of obesity results in metabolic disorders, targeting the gut microbiota through dietary and therapeutic interventions may be valuable. Furthermore, isolating miRNA's may also provide important information regarding EDF or markers of EDF in obese individuals and found their potential link with gut microbiota. Future research targeting improved endothelial function through altered gut microbial health should focus on the role of dietary supplements with high-fiber foods, inulin, or ITF.

11. Publication

This thesis is based on the following original research article with addition of some unpublished data.

Nirmalkar, K., Murugesan, S., Pizano-Zárate, M. L., Villalobos-Flores, L. E., García-González, C., Morales-Hernández, R. M., Nuñez-Hernández, J. A., Hernández-Quiroz, F., Romero-Figueroa, M del S., Hernández-Guerrero, C., Hoyo-Vadillo, C., García-Mena, J. Gut Microbiota and Endothelial Dysfunction Markers in Obese Mexican Children and Adolescents. *Nutrients* **2018**,10 (12), pii: E2009. doi: 10.3390/nu10122009. (IF = 4.19, According to Clarivate Analytics, Thomson Reuters, 2017).

Article

Gut Microbiota and Endothelial Dysfunction Markers in Obese Mexican Children and Adolescents

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12. References

Aradillas-García, C.; Rodríguez-Morán, M.; Garay-Sevilla, M.E.; Malacara, J.M.; Rascon-Pacheco, R.A.; Guerrero-Romero, F. Distribution of the homeostasis model assessment of insulin resistance in Mexican children and adolescents. *Eur. J. Endocrinol.* **2012**, *166*, 301–306, doi:10.1530/EJE-11-0844.

Armougom, F.; Henry, M.; Vialettes, B.; Raccah, D.; Raoult, D. Monitoring bacterial community of human gut microbiota reveals an increase in lactobacillus in obese patients and Methanogens in anorexic patients. *PLoS ONE* **2009**, *4*, e7125, doi:10.1371/journal.pone.0007125.

Benjamini, Y.; Hochberg, Y. Controlling the false discovery rate: A practical and powerful approach to multiple. *J. R. Statist. Soc.* **1995**, *57*, 289–300.

Burguete-García, A.I.; Valdés Villalpando, Y.N.; Cruz, M. Definiciones para el diagnóstico de síndrome metabólico en población infantil. *Gac. Médica México* **2014**, *150*, 79–87.

Carey, V. J.; Walters, E. E.; Colditz, G. A.; Solomon, C. G.; Willett, W. C.; Rosner, B. A.; Speizer, F. E.; Manson, J. E. Body fat distribution and risk of non-insulin-dependent diabetes mellitus in women. The Nurses' Health Study. *Am. J. Epidemiol.* **1997**, *145*, 614–9.

Catry, E.; Bindels, L.B.; Tailleux, A.; Lestavel, S.; Neyrinck, A.M.; Goossens, J.F.; Lobysheva, I.; Plovier, H.; Essaghir, A.; Demoulin, J.B.; et al. Targeting the gut microbiota with inulin-type fructans: Preclinical demonstration of a novel approach in the management of endothelial dysfunction. *Gut* **2018**, *67*, 271–283, doi:10.1136/gutjnl-2016-313316.

CDC BMI-for-age growth charts, 2000. https://www.cdc.gov/growthcharts/data/set2clinical/cj41l074.pdf. [Accessed on April 14, 2019].

Chávez-Carbajal, A.; Nirmalkar, K.; Pérez-Lizaur, A.; Hernández-Quiroz, F.; Ramírez-del-Alto, S.; García-Mena, J.; Hernández-Guerrero, C. Gut Microbiota and Predicted Metabolic Pathways in a Sample of Mexican Women Affected by Obesity and Obesity Plus Metabolic Syndrome. *Int. J. Mol. Sci.* **2019**, *20*, 438,

doi:10.3390/ijms20020438.

Chen, J.; Wright, K.; Davis, J.M.; Jeraldo, P.; Marietta, E.V.; Murray, J.; Nelson, H.; Matteson, E.L.; Taneja, V. An expansion of rare lineage intestinal microbes characterizes rheumatoid arthritis. *Genome Med.* **2016**, *8*, 43, doi:10.1186/s13073-016-0299-7.

Clavel, T.; Desmarchelier, C.; Haller, D.; Gérard, P.; Rohn, S.; Lepage, P.; Daniel, H. Intestinal microbiota in metabolic diseases. *Gut Microbes* **2014**, *5*, 544–551, doi:10.4161/gmic.29331.

Cummings, J.H.; Pomare, E.W.; Branch, W.J.; Naylor, C.P.; Macfarlane, G.T. Short chain fatty acids in human large intestine, portal, hepatic and venous blood. *Gut* **1987**, *28*, 1221–1227.

Davignon, J.; Ganz, P. Role of endothelial dysfunction in atherosclerosis. *Circulation* **2004**, *109*, III-27–III-32, doi:10.1161/01.CIR.0000131515.03336.f8.

De Baere, S.; Eeckhaut, V.; Steppe, M.; De Maesschalck, C.; De Backer, P.; Van Immerseel, F.; Croubels, S. Development of a HPLC–UV method for the quantitative determination of four short-chain fatty acids and lactic acid produced by intestinal bacteria during in vitro fermentation. *J. Pharm. Biomed. Anal.* **2013**, *80*, 107–115, doi:10.1016/j.jpba.2013.02.032.

de La Serre, C.B.; Ellis, C.L.; Lee, J.; Hartman, A.L.; Rutledge, J.C.; Raybould, H.E. Propensity to high-fat diet-induced obesity in rats is associated with changes in the gut microbiota and gut inflammation. *Am. J. Physiol. Liver Physiol.* **2010**, *299*, G440–G448, doi:10.1152/ajpgi.00098.2010.

de Onis, M.; Onyango, A.W.; Borghi, E.; Siyam, A.; Nishida, C.; Siekmann, J. Development of a WHO growth reference for school-aged children and adolescents. *Bull. World Health Organ.* **2007**, *85*, 660–667.

Dron, J.S.; Hegele, R.A. Genetics of triglycerides and the risk of atherosclerosis. *Curr. Atheroscler. Rep.* **2017**, *19*, 31, doi:10.1007/s11883-017-0667-9.

Du, H.; Feskens, E. Dietary determinants of obesity. *Acta Cardiol.* **2010**, *65*, 377–386, doi:10.2143/AC.65.4.2053895.

Duncan, S. H.; Barcenilla, A.; Stewart, C. S.; Pryde, S. E.; Flint, H. J. Acetate utilization and butyryl coenzyme A (CoA):acetate-CoA transferase in butyrate-

producing bacteria from the human large intestine. *Appl. Environ. Microbiol.* **2002**, *68*, 5186–90, doi:10.1128/AEM.68.10.5186-5190.2002.

Eikemo, H.; Sellevold, O.F.M.; Videm, V. Markers for endothelial activation during open heart surgery. *Ann. Thorac. Surg.* **2004**, *77*, 214–219.

ENSANUT 2016.; Salud, D.E. Directorio Secretaría De Salud. https://www.gob.mx/cms/uploads/attachment/file/209093/ENSANUT.pdf. (accessed on 5 October 2018).

Faust, K.; Raes, J. CoNet app: Inference of biological association networks using Cytoscape. *F1000Res.* **2016**, *5*, 1519.

Flammer, A.J.; Anderson, T.; Celermajer, D.S.; Creager, M.A.; Deanfield, J.; Ganz, P.; Hamburg, N.M.; Lüscher, T.F.; Shechter, M.; Taddei, S.; et al. The assessment of endothelial function. *Circulation* **2012**, *126*, 753–767, doi:10.1161/CIRCULATIONAHA.112.093245.

Freedman, D.S.; Mei, Z.; Srinivasan, S.R.; Berenson, G.S.; Dietz, W.H. Cardiovascular risk factors and excess adiposity among overweight children and adolescents: The Bogalusa heart study. *J. Pediatr.* **2007**, *150*, 12–17, doi:10.1016/j.jpeds.2006.08.042.

Friedewald, W.T.; Levy, R.I.; Fredrickson, D.S. Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge, *Clin. Chem.* **1972**, *18*, 499–502.

Gomes, F.; Telo, D.F.; Souza, H.P.; Nicolau, J.C.; Halpern, A.; Serrano, C.V. Obesity and coronary artery disease: Role of vascular inflammation. *Arq. Bras. Cardiol.* **2010**, *94*, 273–279.

Hadi, H.A.R.; Carr, C.S.; Al Suwaidi, J. Endothelial dysfunction: Cardiovascular risk factors, therapy, and outcome. *Vasc. Health Risk Manag.* **2005**, *1*, 183–198.

Holub, C. K.; Elder, J. P.; Arredondo, E. M.; Barquera, S.; Eisenberg, C. M.; Sánchez Romero, L. M.; Rivera, J.; Lobelo, F.; Simoes, E. J. Obesity control in Latin American and U.S. Latinos: a systematic review. *Am. J. Prev. Med.* **2013**, *44*, 529–37, doi:10.1016/j.amepre.2013.01.023.

Jandhyala, S. M.; Talukdar, R.; Subramanyam, C.; Vuyyuru, H.; Sasikala, M.; Nageshwar Reddy, D. Role of the normal gut microbiota. *World J. Gastroenterol.*

2015, *21*, 8787–803, doi:10.3748/wjg.v21.i29.8787.

Kant, R.; Blom, J.; Palva, A.; Siezen, R.J.; de Vos, W.M. Comparative genomics of Lactobacillus. *Microb. Biotechnol.* **2011**, *4*, 323–332, doi:10.1111/j.1751-7915.2010.00215.x.

Karlsson, F.H.; Fåk, F.; Nookaew, I.; Tremaroli, V.; Fagerberg, B.; Petranovic, D.; Bäckhed, F.; Nielsen, J. Symptomatic atherosclerosis is associated with an altered gut metagenome. *Nat. Commun.* **2012**, *3*, 1245, doi:10.1038/ncomms2266.

Kannel, W. B.; D'Agostino, R. B.; Cobb, J. L. Effect of weight on cardiovascular disease. *Am. J. Clin. Nutr.* **1996**, *63*, 419S–422S, doi:10.1093/ajcn/87.6.1602.

Karolina, D.S. Silambarasan, M., Armugam, A., Jeyaseelan K. MicroRNAs and endothelial dysfunction in relation to obesity and type 2 diabetes. *J. Mol. Genet. Med.* **2014**, *S1*, doi:10.4172/1747-0862.S1-011.

Kasai, C.; Sugimoto, K.; Moritani, I.; Tanaka, J.; Oya, Y.; Inoue, H.; Tameda, M.; Shiraki, K.; Ito, M.; Takei, Y.; et al. Comparison of the gut microbiota composition between obese and non-obese individuals in a Japanese population, as analyzed by terminal restriction fragment length polymorphism and next-generation sequencing. *BMC Gastroenterol.* **2015**, *15*, 100, doi:10.1186/s12876-015-0330-2.

Khalyfa, A.; Kheirandish-Gozal, L.; Bhattacharjee, R.; Khalyfa, A.A.; Gozal, D. Circulating microRNAs as potential biomarkers of endothelial dysfunction in obese children. *Chest* **2016**, *149*, 786–800, doi:10.1378/chest.15-0799.

Koren, O.; Spor, A.; Felin, J.; Fak, F.; Stombaugh, J.; Tremaroli, V.; Behre, C.J.; Knight, R.; Fagerberg, B.; Ley, R.E., Bäckhed F. Human oral, gut, and plaque microbiota in patients with atherosclerosis. *Proc. Natl. Acad. Sci.* **2011**, *108*, 4592–4598, doi:10.1073/pnas.1011383107.

Korkmaz, H.; Onalan, O. Evaluation of endothelial dysfunction: Flow-mediated dilation. *Endothelium* **2008**, *15*, 157–163, doi:10.1080/10623320802228872.

Larsen, J.M. The immune response to *Prevotella* bacteria in chronic inflammatory disease. *Immunology* **2017**, *151*, 363–374, doi:10.1111/imm.12760.

Lerman, A.; Burnett, J. C. Intact and altered endothelium in regulation of vasomotion. *Circulation* **1992**, *86*, III12-19.

Ley, R.E.; Turnbaugh, P.J.; Klein, S.; Gordon, J.I. Human gut microbes associated with obesity. *Nature* **2006**, *444*, 1022–1023, doi:10.1038/4441022a.

Liu, Z.; Li, J.; Liu, H.; Tang, Y.; Zhan, Q.; Lai, W.; Ao, L.; Meng, X.; Ren, H.; Xu, D.; Zeng, Q. The intestinal microbiota associated with cardiac valve calcification differs from that of coronary artery disease. *Atherosclerosis* **2019**, *284*, 121–128, doi:10.1016/j.atherosclerosis.2018.11.038.

López-García, E.; Schulze, M.B.; Fung, T.T.; Meigs, J.B.; Rifai, N.; Manson, J.E.; Hu, F.B. Major dietary patterns are related to plasma concentrations of markers of inflammation and endothelial dysfunction. *Am. J. Clin. Nutr.* **2004**, *80*, 1029–1035, doi:10.1093/ajcn/80.4.1029.

Louis, P.; Young, P.; Holtrop, G.; Flint, H. J. Diversity of human colonic butyrate-producing bacteria revealed by analysis of the butyryl-CoA:acetate CoA-transferase gene. *Environ. Microbiol.* **2010**, *12*, 304–314, doi:10.1111/j.1462-2920.2009.02066.x.

Ludmer, P.L.; Selwyn, A.P.; Shook, T.L.; Wayne, R.R.; Mudge, G.H.; Alexander, R.W.; Ganz, P. Paradoxical vasoconstriction induced by acetylcholine in atherosclerotic coronary arteries. *N. Engl. J. Med.* **1986**, *315*, 1046–1051, doi:10.1056/NEJM198610233151702.

Madrigal, J. C.; Correa, S. M. L.; Gómez, V. G.; Ramírez, T. L.; Gamiño, G. B.; Pérez, Y. C. Valores de función endotelial en niños mexicanos. *Med. Interna Mex.* **2011**, *27*, 429–438.

Magge, S.N.; Goodman, E.; Armstrong, S.C. The metabolic syndrome in children and adolescents: Shifting the focus to cardiometabolic risk factor clustering. *Pediatrics* **2017**, *140*, e20171603, doi:10.1542/peds.2017-1603.

Marchesi, J. R.; Adams, D. H.; Fava, F.; Hermes, G. D. A.; Hirschfield, G. M.; Hold, G.; Quraishi, M. N.; Kinross, J.; Smidt, H.; Tuohy, K. M.; Thomas, L. V; Zoetendal, E. G.; Hart, A. The gut microbiota and host health: a new clinical frontier. *Gut* **2016**, *65*, 330–9, doi:10.1136/gutjnl-2015-309990.

Matthews, D.R.; Hosker, J.P.; Rudenski, A.S.; Naylor, B.A.; Treacher, D.F.; Turner, R.C. Homeostasis model assessment: Insulin resistance and beta-cell function

from fasting plasma glucose and insulin concentrations in man. *Diabetologia* **1985**, *28*, 412–419.

Maya-Lucas, O.; Murugesan, S.; Nirmalkar, K.; Alcaraz, L. D.; Hoyo-Vadillo, C.; Pizano-Zárate, M. L.; García-Mena, J. The gut microbiome of Mexican children affected by obesity. *Anaerobe* **2019**, *55*, 11–23, doi:10.1016/j.anaerobe.2018.10.009.

Meyers, M.R.; Gokce, N. Endothelial dysfunction in obesity: Etiological role in atherosclerosis. *Curr. Opin. Endocrinol. Diabetes. Obes.* **2007**, *14*, 365–369, doi:10.1097/MED.0b013e3282be90a8.

Million, M.; Angelakis, E.; Paul, M.; Armougom, F.; Leibovici, L.; Raoult, D. Comparative meta-analysis of the effect of Lactobacillus species on weight gain in humans and animals. *Microb. Pathog.* **2012**, *53*, 100–108, doi:10.1016/j.micpath.2012.05.007.

Morgan, X.C.; Tickle, T.L.; Sokol, H.; Gevers, D.; Devaney, K.L.; Ward, D. V.; Reyes, J.A.; Shah, S.A.; LeLeiko, N.; Snapper, S.B.; et al. Dysfunction of the intestinal microbiome in inflammatory bowel disease and treatment. *Genome Biol.* **2012**, *13*, R79, doi:10.1186/gb-2012-13-9-r79.

Murugesan, S.; Nirmalkar, K.; Hoyo-Vadillo, C.; García-Espitia, M.; Ramírez-Sánchez, D.; García-Mena, J. Gut microbiome production of short-chain fatty acids and obesity in children. *Eur. J. Clin. Microbiol. Infect. Dis.* **2018**, *37*, 621–625, doi:10.1007/s10096-017-3143-0.

Murugesan, S.; Ulloa-Martínez, M.; Martínez-Rojano, H.; Galván-Rodríguez, F.M.; Miranda-Brito, C.; Romano, M.C.; Piña-Escobedo, A.; Pizano-Zárate, M.L.; Hoyo-Vadillo, C.; García-Mena, J. Study of the diversity and short-chain fatty acids production by the bacterial community in overweight and obese Mexican children. *Eur. J. Clin. Microbiol. Infect. Dis.* **2015**, *34*, 1337–1346, doi:10.1007/s10096-015-2355-4.

NCEP (National Cholesterol Education Program), American Academy of Pediatrics: Report of the Expert Panel on Blood Cholesterol Levels in Children and Adolescents; NIH Publication: Bethesda, MD, USA, 1992; p.2732.

Nirmalkar, K.; Murugesan, S.; Pizano-Zárate, M.L.; Romero-Figueroa, S.; Hoyo-Vadillo, C.; García-Mena, J. Endothelial dysfunction in Mexican obese children, is there a role of the gut microbiota ? *Obes. Control Ther.* **2017** doi: 10.15226/2374-8354/2/2/00127.

Obesity and Overweight 2018. Available online: http://www.who.int/news-room/fact-sheets/detail/obesity-and-overweight (accessed on 25 September 2018).

Obesity Update 2017. https://www.oecd.org/els/health-systems/Obesity-Update-2017.pdf (accessed on 25 September 2018).

Ohashi, K.; Shibata, R.; Murohara, T.; Ouchi, N. Role of anti-inflammatory adipokines in obesity-related diseases. *Trends Endocrinol. Metab.* **2014**, *25*, 348–355, doi:10.1016/j.tem.2014.03.009.

Perichart-Perera, O.; Muñoz-Manrique, C.; Reyes-López, A.; Tolentino-Dolores, M.; Espino, Y. Sosa, S.; Ramírez-González, M.C. Metabolic markers during pregnancy and their association with maternal and newborn weight status. *PLoS ONE* **2017**, *12*, e0180874, doi:10.1371/journal.pone.0180874.

Rey, F. E.; Faith, J. J.; Bain, J.; Muehlbauer, M. J.; Stevens, R. D.; Newgard, C. B.; Gordon, J. I. Dissecting the *in Vivo* Metabolic Potential of Two Human Gut Acetogens. *J. Biol. Chem.* **2010**, *285*, 22082–22090, doi:10.1074/jbc.M110.117713.

Rodríguez-Saldaña, J.; Rodríguez-Flores, M.; Cantú-Brito, C.; Aguirre-García, J. A pathological study of the epidemiology of atherosclerosis in Mexico city. *Cardiol. Res. Pract.* **2014**, *2014*, 264205, doi:10.1155/2014/264205.

Rojas, E.; Rodríguez-Molina, D.; Bolli, P.; Israili, Z.H.; Faría, J.; Fidilio, E.; Bermúdez, V.; Velasco, M. The role of adiponectin in endothelial dysfunction and hypertension. *Curr. Hypertens. Rep.* **2014**, *16*, 463, doi:10.1007/s11906-014-0463-7.

Ross, R. Atherosclerosis--an inflammatory disease. *N. Engl. J. Med.* **1999**, *340*, 115–26, doi:10.1056/NEJM199901143400207.

Schächinger, V.; Zeiher, A.M. Atherosclerosis-associated endothelial dysfunction. *Aïrztez. Kardiol.* **2000**, *89*, IX70–IX4.

Scher, J. U.; Sczesnak, A.; Longman, R. S.; Segata, N.; Ubeda, C.; Bielski, C.; Rostron, T.; Cerundolo, V.; Pamer, E. G.; Abramson, S. B.; Huttenhower, C.; Littman, D. R. Expansion of intestinal *Prevotella copri* correlates with enhanced susceptibility to arthritis. *Elife* **2013**, *2*, 1–20, doi:10.7554/eLife.01202.

Scott, K. P.; Martin, J. C.; Campbell, G.; Mayer, C.-D.; Flint, H. J. Whole-Genome Transcription Profiling Reveals Genes Up-Regulated by Growth on Fucose in the Human Gut Bacterium "*Roseburia inulinivorans*." *J. Bacteriol.* **2006**, *188*, 4340–4349, doi:10.1128/JB.00137-06.

Segata, N.; Izard, J.; Waldron, L.; Gevers, D.; Miropolsky, L.; Garrett, W.S.; Huttenhower, C. Metagenomic biomarker discovery and explanation. *Genome Biol.* **2011**, *12*, R60, doi:10.1186/gb-2011-12-6-r60.

Szmitko, P. E.; Wang, C.-H.; Weisel, R. D.; de Almeida, J. R.; Anderson, T. J.; Verma, S. New Markers of Inflammation and Endothelial Cell Activation. *Circulation* **2003**, *108*, 1917–1923, doi:10.1161/01.CIR.0000089190.95415.9F.

Tang, W. H. W.; Wang, Z.; Levison, B. S.; Koeth, R. A.; Britt, E. B.; Fu, X.; Wu, Y.; Hazen, S. L. Intestinal microbial metabolism of phosphatidylcholine and cardiovascular risk. *N. Engl. J. Med.* **2013**, *368*, 1575–84, doi:10.1056/NEJMoa1109400.

Turnbaugh, P.J.; Ley, R.E.; Mahowald, M.A.; Magrini, V.; Mardis, E.R.; Gordon, J.I. An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature* **2006**, *444*, 1027–1031, doi:10.1038/nature05414.

Tremaroli, V.; Bäckhed, F. Functional interactions between the gut microbiota and host metabolism. *Nature* **2012**, *489*, 242–9, doi:10.1038/nature11552.

Vikram, A.; Kim, Y.R.; Kumar, S.; Li, Q.; Kassan, M.; Jacobs, J.S.; Irani, K. Vascular microRNA-204 is remotely governed by the microbiome and impairs endothelium-dependent vasorelaxation by downregulating Sirtuin1. *Nat. Commun.* **2016**, *7*, 1–9, doi:10.1038/ncomms12565.

Weiss, R.; Dziura, J.; Burgert, T.S.; Tamborlane, W.V.; Taksali, S.E.; Yeckel, C.W.; Allen, K.; Lopes, M.; Savoye, M.; Morrison, J.; et al. Obesity and the Metabolic Syndrome in Children and Adolescents. *N. Engl. J. Med.* **2004**, *350*, 2362–2374, doi:10.1056/NEJMoa031049.

Widlansky, M.E.; Gokce, N.; Keaney, J.F.; Vita, J.A. The clinical implications of endothelial dysfunction. *J. Am. Coll. Cardiol.* **2003**, *42*, 1149–1160, doi:10.1016/S0735-1097(03)00994-X.

Wild, S. H.; Byrne, C. D. ABC of obesity. Risk factors for diabetes and coronary heart disease. *BMJ* **2006**, *333*, 1009–11, doi:10.1136/bmj.39024.568738.43.

World Health Organization. (2008). The Global Burden of Disease 2004 UPDATE. World Health Organization.

Wurdemann, D.; Tindall, B.J.; Pukall, R.; Lunsdorf, H.; Strompl, C.; Namuth, T.; Nahrstedt, H.; Wos-Oxley, M.; Ott, S.; Schreiber, S.; et al. Gordonibacter pamelaeae gen. nov., sp. nov., a new member of the Coriobacteriaceae isolated from a patient with Crohn's disease, and reclassification of Eggerthella hongkongensis Lau et al. 2006 as Paraeggerthella hongkongensis gen. nov., comb. nov. *Int. J. Syst. Evol. Microbiol.* **2009**, *59*, 1405–1415, doi:10.1099/ijs.0.005900-0.

Yadav, A.; Kataria, M.A.; Saini, V.; Yadav, A. Role of leptin and adiponectin in insulin resistance. *Clin. Chim. Acta* **2013**, *417*, 80–84, doi:10.1016/j.cca.2012.12.007.

Yamaoka-Tojo, M. Endothelial Function for Cardiovascular Disease Prevention and Management. *Int. J. Clin. Cardiol.* **2017**, 4:103, doi: 10.23937/2378-2951/1410103.

Zitomersky, N.L.; Atkinson, B.J.; Franklin, S.W.; Mitchell, P.D.; Snapper, S.B.; Comstock, L.E.; Bousvaros, A. Characterization of adherent bacteroidales from intestinal biopsies of children and young adults with inflammatory bowel disease. *PLoS ONE* **2013**, *8*, e63686, doi:10.1371/journal.pone.0063686.

13. Appendix

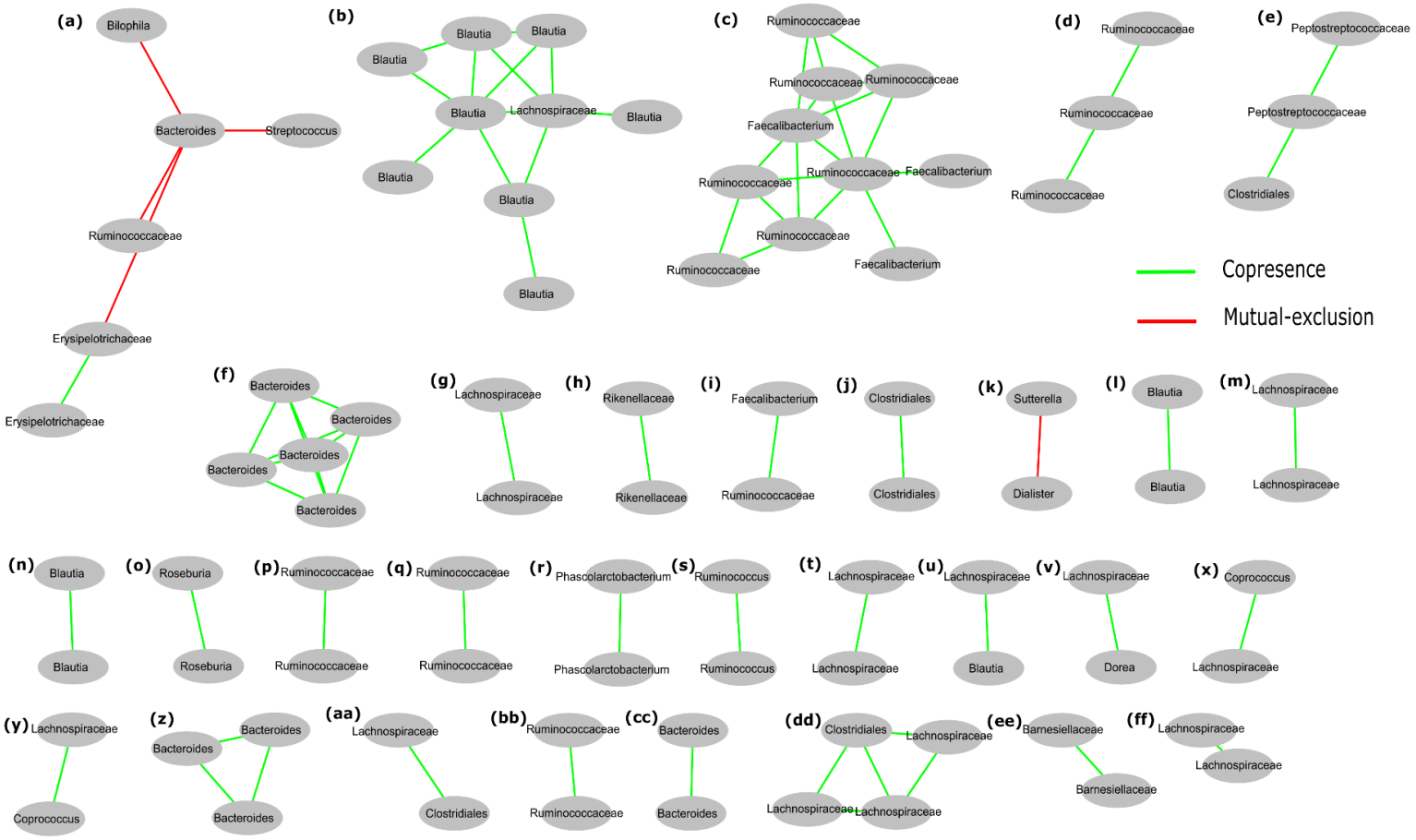

Figure A1. Significant co-occurrence analysis between gut microbiota in normal weight Mexican children (a-ff). This graphic shows copresence (positive interaction; green lines) and mutual exclusion (negative interaction; red lines). Each node indicates a microbial clade (bacterial taxon) belonging to a unique OTUs number. Edges (lines) are connecting two nodes, represent significant correlations ($p < .05$; $q < 0.05$; $R > 0.8$).

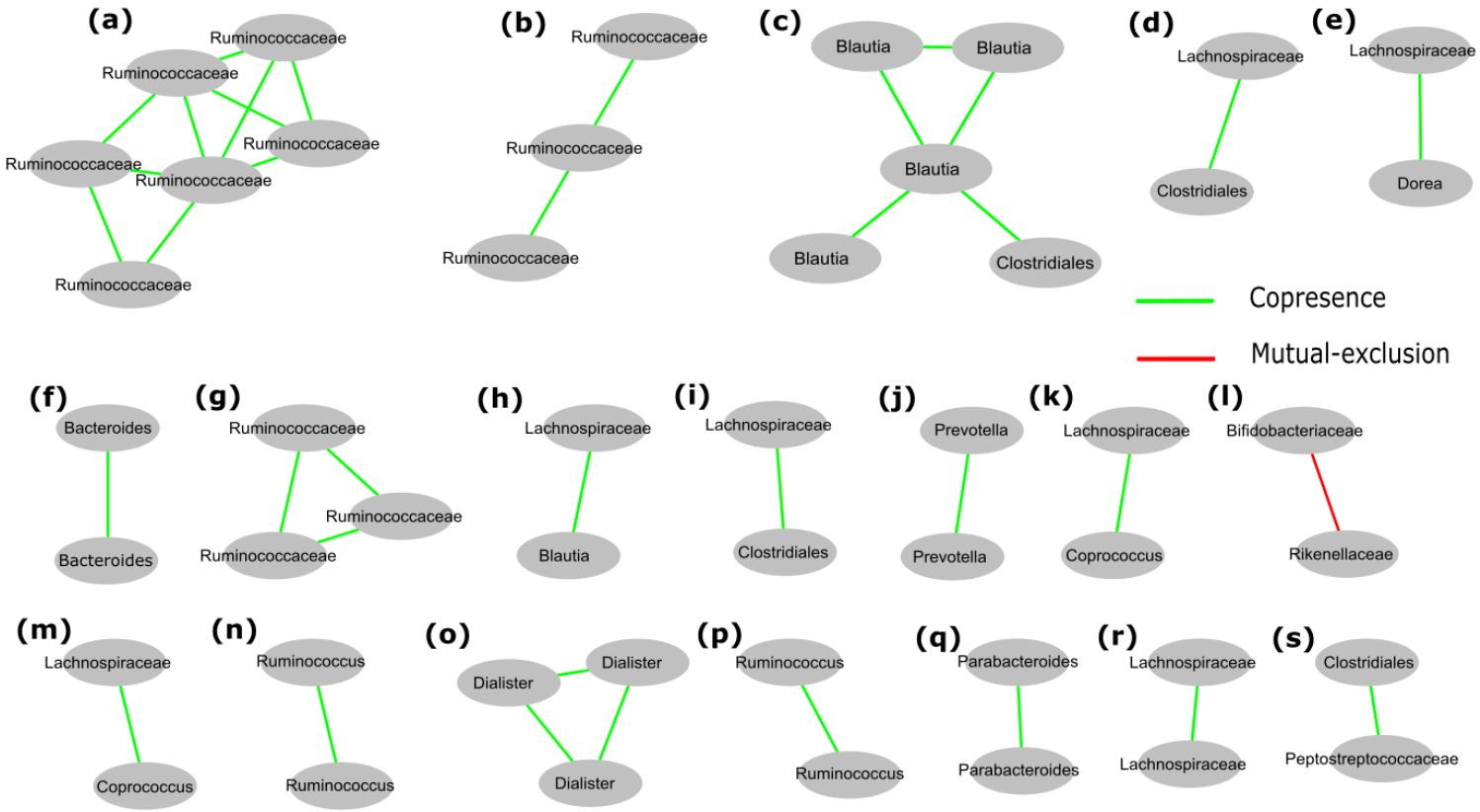

Figure A2. Significant co-occurrence analysis between gut microbiota in normal weight Mexican adolescents (a-s). This graphic shows copresence (positive interaction; green lines) and mutual exclusion (negative interaction; red lines). Each node indicates a microbial clade (bacterial taxon) belonging to a unique OTUs number. Edges (lines) are connecting two nodes, represent significant correlations ($p < .05$; $q < 0.05$; $R > 0.8$).

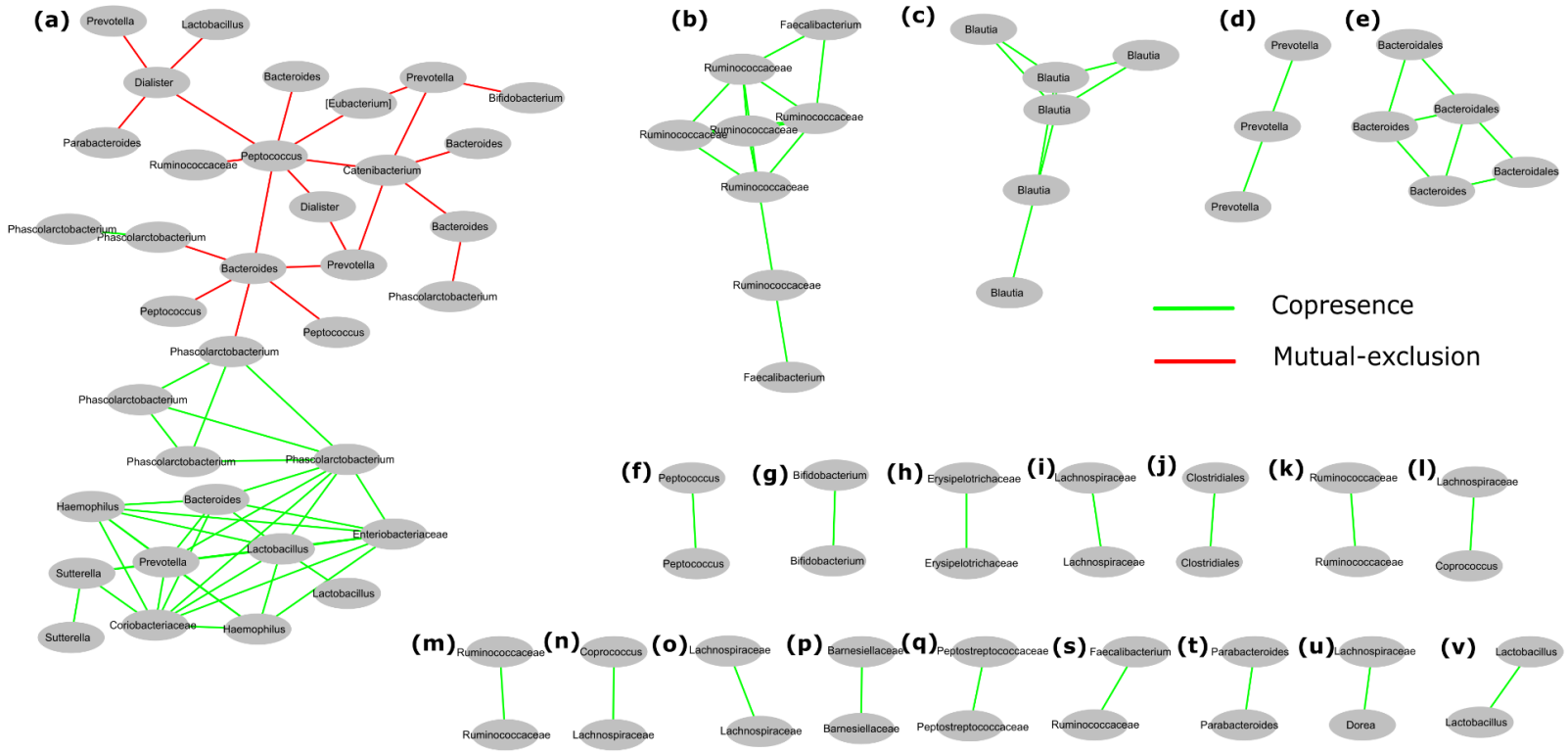

Figure A3. Significant co-occurrence analysis between gut microbiota in obese Mexican children (a-v). This graphic shows copresence (positive interaction; green lines) and mutual exclusion (negative interaction; red lines). Each node indicates a microbial clade (bacterial taxon) belonging to a unique OTUs number. Edges (lines) are connecting two nodes, represent significant correlations ($p < .05$; $q < 0.05$; $R > 0.8$).

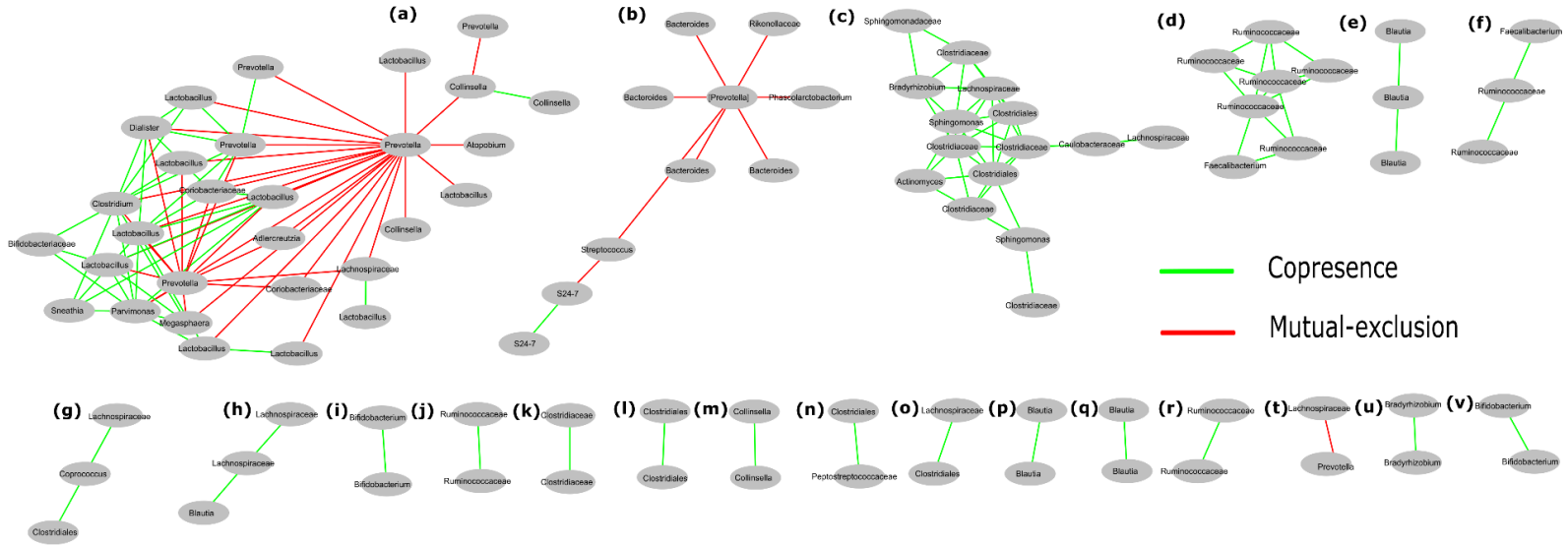

Figure A4. Significant co-occurrence analysis between gut microbiota in obese Mexican adolescents (a-v). This graphic shows copresence (positive interaction; green lines) and mutual exclusion (negative interaction; red lines). Each node indicates a microbial clade (bacterial taxon) belonging to a unique OTUs number. Edges (lines) are connecting two nodes, represent significant correlations ($p < .05$; $q < 0.05$; $R > 0.8$).

Table A1. List of bacterial taxa with their OTUs ID in normal weight children for Figure A1.

No.	OTUs ID	Bacteria	No.	OTUs ID	Bacteria
1	10496	Lachnospiraceae	46	370154	Ruminococcaceae
2	12246	*Bacteroides*	47	409732	*Blautia*
3	12426	*Bilophila*	48	508984	Lachnospiraceae
4	14479	Ruminococcaceae	49	519765	Lachnospiraceae
5	14550	Erysipelotrichaceae	50	523782	*Coprococcus*
6	15355	*Blautia*	51	529740	*Roseburia*
7	22335	Rikenellaceae	52	529940	Ruminococcaceae
8	179384	Lachnospiraceae	53	530094	Ruminococcaceae
9	182107	Lachnospiraceae	54	531582	*Blautia*
10	188887	*Bacteroides*	55	532771	Lachnospiraceae
11	190358	Ruminococcaceae	56	535549	*Bacteroides*
12	190975	Barnesiellaceae	57	548233	*Clostridiales*
13	192406	Ruminococcaceae	58	548503	Lachnospiraceae
14	193041	*Blautia*	59	552380	*Ruminococcus*
15	194341	*Bacteroides*	60	556835	*Phascolarctobacterium*
16	194395	Barnesiellaceae	61	560535	Ruminococcaceae
17	194909	*Bacteroides*	62	570507	*Blautia*
18	195222	Ruminococcaceae	63	577170	*Bacteroides*
19	198145	*Blautia*	64	579561	*Blautia*
20	208739	*Faecalibacterium*	65	580008	Erysipelotrichaceae
21	210371	Ruminococcaceae	66	580629	*Bacteroides*
22	264552	*Dialister*	67	581003	*Clostridiales*
23	267718	*Faecalibacterium*	68	583089	*Blautia*
24	287951	*Clostridiales*	69	591891	Lachnospiraceae
25	297150	Lachnospiraceae	70	593422	*Blautia*
26	301910	Lachnospiraceae	71	659361	*Roseburia*
27	316761	*Bacteroides*	72	696563	*Blautia*
28	318970	*Blautia*	73	700298	*Dorea*
29	322380	Ruminococcaceae	74	702414	Lachnospiraceae
30	325244	Lachnospiraceae	75	708680	*Clostridiales*
31	337167	Ruminococcaceae	76	712677	Peptostreptococcaceae
32	347115	*Blautia*	77	727140	*Clostridiales*
33	351231	*Bacteroides*	78	804526	*Blautia*
34	355837	Ruminococcaceae	79	851865	Ruminococcaceae
35	358410	Ruminococcaceae	80	875709	Lachnospiraceae
36	359359	Ruminococcaceae	81	1105552	Lachnospiraceae
37	360158	Lachnospiraceae	82	1106861	*Coprococcus*
38	361966	*Faecalibacterium*	83	1107327	Lachnospiraceae
39	362968	Ruminococcaceae	84	1111458	Peptostreptococcaceae
40	365496	*Bacteroides*	85	2198356	*Bacteroides*
41	365717	*Faecalibacterium*	86	2388088	*Phascolarctobacterium*
42	368117	Ruminococcaceae	87	3678349	*Streptococcus*
43	369027	Lachnospiraceae	88	4338624	*Sutterella*
44	369109	Ruminococcaceae	89	4476780	Rikenellaceae
45	369555	*Ruminococcus*			

Table A2. List of bacterial taxa with their OTUs ID in normal weight adolescents for Figure A2.

No.	OTUs ID	Bacteria	No.	OTUs ID	Bacteria
1	583	*Dialister*	25	521982	Rikenellaceae
2	1060	*Prevotella*	26	523782	*Coprococcus*
3	8103	Ruminococcaceae	27	527751	Lachnospiraceae
4	9799	Bifidobacteriaceae	28	529940	Ruminococcaceae
5	10094	Lachnospiraceae	29	530094	Ruminococcaceae
6	10254	Ruminococcaceae	30	531582	*Blautia*
7	10888	*Ruminococcus*	31	548503	Lachnospiraceae
8	178664	*Clostridiales*	32	552380	*Ruminococcus*
9	180082	*Parabacteroides*	33	560535	Ruminococcaceae
10	182431	Ruminococcaceae	34	570507	Lachnospiraceae
11	190358	Ruminococcaceae	35	577170	*Bacteroides*
12	192365	*Coprococcus*	36	579561	*Blautia*
13	195222	Ruminococcaceae	37	580629	*Bacteroides*
14	198866	*Parabacteroides*	38	583089	*Blautia*
15	228199	*Ruminococcus*	39	591891	Lachnospiraceae
16	264552	*Dialister*	40	593422	*Blautia*
17	272587	*Dialister*	41	700298	Lachnospiraceae
18	287951	*Clostridiales*	42	702414	Lachnospiraceae
19	325244	Lachnospiraceae	43	708680	*Clostridiales*
20	368117	Ruminococcaceae	44	712677	*Clostridiales*
21	369027	Lachnospiraceae	45	851865	Ruminococcaceae
22	369555	*Ruminococcus*	46	1105552	Lachnospiraceae
23	370086	Ruminococcaceae	47	4314092	*Prevotella*
24	370154	Ruminococcaceae			

Table A3. List of bacterial taxa with their OTUs ID in obese children for Figure A3.

No.	OTUs ID	Bacteria	No.	OTUs ID	Bacteria
1	335	*Phascolarctobacterium*	46	362214	*Bifidobacterium*
2	1899	*Peptococcus*	47	362968	Ruminococcaceae
3	4007	*Peptococcus*	48	365496	*Bacteroides*
4	6529	*Lactobacillus*	49	365717	*Faecalibacterium*
5	7389	*Haemophilus*	50	369027	Lachnospiraceae
6	8433	Clostridiales	51	370086	Ruminococcaceae
7	9453	*Prevotella*	52	370154	Ruminococcaceae
8	11321	*Phascolarctobacterium*	53	403701	*Dialister*
9	11423	*Bacteroides*	54	517282	*Blautia*
10	12345	*Phascolarctobacterium*	55	521982	Clostridiales
11	13188	*Prevotella*	56	522364	*Prevotella*
12	13949	Ruminococcaceae	57	523782	*Coprococcus*
13	14550	Erysipelotrichaceae	58	524371	*Prevotella*
14	19296	*Bacteroides*	59	524884	*Eubacterium*
15	19314	*Parabacteroides*	60	529940	Ruminococcaceae
16	21736	*Phascolarctobacterium*	61	530094	Ruminococcaceae
17	22231	*Haemophilus*	62	531582	*Blautia*
18	22619	*Peptococcus*	63	531928	Clostridiales
19	22894	Ruminococcaceae	64	560535	Ruminococcaceae
20	24722	*Bacteroides*	65	567846	Bifidobacterium
21	41229	*Sutterella*	66	570507	*Blautia*
22	130864	*Lactobacillus*	67	574111	*Prevotella*
23	157424	*Phascolarctobacterium*	68	580008	Erysipelotrichaceae
24	179261	*Sutterella*	69	583746	*Dialister*
25	180082	*Parabacteroides*	70	587753	Coriobacteriaceae
26	183480	Rikenellaceae	71	591891	Lachnospiraceae
27	183603	*Bacteroides*	72	593422	*Blautia*
28	190358	Ruminococcaceae	73	639310	*Bifidobacterium*
29	190975	Barnesiellaceae	74	700298	*Dorea*
30	193723	Bacteroidales	75	702414	Lachnospiraceae
31	194395	Barnesiellaceae	76	708680	Clostridiales
32	195222	Ruminococcaceae	77	712677	Peptostreptococcaceae
33	196604	*Catenibacterium*	78	716286	*Lactobacillus*
34	198866	*Parabacteroides*	79	804526	*Blautia*
35	202162	Ruminococcaceae	80	849535	*Prevotella*
36	208739	Faecalibacterium	81	850218	Phascolarctobacterium
37	215331	*Peptococcus*	82	851865	Ruminococcaceae
38	230403	*Bacteroides*	83	875709	Lachnospiraceae
39	235591	*Lactobacillus*	84	1105328	*Blautia*
40	236308	*Lactobacillus*	85	1105552	Lachnospiraceae
41	269937	*Prevotella*	86	1106861	*Coprococcus*
42	293883	*Phascolarctobacterium*	87	1107327	Lachnospiraceae
43	309133	Enterococcaceae	88	1111458	Peptostreptococcaceae
44	339685	*Peptococcus*	89	4226929	*Bacteroides*
45	361966	*Faecalibacterium*			

Table A4. List of bacterial taxa with their OTUs ID in obese adolescents for Figure A4.

No.	OTUs ID	Bacteria	No.	OTUs ID	Bacteria
1	1002	*Sphingomonas*	51	362214	*Bifidobacterium*
2	1184	*Collinsella*	52	362968	Ruminococcaceae
3	4568	*Bradyrhizobium*	53	363794	*Collinsella*
4	5337	Clostridiaceae	54	365496	*Bacteroides*
5	6613	Bradyrhizobium	55	365717	*Faecalibacterium*
6	7367	*Prevotella*	56	368175	*Collinsella*
7	7786	Clostridiales	57	369109	Ruminococcaceae
8	9022	*Lactobacillus*	58	370154	Ruminococcaceae
9	9328	*Collinsella*	59	383885	*Lactobacillus*
10	10094	Lachnospiraceae	60	469663	*Atopobium*
11	10254	Ruminococcaceae	61	470382	Clostridiales
12	10278	*Lactobacillus*	62	471180	Bifidobacterium
13	10560	*Collinsella*	63	529940	Ruminococcaceae
14	11912	Clostridiaceae	64	530094	Ruminococcaceae
15	12479	*Lactobacillus*	65	531582	*Blautia*
16	12723	Lachnospiraceae	66	556835	*Phascolarctobacterium*
17	12734	Ruminococcaceae	67	560336	*Bacteroides*
18	12974	*Lactobacillus*	68	560535	Ruminococcaceae
19	13485	*Bifidobacterium*	69	566154	Coriobacteriaceae
20	29566	*Sneathia*	70	567846	*Bifidobacterium*
21	128300	*Lactobacillus*	71	568118	*Prevotella*
22	130468	*Lactobacillus*	72	570507	*Blautia*
23	130864	*Lactobacillus*	73	579608	*Streptococcus*
24	133372	*Parvimonas*	74	583089	*Blautia*
25	137183	Bifidobacteriaceae	75	584347	*Actinomyces*
26	177679	S24-7	76	589071	*Bacteroides*
27	183988	Clostridiales	77	593422	*Blautia*
28	188956	*Blautia*	78	663885	*Prevotella*
29	190358	Ruminococcaceae	79	696563	*Blautia*
30	191112	Lachnospiraceae	80	708680	Clostridiales
31	195222	Ruminococcaceae	81	712047	Clostridiaceae
32	197060	Clostridiaceae	82	712677	Clostridiales
33	198915	*Blautia*	83	826270	*Bradyrhizobium*
34	208739	*Faecalibacterium*	84	826382	Lachnospiraceae
35	225846	*Dialister*	85	840914	*Prevotella*
36	236308	*Lactobacillus*	86	844589	S24-7
37	272516	*Adlercreutzia*	87	851726	*Megasphaera*
38	292921	*Prevotella*	88	851865	Ruminococcaceae
39	296420	Clostridiaceae	89	858535	Coriobacteriaceae
40	318990	Lachnospiraceae	90	866365	Caulobacteraceae
41	322367	Clostridiaceae	91	934235	Rhizobiales
42	338273	Clostridiaceae	92	986513	*Clostridium*
43	338381	*Prevotella*	93	1105328	*Blautia*
44	344114	Clostridiales	94	1105552	Lachnospiraceae
45	354905	*Lactobacillus*	95	1106861	*Coprococcus*
46	357046	Rikenellaceae	96	1109964	Sphingomonadaceae
47	358798	Lachnospiraceae	97	1111458	Peptostreptococcaceae
48	359359	Ruminococcaceae	98	1656781	*Prevotella*
49	360158	Lachnospiraceae	99	64179	*Sphingomonas*
50	360518	*Bacteroides*			

Appendix A5. Letter of acceptance from Research and Ethical Committee Boards of the Instituto Nacional de Perinatología, 212250-3310-11402-01-14, Mexico City.

INSTITUTO NACIONAL DE PERINATOLOGÍA
ISIDRO ESPINOSA DE LOS REYES
INPer

DIRECCIÓN DE INVESTIGACIÓN
COMITÉ DE INVESTIGACIÓN

REF.3000.1020.2014

MÉXICO, D.F. A 15 DE SEPTIEMBRE DE 2014

DRA. MA. LUISA PIZANO ZARATE
INVESTIGADORA EN CIENCIAS MÉDICAS "B"
INSTITUTO NACIONAL DE PERINATOLOGÍA
P R E S E N T E.

Me permito informarle que se está fortaleciendo el control de los proyectos, por tal motivo este año la Dirección de Investigación junto con la Unidad Contable de Proyectos, establecieron una reestructuración de número de registro para proyectos, que permitirá que el investigador elabore su propio registro y solo la Dirección de Investigación confirmará que estén correctos los datos, por tal motivo le solicito que a partir de esta fecha cualquier trámite que haga para su proyecto titulado: *"Perfil de inflamación, disfunción endotelial y repercusión subclínica en población infantil con obesidad y síndrome metabólico"* lo haga con el siguiente número definitivo:

DECÍA: No. de Reg. 212250-331011402011

DEBE DECIR: No. de Reg.: 212250-3310-11402-01-14

Sin otro particular de momento, quedo de usted.

Atentamente

DR. HÉCTOR ALFREDO BAPTISTA GONZÁLEZ
PRESIDENTE DEL COMITÉ DE INVESTIGACIÓN

INSTITUTO NACIONAL DE PERINATOLOGÍA
ISIDRO ESPINOSA DE LOS REYES
18 SEP 2014
RECIBIDO
DEPARTAMENTO DE INVESTIGACIÓN EN NUTRICIÓN

HABG/ *PHG

C.C.P. DIRECCIÓN DE INVESTIGACIÓN
C.P. GABRIEL VÁZQUEZ SIERRA.- JEFE DEL DEPARTAMENTO DE CONTABILIDAD Y RESPONSABLE DE LA UNIDAD CONTABLE DE PROYECTOS, INPER

HABC/*PHG

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P.F.

INSTITUTO NACIONAL
DE PERINATOLOGÍA
ISIDRO ESPINOSA DE LOS REYES

DIRECCIÓN GENERAL

MÉXICO, D.F., A 29 DE MAYO DE 2014.

2014.1000 [illegible]

"2014, AÑO DE OCTAVIO PAZ"

DRA. MA. LUISA PIZAÑO ZARATE
INVESTIGADORA EN CIENCIAS MÉDICAS "B"
INSTITUTO NACIONAL DE PERINATOLOGÍA
P R E S E N T E

Me es grato informar a usted y a su grupo de colaboradores que el Comité de Investigación y el Comité de Ética en Investigación han revisado y emitido el dictamen correspondiente a su proyecto:

Perfil de inflamación, disfunción endotelial y repercusión subclínica en población infantil con obesidad y síndrome metabólico
ACEPTADO

Registro: 212250-331011402011

En cuanto al monto económico solicitado por usted para desarrollar el proyecto mencionado, la asignación dependerá estrictamente de la disponibilidad de los recursos fiscales correspondientes y, en su caso, de la disponibilidad de los recursos entregados por agencias financiadoras externas.

Me permito hacer de su conocimiento que, durante el desarrollo de este proyecto, usted deberá entregar informes trimestrales y al concluir el mismo un **informe técnico final**, según el formato institucional disponible en la Dirección de Investigación, para la presentación de productos de investigación, acompañado de los documentos probatorios del mismo.

Le felicito por su desempeño y compromiso institucional y me es grato enviarle un atento saludo.

ATENTAMENTE

DR. JORGE ARTURO CARDONA PÉREZ
DIRECTOR GENERAL

C.C.P. DIRECCION DE INVESTIGACION
C.P. GABRIEL VAZQUEZ SIERRA.- JEFE DEL DEPARTAMENTO DE CONTABILIDAD Y RESPONSABLE DE LA UNIDAD CONTABLE DE PROYECTOS, INPER

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