CENTRO DE INVESTIGACIÓN Y DE ESTUDIOS AVANZADOS DEL INSTITUTO POLITÉCNICO NACIONAL

UNIDAD ZACATENCO

DEPARTAMENTO DE FARMACOLOGÍA

"Estudio de la interacción de las comunidades microbianas y su nivel de ácidos grasos de cadena corta en el colon distal de niños obesos mexicanos"

TESIS QUE PRESENTA

SELVASANKAR MURUGESAN

PARA OBTENER EL GRADO DE

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Director de la Tesis:

Dr. Carlos Hoyo Vadillo

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"Study of interaction of microbial communities and short chain fatty acids level in the distal colon of obese mexican children"

SELVASANKAR MURUGESAN, M.Sc.

A Dissertation

Submitted in Partial Fulfillment of The

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DR. CARLOS HOYO VADILLO

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Dedication

This thesis is dedicated to my parents and brothers who have supported me all the way since the beginning of my studies.

Also, this thesis is dedicated to Dr. V. Alagarsamy who has been a great source of motivation and inspiration.

Finally, this thesis is dedicated to all those who believe in the richness of learning.

Credits

Children for this study were selected under direction of Dr. María Luisa Pizano Zárate from three public primary schools, in the Ecatepec borough in the Greater Mexico City area. The informed consent was signed by parents and children in accordance with the Helsinki Declaration revised in 2000. The research protocol was approved by the Local Ethical Committee Board of Health from the Instituto Mexicano del Seguro Social R-2011–1402 1402–10, Mexico City.

The generation of the bacterial metagenomics data and analysis was developed under the direction of Dr. Jaime García Mena at the Laboratorio de Referencia y Apoyo para la Caracterización de Genomas, Transcriptomas y Microbiomas, Departamento de Genética y Biología Molecular del Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional Unidad Zacatenco.

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Abbreviations

16S rDNA	- 16S ribosomal Deoxy Nucleic Acid
BMI	- Body Mass Index
CDC	- Centre for Disease Control
GI tract	- Gastro Intestinal tract
GWAS	- Genome Wide Association Studies
NCD	- Non-Communicable Diseases
NIH	- National Institute of Health
NLRs	- Nod like Receptors
OECD	- Organization for Economic Co-operation and Development
OTU	- Operational Taxonomy Units
PCA	- Principal Components Analysis
PCoA	- Principal Coordinate Analysis
PRRs	- Pattern Recognizing Receptors
SCFAs	- Short Chain Fatty Acids
TLRs	-Toll like Receptors
WHO	- World Health Organization
LPS	- Lipopolysaccharides

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Resumen

La obesidad y el sobrepeso son problemas de salud de etiología multifactorial, que pueden incluir cambios en el microbioma humano. En México más del 30% de la población infantil de entre 5 y 11 años de edad, sufren de sobrepeso o son obesos, lo que lo convierte en un problema de salud pública en curso. El propósito de este trabajo fue medir la concentración de ácidos grasos de cadena corta por HPLC y caracterizar la diversidad bacteriana por secuenciación masiva semiconductora de bibliotecas 16S rDNA preparadas a partir de heces obtenidas de una muestra de niños mexicanos bien caracterizados para el peso normal, con sobrepeso y condición de obesidad por criterios antropométricos y bioquímicos. Se encontró que los niveles de triglicéridos aumentan en los niños con sobrepeso y obesidad, que presentan concentraciones de ácido propiónico y butírico alterados en las heces. Además, aunque la microbiota del colon no mostró una dramática disbiosis bacteriana entre las tres condiciones, la abundancia de algunas bacterias en particular se encontró alterada con respecto a los controles normales. Llegamos a la conclusión de nuestros resultados que el desequilibrio en la abundancia de por lo menos nueve bacterias diferentes, así como la concentración de ácidos grasos de cadena corta alterada en las heces se asocia a la condición de sobrepeso y obesidad en niños mexicanos.

Abstract

Obesity and overweight are health problems of multifactorial etiology, which may include changes in the microbiome. In Mexico more than 30% of the children population between 5 and 11 years old, suffer of overweight or are obese, which makes it a public health issue in progress. The purpose of this work was to measure the short chain fatty acid concentration by HPLC, and to characterize the bacterial diversity by Ion Torrent Semiconductor Sequencing, of 16S rDNA libraries prepared from stools collected from a sample of well characterized Mexican children for normal weight, overweight and obese condition by anthropometric and biochemical criteria. We found that triglyceride levels are increased in overweight and obese children, which presented altered propionic and butyric acid concentrations in feces. In addition although the colon microbiota did not show a clear bacterial dysbiosis among the three conditions, the abundance of some particular bacteria was changed with respect to normal controls. We conclude from our results that the imbalance in the abundance of at least nine different bacteria as well as altered short chain fatty acid concentration in feces is associated to the overweight and obese condition of Mexican children.

1. Introduction

1.1 Obesity

Obesity is a multifactorial metabolic disease associated with a high risk to develop other chronic diseases (Waalen 2014). The problem of obesity used to be limited to developed countries however; this is no longer the case as the obesity epidemic is now a worldwide issue (James 2008). Excess caloric intake and too little physical activity contribute the most to obesity, but genetic susceptibility and various disorders (eating disorders) may also contribute.

The reference measures of body composition include densitometry, single-cut imaging of the abdomen using computed tomography scan or magnetic resonance imaging, and dual energy X-ray absorptiometry, but these methods are used mostly for research purposes. The World Health Organization (WHO) and the National Institutes of Health (NIH) 1, 2 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 of 25–30 defined as overweight and a BMI >30 classified as obese(WHO, 2008). In the United States, criteria for overweight in children are based on the 2000 U.S. Centers for Disease Control and Prevention (CDC) BMI-for-age growth charts. Overweight is defined as at or above the age-specific 95% BMI percentile. At risk for overweight is defined as having a BMI between 85th–95th percentiles of the BMI-for-age growth charts.

1.2 Obesity and its consequences

Obesity has a profound effect on the body as increased adipose tissue disrupts hormonal balances of leptin, adiponectin and insulin which govern regulation of satiety and food intake, suggesting that chronic food intake characteristic of obesity may not be purely hedonic, but mediated by hormonal imbalances (Blundell and Gillett, 2001). Chronically elevated blood glucose and

lipids; oxidative stress; endotoxemia with low grade systemic inflammation represent additional biomarkers of obesity (Conterno *et al*., 2011).

Obesity can induce complications of physical, social and emotional well-being to a child. Some of those physical complications are high cholesterol, high blood pressure, asthma, sleep disorders and Nonalcoholic fatty liver diseases. Obese youth 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).

Obesity is a process that usually starts in childhood or adolescence, and is set up by an imbalance between energy intake and energy expenditure. This disorder is also a prominent risk factor for the development of chronic diseases, such as Type 2 Diabetes and Metabolic syndrome (Kannel *et al*., 1996; Carey *et al.*, 1997; Wild *et al*., 2006). Obese adolescents are more likely to have prediabetes, a condition in which blood glucose levels indicate a high risk for development of diabetes. Overweight and obesity are associated with increased risk for many types of cancer, including cancer of the breast, colon, endometrium, esophagus, kidney, pancreas, gall bladder, thyroid, ovary, cervix, and prostate, as well as multiple myeloma and Hodgkin's lymphoma. The social and emotional complications are such as Low self-esteem and bullying, Behavior and learning problems and depression among those affect their everyday life.

Obesity epidemic emphasized mainly on the ‘‘obesogenic environment’’ and lifestyle changes, but one should not forget to give attention on heritability. The inherited propensity toward obesity has been supported by numerous family and twin studies, many of which preceded the identification of specific genes by decades. Heritability of fat mass in Finnish twins, for example, has been reported to be 90% among twins with the low physical activity, but only 20% among the most active pairs of twins. (Silventoinen *et al*., 2009). Age effects are also striking, with the greatest heritability BMI, for example, manifesting in adolescence (starting around the age of 11 years) and peaking in young

adulthood (Around the age of 20 years) (Min *et al*., 2013) More detailed exploration of the effects of these and other influences, such as ethnicity and sex, are now being incorporated into studies with increasing emphasis on understanding gene-environment interactions.

Apart from this, identification of genes involved in obesity and other syndromes like Prader-Willi which can cause intense obesity are also have important roles. Recent Genome wide associated studies (GWASs) explored a variety of Single Nucleotide Variants traits related with obesity. Fat mass and obesity-associated (FTO) gene, TMEM18 (transmembrane protein 18), and Melanocortin4 Receptor (MC4R) are those few Single Nucleotide Polymorphisms for BMI among populations of European ancestry identified in multiple large GWASs meta-analysis (Speliotes *et al*., 2010). But different set of loci have been identified in a GWASs study associated BMI-adjusted WHR, a trait directly linked with adiposity. Among the 13 traits, 7 were significant in women. The sex-specific effects were found among genes generally involved in insulin sensitivity (PPARG, VEGFA, ADAMTS9, and GRB14) and lipid-related traits (LYPLAL1, MAP3K1, and GRB14) (Heid *et al*., 2010). Understandings of the genetic contribution to obesity, including genetic interactions with the environment are rapidly drawing attention with information which involves in developing treatment methods to control obesity epidemic.

1.3 Prevalence of Obesity

Obesity has received considerable attention after its rise over the past three decades worldwide (Ng *et al*., 2014). At present more than 1.9 billion adults, 18 years and older, were overweight. Of these over 600 million were obese. Most of the world's population live in countries where overweight and obesity kills more people than underweight. 42 million children under the age of 5 were overweight or obese in 2013. The complications caused by overweight and obesity are the fıfth-leading cause of all deceases in the world, at least 2.8 million annual deaths attributed to it. In 2008, 35% of adults aged more than 20 were overweight (BMI

≥ 25 kg/m2) (34% men and 35% of women). The global prevalence of obesity has nearly doubled in last three decades. In 2008, 10% of men and 14% of women in the world were obese (BMI ≥30 kg/m2), compared with 5% for men and 8% for women in 1980. An estimated 205 million men and 297 million women over the age of 20 were obese – a total of more than half a billion adults worldwide (WHO, 2015). According to National Health and Nutrition Examination Survey (NHANES) data in 2003–2004, 66.2% of U.S. adults 20–74 years old were either overweight or obese, 33.4% were overweight and 32.9% were obese. The obesity prevalence was relatively low and stable between 1960 and 1980, but more than doubled from 15% in 1980 to 34% in 2006 (Ogden *et al*., 2007). In the recent survey in 2012, the prevalence remained stable between 2003-2004 and 2009-2010, but obesity prevalence remains high and thus it is important to continue surveillance (Ogden *et al*., 2014).

The prevalence of overweight and obesity were highest in the WHO Regions of the Americas (62% for overweight in both sexes, and 26% for obesity) and lowest in the WHO Region for South East Asia (14% overweight in both sexes and 3% for obesity). In the WHO Region for Europe and the WHO Region for the Eastern Mediterranean and the WHO Region for the Americas over 50% of women were overweight. For all three of these regions, roughly half of overweight women are obese (23% in Europe, 24% in the Eastern Mediterranean, 29% in the Americas). In all WHO regions women were more likely to be obese than men. In the WHO regions for Africa, Eastern Mediterranean and South East Asia, women had roughly double the obesity prevalence of men. The prevalence of raised body mass index increases with income level of countries up to upper middle income levels. The prevalence of overweight in high income and upper middle income countries was more than double that of low and lower middle income countries. For obesity, the difference more than triples from 7% obesity in both sexes in lower middle income countries to 24% in upper middle income countries. Women's obesity was significantly higher than men's, with the exception of high income countries

where it was similar. In low and lower middle income countries, obesity among women was approximately double that among men (WHO, 2008).

Mexico has experienced a rapid increase in wealth in recent era, which brings a significant shift in socio-economic status and a geographic shift from rural to urban among its population. This has led to changes in diet that are harmful to health: an increase in physical inactivity and increased access to low-priced highly energy-dense foods. As a result, rapid growth in the prevalence of obesity and obesity-related non-communicable diseases (NCD) has been observed with a lack of preventive steps to curb this rise. In 2010, 32 % of men and 26 % of women were normal weight. By 2050, the proportion of normal weight will decrease to 12 % and 9 % for males and females, respectively, and more people will be obese than overweight (Rtveladze *et al*., 2014).

1.4 Prevalence of Childhood obesity

Childhood obesity has been called one of the most serious public health challenges of the 21st century, The prevalence in the adolescent population (12-17 years old) is also growing at an alarming rate, as has been documented in national surveys conducted in 1988, 1999 and 2006 (Secretaría de Salud, 2010). In Latin America, the ever-rising rates of overweight and obesity observed over the past three decades have not spared children and adolescents. The most recent data estimate that between 42.4 and 51.8 million children and adolescents (0-18 years) are overweight or obese, representing 20-25% of the total population of children and adolescents in the region (Rivera, 2014) It is responsible for about 8-10% of premature deaths in Mexico. Approximately 70% of the adult Mexican population, has excessive body weight (Latnovic *et al*., 2013) and according to the Mexican National Survey of Health 2012 more than 30% of the child population between 5 and 11 years old, presents problems of overweight or obesity, which makes it a problem of public health (Figure 2) (Gutiérrez *et al*., 2012) and Mexico is now just behind the United States experiencing the worst epidemic of adolescent obesity in the world (Holub *et al*.,

2013). From the survey of the Organization for Economic Co-operation and Development (OECD) reveals that obesity epidemic has spread further in the past five years, but rates have been increasing at a slower pace than before. Obesity and overweight have been virtually stable, or have grown modestly, in Canada, England, Italy, Korea, Spain and the United States, but have increased by a further 2-3% in Australia, France, Mexico and Switzerland. The economic crisis is likely to have contributed to further increase in obesity. In Mexico, growth rate of overweight and obesity continues to be robust and no signs of convergence and retrenchment of the epidemic (Figure 1) (OECD, 2010). The improvement in health condition, income, education than 18th century influences beneficially in weight gaining, have now crossed the line beyond which further gains are risky. Life expectancy of the people have been reduced up to 10 years than normal weight people. Child overweight rates in Mexico are among the highest in the OECD area. It showed that almost 1 in 3 children is overweight in Mexico, compared with 23% of boys and 21% of girls, on average, in OECD countries (OECD, 2014). Since 2000, obesity and overweight rate increases steadily in both boys and girls.

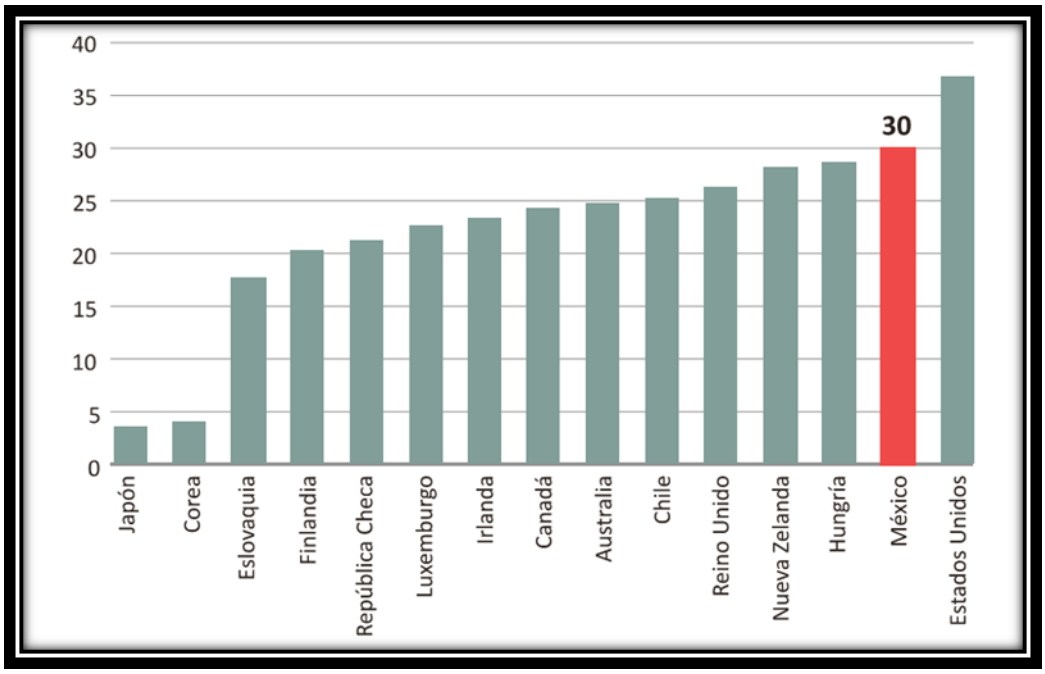


Figure 1. Percentage of adult obesity among countries according to OECD 2010.

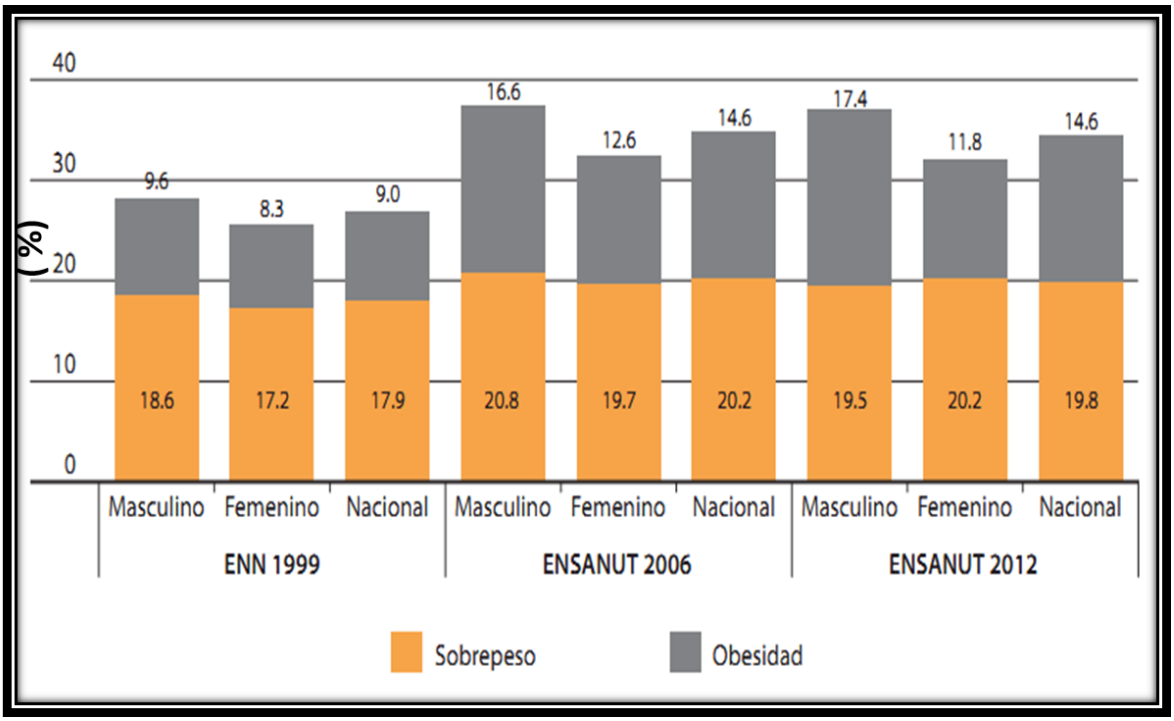


Figure 2. Comparison of the National prevalence of overweight and obesity in population 5 to 11 years old children (ENSANUT, 2012).

1.5 Structure and Digestive Function of the GI Tract

The human gastrointestinal (GI) tract (gut) is the largest organ in the human body, extending from the oral cavity to the anus. This axis is defined by sharp and functional anatomical boundaries between continuous segments comprised of the esophagus, stomach, small intestine and colon (San Roman *et al*., 2011). The stomach is composed of three distinct regions with the fundus separated from the corpus and antrum-pylorus regions. The pyloric sphincter is the definite boundary separating stomach processes from the small intestinal region. Entry into the small intestine starts at the duodenum, with subsequent movement to the jejunum and ileum regions. At the ileo-cecal valve the villous small intestinal epithelium transitions to a non-villous, flat epithelium encompassing the caecum and proximal, transverse and distal colon regions (Figure 3) (San Roman *et al.,* 2011).

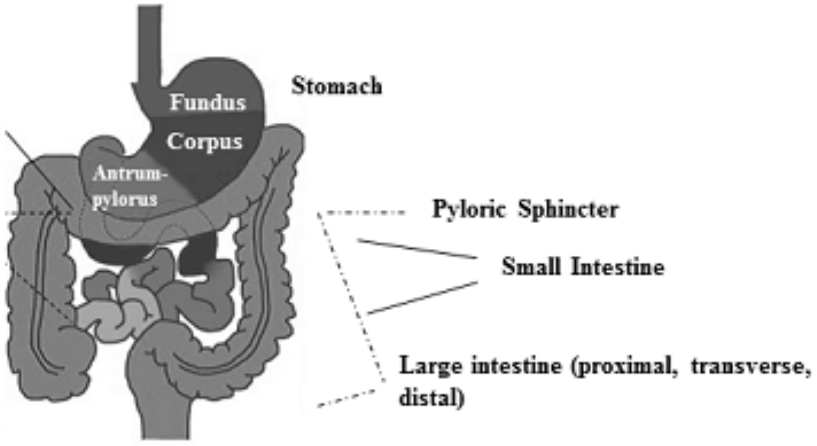


Figure 3. Structure and digestive functionality of the human GI tract. Left: View of the liver, small and large colon regions. Right: Specific stomach and intestinal regions, adapted from San Roman *et al*., 2011.

The GI tract is responsible for digestion and absorption of solid and liquid food entering the oral cavity. Upon ingestion, food is mechanically grinded into smaller particles during chewing and moistened by saliva, facilitating its ease of passage down the esophagus. Chemical degradation of polysaccharides is also mediated in the mouth by the enzyme amylase. Passage of food from the mouth to stomach results in formation of chyme, a mixture of partially degraded, moist food particles, gastric juice (an enzyme mixture of gastric lipase and pepsin) and hydrochloric acid. Chyme is moved through the pyloric sphincter to the duodenum where liver-derived bile emulsifies dietary fat for pancreatic lipase degradation to fatty acids and glycerol. Digestive processes are further mediated by peristaltic movement of chyme down the small intestinal epithelium. Starch and other polysaccharides are enzymatically cleaved into di and monosacchride moieties by pancreatic amylase and trypsin digestion of peptones and other proteins results in liberation of free amino acids.

The small intestinal epithelium is composed of finger-like appendages known as villi which promote nutrient absorption from the lumen and nutrient passage to the systemic and lymphatic circulatory systems. The small intestine represents the primary site of GI-dependent nutrient absorption as only alcohol or simple monosaccharides are readily absorbed directly in the stomach. Physiological conditions of the large intestine are more diverse in comparison to other GI tract compartments. Colonic pH is highly stratified, ranging from 5.4 to 7.9, with acidic conditions confined to the proximal colon region and pH gradually increasing to circumneutral and neutral conditions in the distal region (Cummings *et al*., 1991).

The large intestine is composed of three colorectal epithelial cell lineages: goblet, absorptive and enteroendocrine cells which are smooth and non-villous, containing numerous invaginations known as crypts (Kirkland and Henderson 2001). Upper GI tract digestive and absorptive processes remove approximately 90% of degraded carbohydrate and protein from chyme before reaching the large intestine (O'Keefe 2008). However, a large proportion of ingested food is resistant to human GI processes, resulting in ~1.5 kg of undigested food reaching the colon daily. This undigested food is primarily composed of complex plant polysaccharides such as cellulose as well as starch, resistant starch, both soluble and insoluble dietary fiber, mucins and proteins. The process of digestion in the large intestine is completed by hydrolytic resident gut microbiota, which hydrolyzes these otherwise indigestible polysaccharides. Subsequent microbial fermentation of hydrolyzed polysaccharides in the large intestine results in production of short chain fatty acids (SCFA) such as acetate, propionate and butyrate; branched chain fatty acids (BCFA); lactate, formate, ethanol and mixed gases (e.g. CH4, CO2 and H2) (Robert *et al*., 2003). Dissimilatory metabolism of proteinaceous material in the intestine further produces ammonia, amines, mercaptans, H2S as well as some toxic indolic and phenolic compounds (Smith *et al*., 1996). The total of gut transit ranges from 20 to 140 h, with an average time of 60 h in healthy adults (Cummings *et al*., 1991). Solid matter escaping through to the anus is ultimately excreted as feces.

1.6 The Human microbiota

A diverse microbiota is associated with the skin and mucous membranes of every human being from shortly after birth until death. The human microbiota is composed of bacteria, archaea, viruses, and eukaryotic microbes that reside in and on our bodies. These microbes have tremendous potential to impact our physiology, both in health and in disease. They contribute metabolic functions, protect against pathogens, educate the immune system, and, through these basic functions, affect directly or indirectly most of our physiologic functions. The human body, which contains about 10^{13} cells, routinely harbors about 10^{14} bacteria. This bacterial population constitutes human microbiota, is relatively stable, with specific genera populating various body regions during particular periods in an individual's life. Healthy adult humans each typically harbor more than 1000 species of bacteria belonging to a relatively few known bacterial phyla with Bacteroidetes and Firmicutes being the dominant phyla (Lozupone *et al*., 2012). We humans are made-up of only 10% of human cells and the rest of 90% are of microbial cells (Cani 2013), so we can call ourself as “Metaorganisms”. Trillions of bacteria reside in the different body sites like skin, mouth, airways and gastro intestinal tract (gut).

Human microbiota may aid the host (by competing for microenvironments more effectively than such pathogens as *Salmonella* spp or by producing nutrients the host can use), may harm the host (by causing dental caries, abscesses, or other infectious diseases), or may exist as commensals (inhabiting the host for long periods without causing detectable harm or benefit). Even though most elements of the normal microbial flora inhabiting the human skin, nails, eyes, oropharynx, genitalia, and gastrointestinal tract are harmless in healthy individuals, these organisms frequently cause disease in compromised hosts. Viruses and parasites are not considered members of the normal microbiota by most investigators because they are not commensals and do not aid the host (Davies 1996).

1.7 The Human Gut microbiota

The human gut is home to an estimated 100 trillion microorganisms, which thought to be more than the cells of their host by 10 fold and represent by far the largest microbial community associated with the human body (Savage 1977). Gut has rich and complex microbial consortium which can be considered as a microbial organ inside host organ (Bäckhed *et al*., 2005), collectively referred as the gut microbiota.

Dietary components with biological effects are susceptible to be metabolized by intestinal bacteria during the gastrointestinal passage, prior being absorbed. The colon has the highest bacterial load and constitutes an active site of metabolism rather than a 25 simple excretion route (Aura 2008). The metabolic activity of the gut microbiota on bioactive food components can modify the host exposure to these components and their potential health effects. Furthermore, some functional food components influence the growth and metabolic activity of the gut microbiota and, thereby, its composition and functions (Campbell *et al*., 1997; Gibson *et al*., 2005). Therefore, the intestinal microbiota is both a target for nutritional intervention to improving health and a factor influencing the biological activity of other food compounds acquired orally.

Furthermore, this large and dynamic community undergoes dramatic changes after initial colonization of the host at birth, and develops through weaning and maturity (Hooper 2004). Due to the potential for beneficially influencing function and health by manipulating the intestinal microbiota, there is much interest in the use of dietary supplements. In particular, engineering the microbiota through dietary intervention in the young when the large bowel community is in transition and still undergoing major changes may be a useful strategy for promoting health benefits later in life. Commonly used dietary supplements include prebiotics and probiotics. Prebiotics are a term used to describe food ingredients that resist degradation and absorption in the upper digestive tract and selectively enrich the population or metabolic activity of one or a limited number of resident bacteria that are beneficial to the host (Gibson *et*

al., 1995), while probiotics are live microorganisms that are thought to confer a health benefit when administered in adequate amounts (Fuller 1989). The most commonly used probiotics are strains of lactobacilli and *Bifidobacteria*, but others may also include some bacilli and yeasts. However, the mechanisms of action for the effects of dietary supplements such as prebiotics or probiotics on host physiology are poorly understood, and many health claims for supplement use are not adequately supported by scientific evidence.

The gut microbiota enables hydrolysis of indigestible polysaccharides to easily absorbable monosaccharides and activation of lipoprotein lipase by direct action on the villous epithelium (Bäckhed *et al*., 2004) and affect nutrient acquisition and energy regulation (DiBaise *et al*., 2008). The gut microbiota could contribute to obesity by increase in harvesting energy from the diet, through its metabolites and microbial cells induced signal on host metabolic pathways that affects lipid metabolism and energy regulating homoeostasis (Flint 2011).

1.8 Establishment of Gut microbiota from the time of birth

The fetal gut is sterile and is colonized at birth with microbes from the mother's vaginal and fecal microbiota as well as with other environmental microbes encountered in the first days of life. Early colonization depends on the mode of delivery, diet (breast- vs. formula-feeding), hygiene, and antibiotic treatment (Wall *et al*., 2009). The first colonizers are facultative anaerobes, such as *Escherichia coli* and *Streptococcus* spp., and obligate anaerobic species colonize as the oxygen levels in the gut decrease. A large study involving three populations in different geographic locations found that a child's microbiota stabilize and become adult-like at ~3 years of age (Yatsunenko *et al*., 2012). The metagenome of the infant gut is characterized by enrichment of genes for simple sugar breakdown, such as lactose and galactose, whereas the weaned microbiota are enriched in genes for polysaccharide breakdown and vitamin production (Koenig *et al*., 2011).

In the elderly, changes in the microbiota occur, resulting in reduced microbial diversity, which is accompanied by increased inflammation (Claesson *et al*., 2012). Compared with elderly subjects in long-stay residential care units, elderly individuals living in the community have higher levels of fecal butyrate and other short-chain fatty acids (SCFAs), such as acetate and propionate; the presence of SCFAs is generally believed to be associated with diminished inflammation.

1.9 Role of Gut microbiota on Immune system

Our immune system recognizes bacterial components (Figure. 4) through pattern recognizing receptors (PRRs) (Harris *et al*., 2012). There are two types of PRRs, Toll like Receptors (TLRs) and Nod like Receptors (NLRs). TLR recognizes specific ligands and activates inflammatory responses, TLRs (1, 2, 4, 5, 6 and 10) are expressed in cell surface but the remaining TLRs (3, 7, 8 and 9) locate in the membrane of endolysosomal compartments (Barton 2009). Among 22 NLRs in human, only two (NOD1 and NOD2) are well characterized and located in cytoplasm (Clarke *et al*., 2011). PRRs not only recognizes the structures of microbiota (LPS, Flagella, Peptidoglycan and Lipoproteins) by signaling immune system but also involves in the development of metabolic diseases like Insulin resistance and cardiovascular diseases.

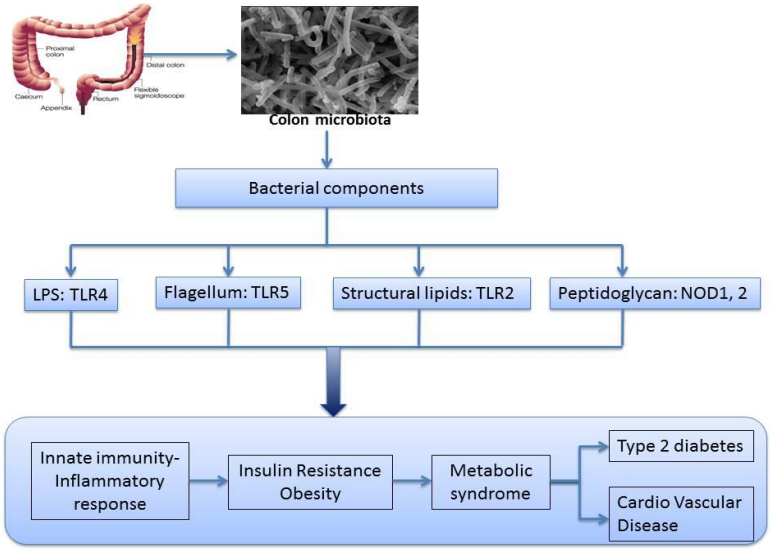


Figure 4. Gut microbiota- Bacterial components cause an innate immune response.

In Gut microbiota family, Stomach (10^1–10^3 cfu/ml), duodenum (10^1–10^3 cfu/ml), ileum (10^4–10^7cfu/ml) and colon microbiota (10^{11}–10^{12} cfu/ml) (Figure. 5) (O'Hara and Shanahan, 2006) are vital members. Among that colon microbiota has higher colonization high diversity of microbiota. Colon Microbiota provides an additional metabolic energy through undigested carbohydrate fibers. Countering this metabolic activity will be more relevant to development of obesity. The Main metabolic products of colon microbiota are Short Chain Fatty Acids (SCFAs) such as Acetic acid, Propionic acid and Butyric acid (Gill *et al*., 2006), which can be utilized for lipid or glucose denovo synthesis (Eckburg *et al*., 2005; Palmer *et al*., 2007). Alteration in the levels of SCFAs in obesity might be due to dysbiosis in the colon microbiota. So it is essential to explore the distal colon community members and SCFAs level to comprehend its role in development of obesity. This complex ecosystem represents a huge reservoir of metabolic capability and plays a crucial role in a number of developmental and nutritional processes in the intestine. The adaptive co-evolution of the microbial community and their hosts has enabled humans to harvest nutrients from sources that they would otherwise lack the ability to utilize (Backhed *et al*., 2005). The intestinal microbiota also stimulates the development of the immune system and assists the host by excluding pathogens. Studies have revealed how members of the intestinal microbiota are able to manipulate host physiology to the benefit of both microbe and host (Kelly *et al*., 2004). Community composition and metabolic activity of the microbiota are influenced by a variety of factors including the diet and health status of the host (De Filippo *et al*., 2010; Lindsay *et al*., 2006). Likewise, the resident microbiota may also be an important factor in a number of diseases or conditions, including colon cancer, inflammatory bowel diseases, and obesity (O'Keefe *et al*., 2009; Sokol *et al*., 2008; Turnbaugh *et al*., 2009a).

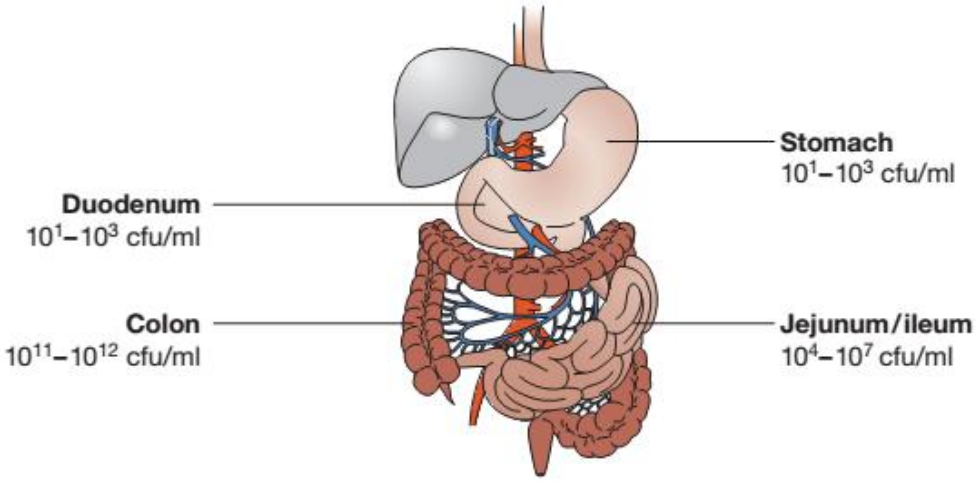


Figure 5. Bacteria density increases in the jejunum/ileum from the stomach and duodenum, and in the large intestine, colon residing bacteria achieve the highest cell densities recorded for any ecosystem. (O'Hara *et al*., 2006).

1.10 The role of the gut microbiota in diseases and conditions

The gut microbiota have the capacity to affect host physiology within and outside the gut. For example, the gut microbiota are essential for normal development and homeostasis of the immune system in the gut, modulate epithelial cell proliferation, protect against pathogenic bacteria, and modulate villus architecture and angiogenesis within the intestine. Furthermore, the gut microbiota affect xenobiotic metabolism, bone mineral density, behavior, and several metabolic functions (Sommer *et al*., 2013; Tremaroli *et al*., 2012), and emerging data from humans and mouse models suggest that the gut microbiota play a role in the development of metabolic diseases. It is often not clear what microbiota changes associated with disease are meaningful, and distinguishing between cause and effect is inherently challenging. Although it is intriguing to speculate that dysbiosis may cause disease as more research is going about how the microbiota can influence the host, it is also noted that the diseased state can lead to changes to the microbiota through various mechanisms, including changes in eating habits and bowel function as well as through the addition of medications such as antibiotics. In this section, we highlight a few of the recent

findings on the role of the microbiota in particular diseases or conditions, but we cannot touch on all of the emerging findings in a multitude of other diseases both inside and outside the gut, including but not limited to rheumatoid arthritis (Scher *et al*., 2013), colorectal cancer (Kostic *et al*., 2013), obesity (Le Chatelier *et al*., 2013), and diabetes (Karlsson *et al*., 2013).

1.10.1 Type 2 diabetes

The incidence of type 2 diabetes (T2D) is increasing in parallel with obesity, and environmental factors that are associated with T2D risk include diet and the gut microbiota (Larsen *et al*., 2010). Low-grade inflammation is observed in T2D patients, and diabetic mice and humans have increased plasma levels of lipopolysaccharide (LPS), a membrane component of Gram-negative bacteria, which has been shown to impair glucose metabolism in mice (Creely *et al*., 2007). Germ-free mice have fewer macrophages in their adipose tissue and improved glucose metabolism compared with colonized mice (Caesar *et al*., 2012).

1.10.2 Atherosclerosis

Accumulation of cholesterol and recruitment of macrophages to the arterial wall promote the formation of atherosclerotic plaques, which may lead to myocardial infarction and stroke. Bacterial species from the genera *Chryseomonas, Veillonella*, and *Streptococcus* have been found in plaques and are also present in the oral cavity or the gut (Koren *et al*., 2011). We recently demonstrated that patients who had experienced an atherosclerotic event had higher levels of *Collinsella* and lower levels of *Eubacterium* and *Roseburia* in their gut microbiota than healthy control subjects. The health status in these patients correlated with several aspects of the functional metagenome, such as an increase in pro-inflammatory peptidoglycan genes and a decrease in genes involved in the synthesis of anti-inflammatory molecules (e.g., butyrate). A

particularly interesting finding was the increased prevalence of genes involved in biosynthesis of the antioxidant β-carotene, together with increased blood levels of β-carotene in healthy control subjects (Karlsson *et al*., 2012). These observations suggest that it may be possible to develop strategies to prevent atherosclerotic events based on the gut microbiota.

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 cardiovascular events (Tang *et al*., 2013). The authors also showed that feeding mice with choline promoted the formation of atherosclerotic plaques and that plaque formation could be prevented by antibiotic treatment (Wang *et al*., 2011). Dietary L-carnitine, which is abundant in red meat and has a similar polar head group to choline, was recently shown to be metabolized by the gut microbiota and to contribute to atherosclerosis and cardiovascular disease (Koeth *et al*., 2013).

1.10.3 Role of Gut microbiota in obesity

The basis that lead to the development of obesity, shows that in addition to the genetic component of the human genome; in many cases there is a clear influence of the human microbiome (Devaraj *et al*., 2013). The microbiome is the full set of genes in the genomes of all microbes that live in the human body, and whose expression has influence on its systemic function (Ursell *et al*., 2012). Recent reports in mice model have shown that overweight and obesity are associated with a particular type of bacteria that inhabit the digestive tract; other studies in adult humans, have shown that variations in the relative abundance of two phyla Firmicutes and Bacteriodetes, are related to the condition of accumulation of body fat (Ley 2010).

The digestive tract is the largest immunological organ in the adult, living in it approximately 100 trillion bacteria (Caricilli *et al*., 2014); however, usually there are no health problems, since this organ sustains a balanced mutualistic

relationship with the commensal bacteria (Michelsen *et al*., 2007). The proper association between bacteria and the wall of the digestive tract occurs through the interaction of bacterial structural components and metabolites, with specialized receptors in the gut (Rooks *et al*., 2011). The short-chain fatty acids (SCFAs), product of the anaerobic fermentation by some species of bacteria, have been used traditionally as a therapy for colitis and ulcerative colitis, due to their anti-inflammatory effect (Whelan *et al*., 2004). It is presumed that inhibition of the NFκB factor is involved in its mechanism of action (Segain *et al*., 2000); thus, these metabolites would be related with signal transduction pathways with influence in systemic inflammation state (Dengler *et al*., 2014).

Obesity is a chronic inflammatory process of low intensity of multifactorial etiology, where a large proportion of cases, might be due to a dysfunction in the genetic expression of the microbiome, affecting the systemic signaling in the human body. The microbiota colonizes the human body at birth, and there is a succession in the bacterial diversity with abundance of Lactobacilli or Staphylococci according to the way of birth; to Enterobacteria about the first month, which moves to *Bifidobacteria* and *Bacteriodes* before the sixth month, and finally to abundance of Bacteroidetes and Firmicutes from age two (Clemente *et al*., 2012).

The gut microbiota participates in the saccharification of undigested polysaccharides to easily absorbable monosaccharides, and activation of lipoprotein lipase by direct action on the villous epithelium (Bäckhed *et al*., 2004), playing a role in nutrient acquisition and energy regulation by the host (DiBaise et al., 2008). It has been postulated that gut microbiota contributes to obesity, by increasing energy harvesting from diet, and modulating through its metabolites, the host metabolic pathways involved in lipid metabolism and energy regulating homoeostasis (Figure 6) (Flint *et al*., 2011). To date, the main reported metabolic products of colon microbiota are acetic, propionic, and butyric acids or short chain fatty acids (Gill *et al*., 2006), which can be utilized for de novo lipid or glucose synthesis (Eckburg *et al*., 2005; Palmer et al., 2007). Alteration in the levels of SCFAs in obesity, might be associated to bacterial dysbiosis in the

colon; making essential to explore the diversity of bacterial communities and the SCFAs level to comprehend its role in the development of this disease.

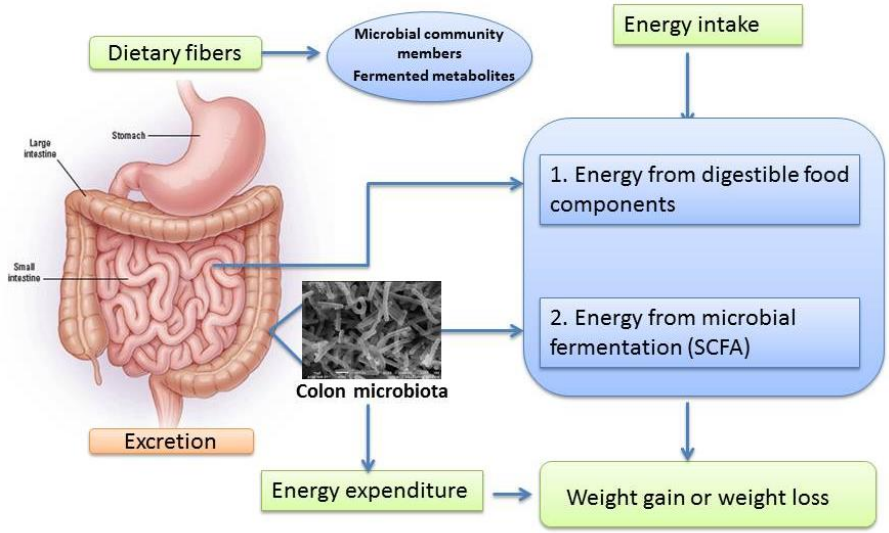


Figure 6. Effect of Colon microbiota in human weight.

Culture independent technique to explore colon microbiota has advanced very much in the recent years (Qin *et al*., 2010). Colon microbiota profiling can be done using fecal microbial genomic DNA from 16S rDNA fingerprint has shown that this community is of 100 trillions of archaeas and bacterias. Distal colon microbiota has been dominated mainly by four types of phylums like Bacteroidetes, Firmicutes, Actinobacteria and Proteobacteria (Tremaroli *et al*., 2012).

2. RATIONALE

Obesity is a growing epidemic problem in developed countries and currently is a raising concern of developing countries that have historically charged in addition to malnutrition. The combined prevalence of overweight and obesity in adults above 50% while increasing levels of childhood overweight is an alarm to take right step. Obesity is a major risk factor in other health disorders such as type 2 diabetes, cardiovascular disease and a variety of cancers, which further

complicates a number of psychosocial concerns (increasing social and economic costs) increasingly overwhelming systems health and oversaturated.

Evidence suggests that bacteria in the human microbiota (intestinal microbiota) affect the acquisition and energy regulation, in addition, the composition of the microbiota differs between obese and lean subjects (Ley *et al*., 2006). Thus, it is considered that the intestinal microbiota plays an important role in weight regulation and may even be partly responsible for the development of obesity in some people, but these hypotheses have not been confirmed for the children. Also, the need for new tools for diagnosis and early management of obesity makes the study of intestinal microbiota a novel alternative way to control obesity. There are no reported systematic studies about the role of microbiota associated with obesity in Mexican children.

3. SCOPE

This work focuses on the analysis of fecal samples obtained from Mexican children of both sexes from Institute of Perinatology, and they are grouped as obese and healthy each of them having 100 in number. Assessment of composition of the microbiota in samples and SCFAs analysis using fecal sample is not an invasive procedure for the infant and provides useful information on the microbial communities present in the intestine. This will assess the relationship between the colon microbiotas such as Firmicutes and bacteriodes with the SCFAs level in both healthy and obese Mexican children.

4. HYPOTHESIS

In the obese Mexican children the relative abundance of the phylum Firmicutes and the concentration of the SCFA in feces will be larger than in healthy Mexican children.

5. OBJECTIVE

The general objective of this project is a description of the diversity and dynamics of interaction of the distal colon microbiota of Mexican children and its SCFAs level associated with obesity in terms of abundance of its members. This general objective is achieved through the following specific objectives:

5.1 Specific objective 1.

To select subjects by phenotypic, biochemical profile of a sample of 100 obese children and 100 healthy children each category for further studies.

5.2 Specific objective 2.

To characterize three SCFAs of colon microbiota by HPLC from the faecal samples of Normal weight and obese Mexican children.

5.3 Specific objective 3.

To prepare 16S rDNA libraries massive pool of Normal weight and Obese Mexican children to sequence in Ion semiconductor sequencer.

5.4 Specific objective 4.

To develop the database of the microorganisms and to describe the microbial diversity using sequencing data and biochemical profile for healthy and obese phenotype using multivariate statistical analysis show a dynamic model of interaction and obesity associated with residents of colon microorganisms in Mexican children.

6. MATERIALS AND METHODS

6.1 Experimental Strategy

The work has been divided into three stages and 9 activities (Figure. 7) in a manner to fulfill steps such as selection of children, SCFA analysis, Preparation of amplicons for sequencing, Microbial diversity analysis.

6.1.1 Stage. 1 Selection of Children and SCFA analysis

Activities

1. Selection of children based on anthropometric method
2. Clinical analysis of metabolic factors from blood samples
3. SCFA analysis from fecal samples of children

6.1.2 Stage. 2 Preparation of 16s rDNA libraries for massive sequencing

Activities

4. Extraction of bacterial genomic DNA from fecal samples
5. Preparation of 16s rDNA amplicon libraries
6. Massive pool preparation of amplicons to sequence in ion torrent semi-conductor sequencer.

6.1.3 Stage. 3 Metagenomic analysis of colon microbiota of Mexican children

Activities

7. Taxonomy assignment using Bioinformatics analysis- QIIME pipeline
8. Microbial diversity analysis for both alpha and beta diversity
9. Data processing using multivariate analysis

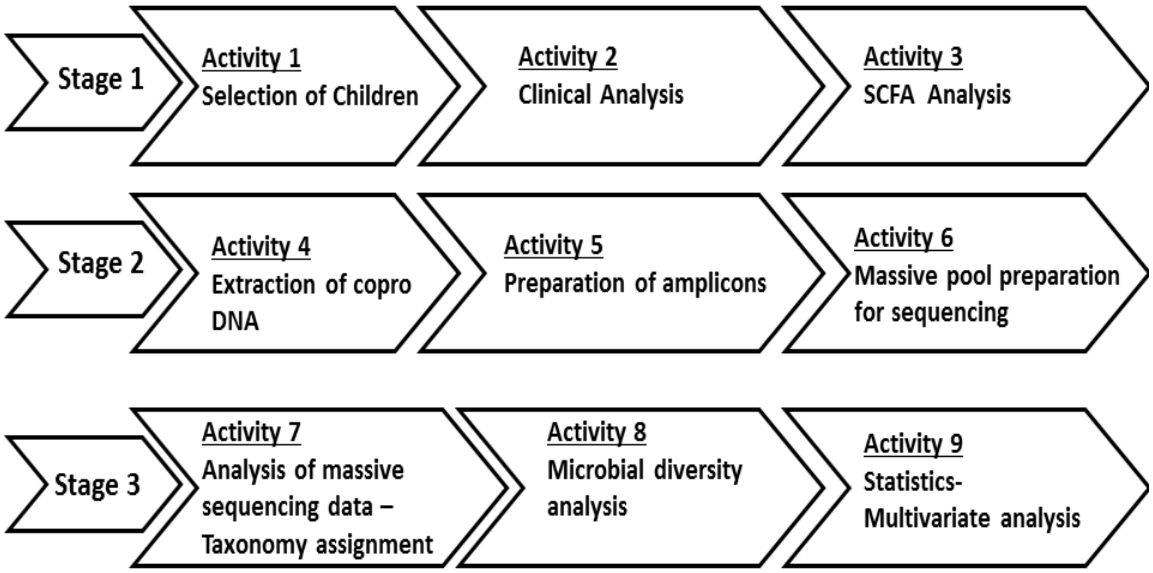


Figure 7. Work plan for SCFA and Metagenomics analysis

6.2 Methods

6.2.1 Stage. 1 Selection of children and SCFA analysis

Activity 1. Selection of children based on anthropometric method

A total number of 190 unrelated children (81 normal, 29 overweight and 80 obese) between the ages of 9 to 11 years were selected from a public primary school, in the Ecatepec borough in the Greater Mexico City area. Children were classified into three groups using anthropometric, body mass index and biochemical profile studies. Selected children were healthy and had not received any antibiotics in the immediate previous 3 months period. The informed consent was signed out by parents and children in accordance with the Helsinki Declaration revised in 2000. The research protocol was approved by the Local Ethical Committee Board of Health from the Instituto Mexicano del Seguro Social R-2011–1402 1402–10, Mexico City.

Children were weighed using a scale, and measured using a stadiometer. The body mass index (BMI) was calculated and classified based on the World Health Organization Norms (de Onis *et al.,* 2007). According to the tables and graphics, for ages ranging from 2 to 20 years, normal children have a BMI between the 10th and the 85th percentile; overweight above 85th and up to 95th; while obese children have a BMI greater than the 95th percentile. The waist was measured at the midpoint between the lower rib and iliac crest.

Activity 2. Clinical analysis of metabolic factors from blood samples

Biochemical studies

Two blood samples were taken with 12 h fasting, in a tube with EDTA and in a Vacutainer Rapid Serum Tube. Glucose, total cholesterol, high lipoprotein density (HDL), low lipoprotein density (LDL) and triglycerides were measured in mg/dL using an ILab 350 System (García-Cuartero *et al*., 2007).

Dietary diversity assessment

Diversity in the diet was estimated using a 7-d recall (Arimond M *et al*., 2004). Information was collected from children in our data base supervised by their parents. A set of seven foods/food groups diversity indicators were selected 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. Foods/food groups that the child consumed ≥3 days in the previous week received a score of “1”, and those the child consumed <3 days in the past week scored “0.” A final score was calculated for each child adding the values of all consumed groups, thus, a score of 7 was the maximum possible value for each individual.

Activity 3. SCFA analysis from fecal samples of children

Sample collection and processing

Fecal samples were collected aseptically in a sterile stool container from normal weight, overweight and obese children. Samples were transported to the laboratory using frozen ice packs and were immediately stored at -70° C, and aliquoted until further processing. For SCFA analysis, fecal samples were dried up to constant weight.

Measurement of short chain fatty acids concentration by HPLC

Fecal samples to be analyzed for short chain fatty acids (SCFA) content were dried up to constant weight, and subsequently processed using solid-phase extraction method, to analyze through a High Performance Liquid Chromatography (HPLC-Agilent Technologies 1260). 100 mg of dried feces were suspended completely in 1 ml of deionized water by vigorous vortexing, maximum speed, during 5 min. The suspension was centrifuged at 15,800 rcf for 5 min, the supernatant was transferred to a fresh tube and the pH was adjusted to 6 using 0.1 M HCl. This solution was passed through activated C-18 max 100 mg/ 1 mL GracePure™ Reversed-Phase SPE Columns. The SCFAs were eluted using 1 mL 100 % absolute ethanol, and analyzed through HPLC (De Baere *et al*., 2013), using 0.1 M Glycine-HCl as mobile phase. Methimazole at 10 mM was used as internal standard.

6.2.2 Stage. 2 Preparation of 16s rDNA libraries for massive sequencing

Activity 4. Extraction of bacterial genomic DNA from fecal samples

DNA extraction from feces

- DNA was extracted from of feces of Mexican children using QiaAmp Stool DNA mini kit (Qiagen) as follows,

- 200 mg of Feces were weighed in a 2 mL microcentrifuge tube and placed on ice.
- 1.4 ml Buffer ASL was added to each stool sample and Vortexed continuously (upto 5 mins) for until the stool sample was thoroughly homogenized.
- Samples were centrifuged at full speed (13000 RPM) for 1 min to pellet stool particles.
- The supernatants were collected into a new 2 ml microcentrifuge tube and the pellets were discarded.
- InhibitEX Tablet was added to each sample and vortexed immediately and continuously for 1 min or until the tablet is completely suspended. The suspension was incubated for 1 min at room temperature to allow inhibitors to adsorb to the InhibitEX matrix.
- Samples were centrifuged at full speed for 3 min to pellet stool particles and inhibitors bound to InhibitEX matrix.
- Immediately after the centrifuge stopped, pipetted out all of the 600 µL of supernatant into a new 1.5 mL microcentrifuge tube and the pellets were discarded, then samples were centrifuged at full speed for 3 min
- In a new 1.5 mL microcentrifuge tube 25 µL of Proteinase K were added, to which the supernatant solution from the previous step were also added.
- To the above mixture 600 µL of AL buffer was added and vortexed for 15 seconds. It was incubated at 70^{o}C for 10 min.
- 600 µl of ethanol (96–100%) were added to the lysate (above mixture), and mixed well by vortexing.
- The lid of a new QIAamp spin column was labeled and to this Carefully 600 µl lysate was applied from the last step to the QIAamp spin column without moistening the rim. The cap was closed and centrifuged at full speed for 1 min. QIAamp spin column was placed in a new 2 ml collection tube, and the tube containing the filtrate was discarded. This step was repeated until all the lysates were passed through the spin column.

- To the QIAamp spin column, 500 µl Buffer AW1 was added carefully and was centrifuged at full speed for 1 min. The QIAamp spin column was placed in a new 2 ml collection tube, the collection tube containing the filtrate was discarded.
- To the QIAamp spin column, 500 µl Buffer AW2 was added carefully and was centrifuged at full speed for 1 min. The QIAamp spin column was placed in a new 2 ml collection tube, the collection tube containing the filtrate was discarded.
- The QIAamp spin column was transferred into a new, labeled 1.5 ml microcentrifuge tube. The QIAamp spin column was opened carefully and 200 µl Buffer AE was pipetted directly onto the QIAamp membrane. The cap was closed and incubated for 1 min at room temperature, then was centrifuge at full speed for 1 min to elute DNA.
- Eluted DNA samples were stored in -70^{o}C before further use.
- The quantity of purified DNA was measured at 260/280 absorbance using a Nanodrop LITE (Thermo Scientific), and the quality was evaluated by electrophoretic fractionation in 0.5 % agarose.

Activity 5. Preparation of 16s rDNA amplicon libraries

For each copro DNA sample, an amplicon of approximately 263 bp containing the V3 polymorphic region of the bacterial 16S rDNA (Figure 8.), was amplified using a sense V3-341F primer containing a particular 12 bp Golay barcode (Fierer *et al*., 2008), an A–adapter for massive sequencing in Ion Torrent PGM (Life Technologies), and an antisense V3-518R containing Truncated P1 (TrP1)–adapters (Whiteley *et al*., 2012). The complete list of the primers used in this study is reported in Table 1. The 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 6. Massive pool preparation of amplicons to sequence in ion torrent semi-conductor sequencer.

For sequencing, equivalent amounts (10 µg each) of amplicons, were combined in groups of 50 individuals, regardless of their normal, overweight, or obese phenotype. Each 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 26 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. The sequencing was made using Ion 316 v2 Chips and Ion Torrent PGM system. 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 auto

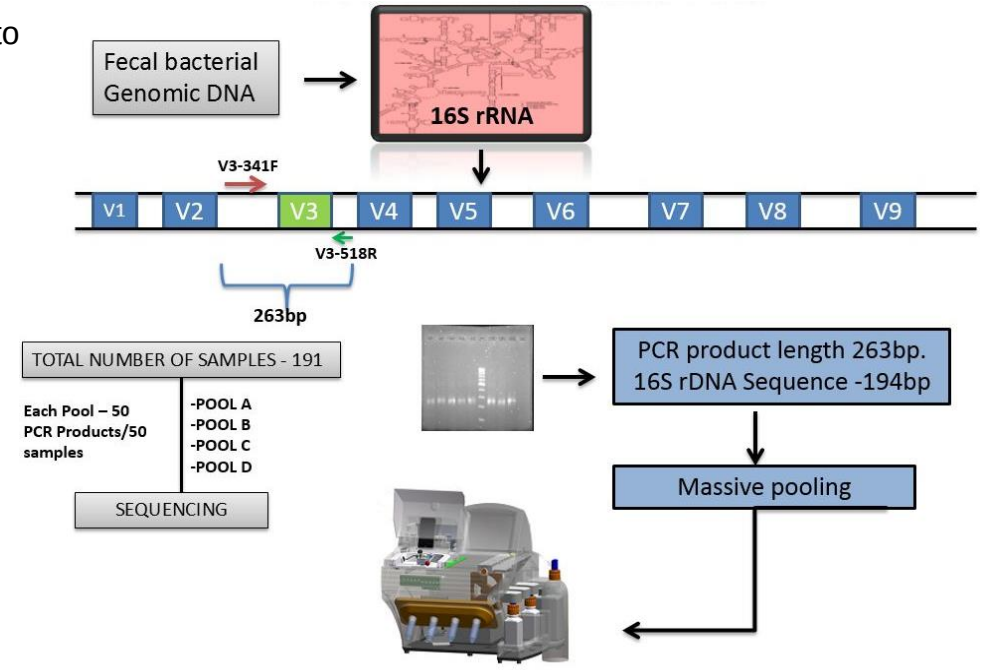


Figure 8. Strategy for sequencing colon microbiota using ion torrent PGM

Table. 1 List of primers used in amplicon preparation

NAME OF PRIMER	ION TORRENT LINKER	GOLAY BARCODE	SPACER	16S rRNA Primer (5'-3')
V3-341 F1	CCATCTCATCCCTGCGTGTCTCCGACTCAG	GATCTGCGATCC	GT	CCTACGGGAGGCAGCAG
V3-341 F2	CCATCTCATCCCTGCGTGTCTCCGACTCAG	CAGCTCATCAGC	GT	CCTACGGGAGGCAGCAG
V3-341 F3	CCATCTCATCCCTGCGTGTCTCCGACTCAG	CAAACAACAGCT	GT	CCTACGGGAGGCAGCAG
V3-341 F4	CCATCTCATCCCTGCGTGTCTCCGACTCAG	GCAACACCATCC	GT	CCTACGGGAGGCAGCAG
V3-341 F5	CCATCTCATCCCTGCGTGTCTCCGACTCAG	GCGATATATCGC	GT	CCTACGGGAGGCAGCAG
V3-341 F6	CCATCTCATCCCTGCGTGTCTCCGACTCAG	CGAGCAATCCTA	GT	CCTACGGGAGGCAGCAG
V3-341 F7	CCATCTCATCCCTGCGTGTCTCCGACTCAG	AGTCGTGCACAT	GT	CCTACGGGAGGCAGCAG
V3-341 F8	CCATCTCATCCCTGCGTGTCTCCGACTCAG	GTATCTGCGCGT	GT	CCTACGGGAGGCAGCAG
V3-341 F9	CCATCTCATCCCTGCGTGTCTCCGACTCAG	CGAGGGAAAGTC	GT	CCTACGGGAGGCAGCAG
V3-341 F10	CCATCTCATCCCTGCGTGTCTCCGACTCAG	CAAATTCGGGAT	GT	CCTACGGGAGGCAGCAG
V3-341 F11	CCATCTCATCCCTGCGTGTCTCCGACTCAG	AGATTGACCAAC	GT	CCTACGGGAGGCAGCAG
V3-341 F12	CCATCTCATCCCTGCGTGTCTCCGACTCAG	AGTTACGAGCTA	GT	CCTACGGGAGGCAGCAG
V3-341 F13	CCATCTCATCCCTGCGTGTCTCCGACTCAG	GCATATGCACTG	GT	CCTACGGGAGGCAGCAG
V3-341 F14	CCATCTCATCCCTGCGTGTCTCCGACTCAG	CAACTCCCGTGA	GT	CCTACGGGAGGCAGCAG
V3-341 F15	CCATCTCATCCCTGCGTGTCTCCGACTCAG	TTGCGTTAGCAG	GT	CCTACGGGAGGCAGCAG
V3-341 F16	CCATCTCATCCCTGCGTGTCTCCGACTCAG	TACGAGCCCTAA	GT	CCTACGGGAGGCAGCAG
V3-341 F17	CCATCTCATCCCTGCGTGTCTCCGACTCAG	CACTACGCTAGA	GT	CCTACGGGAGGCAGCAG
V3-341 F18	CCATCTCATCCCTGCGTGTCTCCGACTCAG	TGCAGTCCTCGA	GT	CCTACGGGAGGCAGCAG
V3-341 F19	CCATCTCATCCCTGCGTGTCTCCGACTCAG	ACCATAGCTCCG	GT	CCTACGGGAGGCAGCAG
V3-341 F20	CCATCTCATCCCTGCGTGTCTCCGACTCAG	TGGACATCTCTT	GT	CCTACGGGAGGCAGCAG
V3-341 F21	CCATCTCATCCCTGCGTGTCTCCGACTCAG	GAACACTTTGGA	GT	CCTACGGGAGGCAGCAG
V3-341 F22	CCATCTCATCCCTGCGTGTCTCCGACTCAG	GAGCCATCTGTA	GT	CCTACGGGAGGCAGCAG
V3-341 F23	CCATCTCATCCCTGCGTGTCTCCGACTCAG	TTGGGTACACGT	GT	CCTACGGGAGGCAGCAG
V3-341 F24	CCATCTCATCCCTGCGTGTCTCCGACTCAG	AAGGCGCTCCTT	GT	CCTACGGGAGGCAGCAG
V3-341 F25	CCATCTCATCCCTGCGTGTCTCCGACTCAG	TAATACGGATCG	GT	CCTACGGGAGGCAGCAG
V3-341 F26	CCATCTCATCCCTGCGTGTCTCCGACTCAG	TCGGAATTAGAC	GT	CCTACGGGAGGCAGCAG
V3-341 F27	CCATCTCATCCCTGCGTGTCTCCGACTCAG	TGTGAATTCGGA	GT	CCTACGGGAGGCAGCAG
V3-341 F28	CCATCTCATCCCTGCGTGTCTCCGACTCAG	CATTCGTGGCGT	GT	CCTACGGGAGGCAGCAG
V3-341 F29	CCATCTCATCCCTGCGTGTCTCCGACTCAG	AACGCACGCTAG	GT	CCTACGGGAGGCAGCAG
V3-341 F30	CCATCTCATCCCTGCGTGTCTCCGACTCAG	ACACTGTTCATG	GT	CCTACGGGAGGCAGCAG
V3-341 F31	CCATCTCATCCCTGCGTGTCTCCGACTCAG	ACCAGACGATGC	GT	CCTACGGGAGGCAGCAG
V3-341 F32	CCATCTCATCCCTGCGTGTCTCCGACTCAG	ACGCTCATGGAT	GT	CCTACGGGAGGCAGCAG
V3-341 F33	CCATCTCATCCCTGCGTGTCTCCGACTCAG	ACTCACGGTATG	GT	CCTACGGGAGGCAGCAG
V3-341 F34	CCATCTCATCCCTGCGTGTCTCCGACTCAG	AGACCGTCAGAC	GT	CCTACGGGAGGCAGCAG
V3-341 F35	CCATCTCATCCCTGCGTGTCTCCGACTCAG	AGCACGAGCCTA	GT	CCTACGGGAGGCAGCAG
V3-341 F36	CCATCTCATCCCTGCGTGTCTCCGACTCAG	ACAGACCACTCA	GT	CCTACGGGAGGCAGCAG
V3-341 F37	CCATCTCATCCCTGCGTGTCTCCGACTCAG	ACCAGCGACTAG	GT	CCTACGGGAGGCAGCAG
V3-341 F38	CCATCTCATCCCTGCGTGTCTCCGACTCAG	ACGGATCGTCAG	GT	CCTACGGGAGGCAGCAG
V3-341 F39	CCATCTCATCCCTGCGTGTCTCCGACTCAG	AGCTTGACAGCT	GT	CCTACGGGAGGCAGCAG
V3-341 F40	CCATCTCATCCCTGCGTGTCTCCGACTCAG	AACTGTGCGTAC	GT	CCTACGGGAGGCAGCAG
V3-341 F41	CCATCTCATCCCTGCGTGTCTCCGACTCAG	ACCGCAGAGTCA	GT	CCTACGGGAGGCAGCAG
V3-341 F42	CCATCTCATCCCTGCGTGTCTCCGACTCAG	ACGGTGAGTGTC	GT	CCTACGGGAGGCAGCAG
V3-341 F43	CCATCTCATCCCTGCGTGTCTCCGACTCAG	ACTCGATTCGAT	GT	CCTACGGGAGGCAGCAG
V3-341 F44	CCATCTCATCCCTGCGTGTCTCCGACTCAG	AGACTGCGTACT	GT	CCTACGGGAGGCAGCAG
V3-341 F45	CCATCTCATCCCTGCGTGTCTCCGACTCAG	AGCAGTCGCGAT	GT	CCTACGGGAGGCAGCAG
V3-341 F46	CCATCTCATCCCTGCGTGTCTCCGACTCAG	AGGACGCACTGT	GT	CCTACGGGAGGCAGCAG
V3-341 F47	CCATCTCATCCCTGCGTGTCTCCGACTCAG	AAGAGATGTCGA	GT	CCTACGGGAGGCAGCAG
V3-341 F48	CCATCTCATCCCTGCGTGTCTCCGACTCAG	ACAGCAGTGGTC	GT	CCTACGGGAGGCAGCAG
V3-341 F49	CCATCTCATCCCTGCGTGTCTCCGACTCAG	ACGTACTCAGTG	GT	CCTACGGGAGGCAGCAG
V3-341 F50	CCATCTCATCCCTGCGTGTCTCCGACTCAG	ACTCGCACAGGA	GT	CCTACGGGAGGCAGCAG
V3-518 R	CCTCTCTATGGGCAGTCGGTGAT	NOT APPLICABLE	CC	ATTACCGCGGCTGCTGG

6.2.3 Stage. 3 Metagenomic analysis of colon microbiota of Mexican children

Activity 7. Taxonomy assignment using Bioinformatics analysis- QIIME pipeline.

Ion torrent PGM software, Torrent Suite v4.0.2 was used to demultiplex the sequenced data based on their barcodes in normal, overweight and obese phenotypes. Poor quality reads were eliminated from the datasets, i.e. quality score <20, containing homopolymers >6, length <200 nt, and containing errors in primers and barcodes. Filtered data were exported as FASTQ files. Demultiplexed sequencing data were analyzed using QIIME software v1.8.0 pipeline (Caporaso *et al*., 2010). FASTQ files were converted into FASTA files, and all demultiplexed files were concatenated into a single file. Closed reference Operational Taxonomic Units (OTU) were determined at 97 % similarity level with UCLUST Algorithm (Edgar 2010). Chimeras were detected and removed from the datasets using the Chimera Slayer (Haas *et al*., 2011). Sequence alignments were done against the Greengenes core set (DeSantis *et al.*, 2006).

Activity 8. Microbial diversity analysis for both alpha and beta diversity.

Microbial diversity had been assessed through both alpha and beta diversity. Using rarefied OTU tables, alpha diversities were calculated using various matrices like Shannon, PD whole tree, chao1, observed species. The beta diversity analysis was calculated using UniFrac analysis (Vázquez-Baeza *et al*., 2013), by phylogenetic tree computed with FastTree and a rarefiled biom table as inputs. Abundance of the bacterial groups at different taxonomic levels (phylum, order, and genus) was separately explored with a Principal Coordiniate Analysis (PCoA) and Unweighted Pair Group Method with Arithmetic mean (UPGMA) Clustering.

Activity 9. Data processing using multivariate analysis.

The anthropometric characteristics and SCFAs profiles were statistically analyzed using chi-square, analysis of variance (ANOVA) and Kruskal–Wallis one-way analysis of variance. Statistical analysis of Relative abundance of colon microbiota among the Mexican children were done using Kruskal-Wallis test. For Multivariate - Principal components analysis were used to see their variance. Furthermore, we demonstrate a multivariate correlation between the colon microbiota with SCFAs and the other clinical factors such as blood glucose, Triglycerides, LDL, HDL and Cholesterol levels to find the best model of obesity.

7. RESULTS

7.1 Activity 1. Selection of children based on anthropometric method

A total number of 190 children 9 to 11 years old, were selected from the database, and classified into normal weight (N=81), overweight (N=29) and obese (N=80) phenotype based on World Health Organization norms. The anthropometric data showed a significant difference in weight and height; the statistical analysis indicated the BMI was increased in overweight and obese children with respect to normal ($P<0.001$) (Table 2).

7.2 Activity 2. Clinical analysis of metabolic factors from blood samples

The biochemical studies revealed the bloodstream triglycerides levels were significantly elevated in overweight and obese children ($P=0.0001$). The fasting glucose levels were below 100 mg/dL in the normal, overweight and obese children, and there was not difference among them ($P=0.192$); in the same manner, there was not difference in the low ($P=0.246$) and high density lipoprotein levels ($P=0.104$). On the other hand, although there was not difference in cholesterol levels among the normal, overweight and obese children ($P=0.194$), the bloodstream concentration for overweight and obese was

above 170 mg/dL (Table 2). We explored the dietary diversity in all children using a 7-d recall study as described in materials and methods, without finding a significant difference among the three groups, that could explain the increase in triglycerides levels we observed (Table 3).

Table 2. Clinical characteristics of children

Characteristics	Normal	Overweight	Obese	P
Number of children	N=81 (42.6%)	N=29 (15.3%)	N=80 (42.1%)	nd
Female (%)	57.3	62.1	52.5	0.641[#]
Age (years)	9.85 ± 0.83	9.86 ± 0.92	10.04 ± 0.85	0.352[*]
Anthropometric				
Weight (kg)	31.68 ± 5.10	38.13 ± 3.94	46.48 ± 6.94	<0.001[&]
Height (cm)	1.37 ± 0.06	1.39 ± 0.06	1.41 ± 0.06	0.020[*]
BMI (kg/m^2)	16.77 ± 1.74	19.82 ± 1.02	23.40 ± 2.78	<0.001[&]
BMI (percentile)	51.08 ± 25.47	89.12 ± 2.89	97.82 ± 3.67	<0.001[&]
BMI	<85 pc	85 pc ≤ and ≤95	>95 pc	nd
Metabolic factors				
Fasting glucose (mg/dL)	96.09 ± 10.33	96.57 ± 7.73	93.79 ± 8.25	0.192[*]
Triglycerides (mg/dL)	97.83 ± 38.36	111.76 ± 43.41	134.18 ± 49.47	0.0001[*]
Total cholesterol (mg/dL)	166.70± 27.32	175.28 ± 25-15	174.95 ± 36.72	0.194[*]
HDL (mg/dL)	51.94 ± 10.45	49.53 ± 9.60	48.41 ± 11.06	0.104[*]
LDL (mg/dL)	95.22 ± 23.41	103.44 ± 22.14	99.68 ± 34.64	0.246[&]

N, number of individuals; HDL, high density lipoprotein; LDL, low density lipoprotein; Data are means ± standard deviation. P values were calculated according to [#]Chi-Square Test; *ANOVA test for equal variances, and [&]Kruskall Wallis test for different variances. P<0.05 are considered statistically significant; pc, percentile; nd, not determined.

Table 3. Dietary diversity for children by phenotypic classification

	Mean diversity score (range 0–7)	% with low diversity 0–2 food groups	% with middle diversity 3–4 food groups	% with high diversity 5–7 food groups
Normal	2.8	41.3	39.1	19.6
Overweight	2.5	53.3	40.0	6.7
Obese	2.8	44.7	36.2	19.1

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 (Arimond *et al*., 2004).

7.3 Activity 3. SCFA analysis from fecal samples of children.

Overweight and obese children present altered propionic and butyric concentration in feces.

The short chain fatty acids (butyric, propionic and acetic) concentration was measured in feces by HPLC as described in Materials and methods. We found that feces collected from overweight and obese children contained a significant lower butyric acid concentration compared to normal weight children (P=0.023) (Figure 7). In the case of propionic acid, another important fatty acid; the feces of obese children had a significant lower concentration in comparison to overweight (P=0.025), and at the same time, the concentration of propionic acid in obese children was lower than the concentration found in normal children (P=0.048). On the other hand, there was not difference in the concentration of acetic acid among the three groups (Figure 17).

7.4 Activity 4. Extraction of bacterial genomic DNA from fecal samples.

Extracted fecal bacterial genomic DNA concentration of normal weight, overweight and obese Mexican children were measured using Nanodrop LITE (Thermo Scientific). The quality of the extracted DNA were checked through 0.5% agarose gel electrophoresis. The list of DNA concentration of normal weight, overweight and Obese were mentioned in Table 3-5. The concentration of fecal bacterial DNA of normal weight, overweight and obese children were 212.9 ± 134.4 ng/µL, 240.7 ± 95.06 ng/µL and 227.1 ± 120.4 ng/µL, respectively. Eventhough, there was no significant difference but a slight increase in DNA concentraton in both overweight and obese group compared to normal weight children.

Table 4. Fecal bacterial DNA Concentration of Normal weight children

Folio	Phenotype	DNA conc(ng/µl)	168	Normal	200.6
006	Normal	139.2	173	Normal	201.6
007	Normal	144.8	175	Normal	196.5
009	Normal	29.6	177	Normal	232.1
013	Normal	69.2	181	Normal	247.1
018	Normal	641.3	185	Normal	401.7
022	Normal	183.4	189	Normal	158.5
024	Normal	46.3	190	Normal	196.5
029	Normal	98	192	Normal	262.4
030	Normal	326.8	195	Normal	107.9
032	Normal	10.1	199	Normal	179.4
033	Normal	53.5	205	Normal	619
036	Normal	141.2	207	Normal	195.3
046	Normal	288.5	209	Normal	195.9
048	Normal	597.7	211	Normal	275.8
053	Normal	167.2	217	Normal	163.1
071	Normal	133.2	221	Normal	242.7
078	Normal	93.2	222	Normal	151.8
085	Normal	324.3	225	Normal	324.1
091	Normal	97.9	227	Normal	152.6
093	Normal	107.3	235	Normal	457.1
099	Normal	167.7	236	Normal	322
107	Normal	48.9	240	Normal	206.1
111	Normal	273.2	242	Normal	166.6
114	Normal	250.8	243	Normal	253.4
117	Normal	661.1	247	Normal	151.5
125	Normal	84.3	249	Normal	184.8
130F	Normal	57.4	250	Normal	171
132	Normal	162.4	253	Normal	165
133I	Normal	203.2	254	Normal	145.3
140	Normal	175.2	261	Normal	232.4
145	Normal	78.5	262	Normal	129.9
152	Normal	186.2	264	Normal	236.2
156	Normal	62.9	270	Normal	304.3
157	Normal	121.9	274	Normal	471.3
159	Normal	244.6	277	Normal	354.3
160	Normal	370.4	284	Normal	183.7
161	Normal	142.8	288	Normal	289.5
164	Normal	192.3	290	Normal	89.7
166	Normal	108.9	291	Normal	293.6
167	Normal	88.6	293	Normal	358.8

Table 5. Fecal bacterial DNA Concentration of Overweight children

Folio	Phenotype	DNA conc(ng/µl)	Folio	Phenotype	DNA conc(ng/µl)
10	Over	87.7	150	Over	390.6
12	Over	166.3	155	Over	256.6
15	Over	299.8	158	Over	176.7
20	Over	137.7	165	Over	425.2
26	Over	97	169	Over	206.6
38	Over	219.3	179	Over	253.4
67	Over	165.5	197	Over	224.6
73	Over	229.3	203	Over	323.7
75	Over	380.9	238	Over	351.8
86	Over	173.2	245	Over	337.7
89	Over	64.8	273	Over	352.7
95	Over	115.8	280	Over	309
105	Over	166	282	Over	301.1
110	Over	300.5	283	Over	224.1
146	Over	244.7			

Table 6. Fecal bacterial DNA Concentration of Obese children

Folio	Phenotype	DNA conc(ng/µl)	Folio	Phenotype	DNA conc(ng/µl)
001	Obese	138.6	162	Obese	429.7
002	Obese	117.3	170	Obese	248.3
003	Obese	225.3	171	Obese	196.5
005	Obese	222.4	172	Obese	479.1
011	Obese	107.1	178	Obese	225.6
014	Obese	319.7	180	Obese	226.8
017	Obese	266.1	184	Obese	190.6
019	Obese	662.4	193	Obese	375.4
027	Obese	198.4	194	Obese	179.7
028	Obese	86.3	196	Obese	100
049	Obese	38.1	200	Obese	197.6
051	Obese	383.5	201	Obese	256.5
052	Obese	212.3	202	Obese	254.5
062	Obese	217.3	204	Obese	202.9
064	Obese	125.4	208	Obese	262.7
070	Obese	409	210	Obese	70.6
076	Obese	133.5	212	Obese	294.1
080	Obese	378	213	Obese	554
082	Obese	445.1	214	Obese	200
083	Obese	88.3	215	Obese	94.9
087	Obese	176.1	216	Obese	247.7
092	Obese	88.1	223	Obese	66.3

096	Obese	182.8	224	Obese	427.4
097	Obese	67	226	Obese	257.4
098	Obese	152.7	228	Obese	401.8
100	Obese	164	229	Obese	337.7
101	Obese	177.1	230	Obese	277.7
112	Obese	172.7	232	Obese	331.1
116	Obese	166.4	233	Obese	170.6
123	Obese	51.4	241	Obese	454.8
127	Obese	278	244	Obese	314.6
129	Obese	288.6	248	Obese	297.1
131	Obese	211	256	Obese	168.8
134	Obese	105.3	257	Obese	223.7
135I	Obese	102.4	259	Obese	96.5
136	Obese	95.2	268	Obese	155.9
138	Obese	118.3	276	Obese	247.6
141	Obese	212.8	278	Obese	115.3
142	Obese	219.3	286	Obese	233.6
154	Obese	229.8	287	Obese	277.3

7.5 Activity 5. Preparation of 16s rDNA amplicon libraries

16S rDNA amplicon libraries were prepared using PCR (GeneAmp PCR System 2700 Thermocycler (Applied Biosystems)). The quality of amplicon libraries were fractionated using 2% agarose gel electrophoresis. 16S rDNA libriaries concentration of normal weight, overweight and obese Mexican children were measured using Nanodrop LITE (Thermo Scientific).

7.6 Activity 6. Massive pool preparation of amplicons to sequence in ion torrent semi-conductor sequencer.

From the measured DNA concentration of Amplified 16S rDNA libraries, massive pool was prepared. From each sample 10µg of amplicon was used to prepare the pool. The total number of 190 samples were divided into 4 Pools such as A, B, C and D. Each pool was made of 50 amplicon with different barcode per sample. 100µL Massive pool was fractionated using 2% agarose preparative gel, and then gel slice was made at amplicon of 263bp size (Figure.

9-12). From the gel slice amplicons for massive sequencing were purified using Wizard SV Gen PCR Clean-Up System (Promega). Purified amplicon concentrations of Pool A, B, C and D were 17.1 ng/µL, 19.6 ng/µL, 15.8 ng/µL and 24.9 ng/µL respectively.

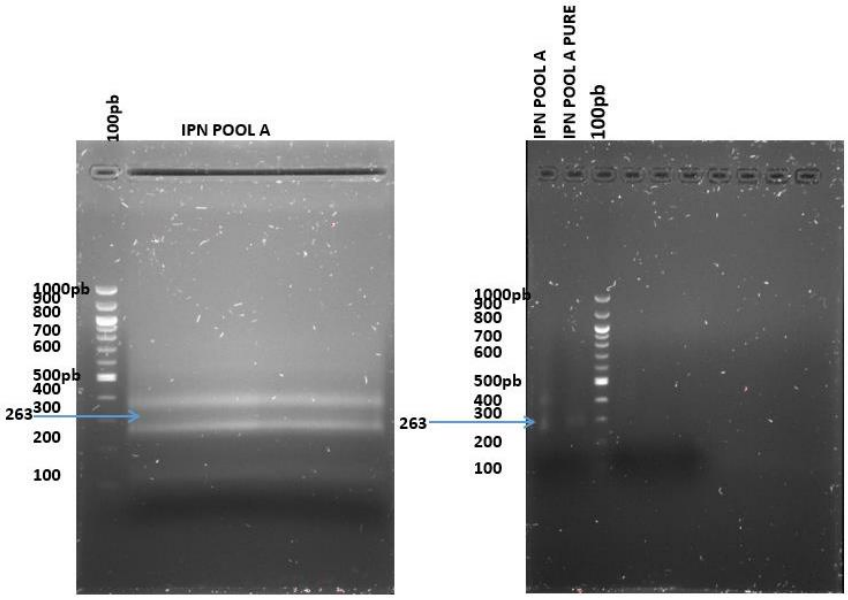


Figure 9. Massive amplicon pool-A preparation and purification. Preparative and analytic 2% Agarose Gel with 0.5µL of Midori Green- run at 100v.

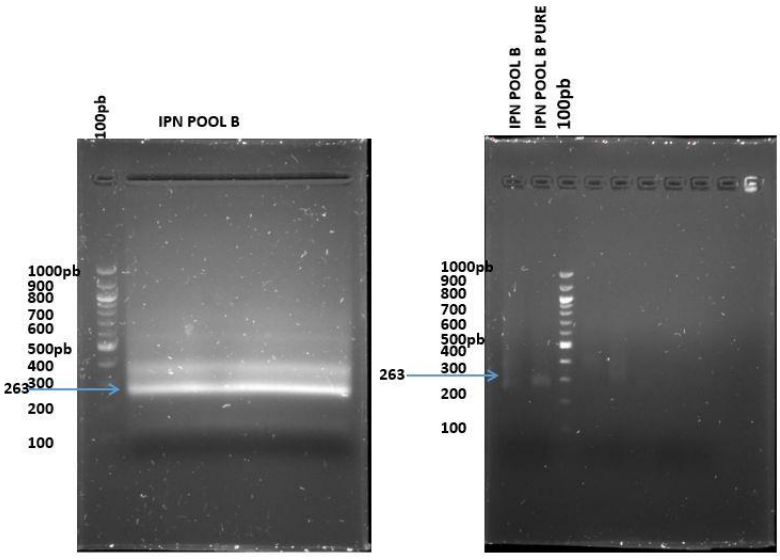


Figure 10. Massive amplicon pool-B preparation and purification. Preparative and analytic 2% Agarose Gel with 0.5µL of Midori Green- run at 100v.

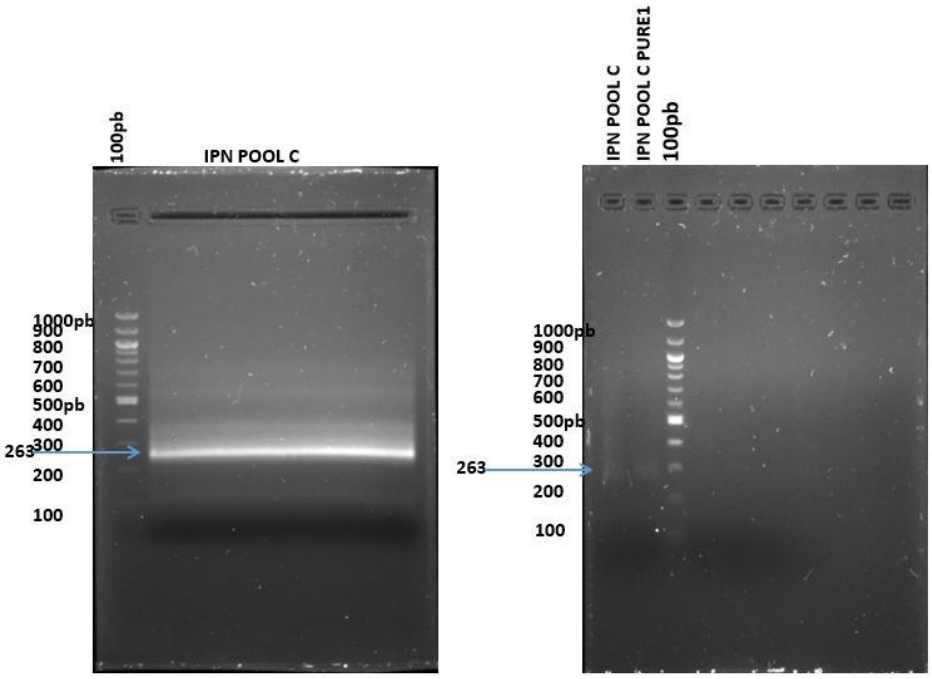


Figure 11. Massive amplicon pool-C preparation and purification. Preparative and analytic 2% Agarose Gel with 0.5µL of Midori Green- run at 100v.

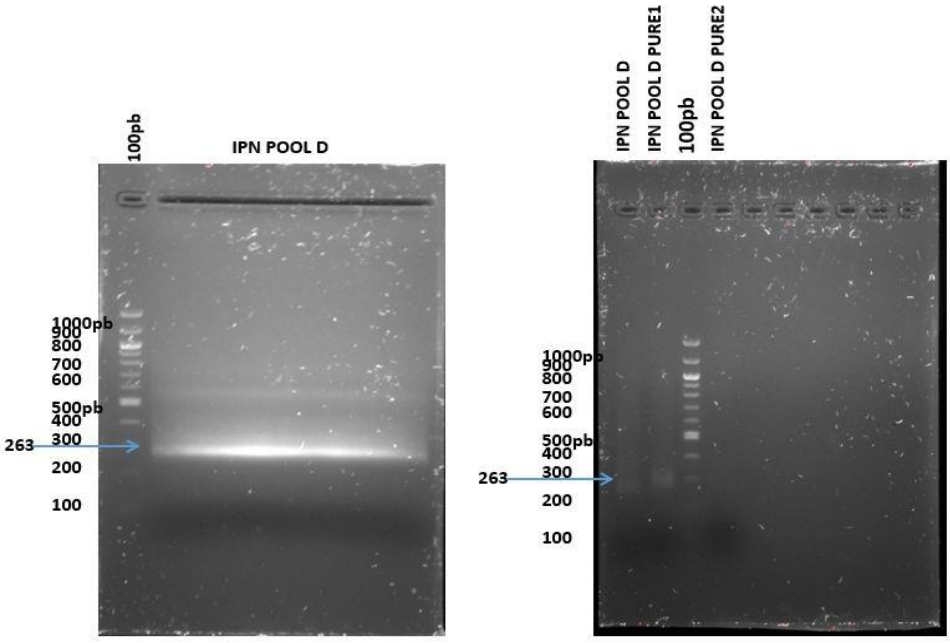


Figure 12. Massive amplicon pool-D preparation and purification. Preparative and analytic 2% Agarose Gel with 0.5µL of Midori Green- run at 100v.

7.7 Activity 7. Taxonomy assignment using Bioinformatics analysis-QIIME pipeline

Colon microbiota in Mexican overweight and obese children does not show a clear imbalance in the bacterial diversity

We next determined the relative abundance of dominant bacterial phyla in feces collected from normal, overweight and obese Mexican children. The bacterial diversity of 16S rDNA libraries was determined by ion semiconductor DNA sequencing, and the data was processed as described in Materials and methods. The results showed a lower abundance of Proteobacteria phylum in overweight (8%) and obese (4%) with respect to the normal control (10%) children; and a slight increase in the abundance of phylum Firmicutes in overweight (52%) and obese children (50%) with respect to normal weight (46%); however, the differences were not of statistical significance. In contrast, there was not a difference in the percentage of abundance of Actinobacteria and Bacteroidetes phyla among the groups (Figure13).

The abundance of particular bacteria is altered in overweight and obese Mexican children with respect to normal controls

The Relative abundance of dominant bacterial genus in Normal, Overweight and Obese Mexican children was shown in Figure 14. When data were analyzed looking for particular genera or family whose abundance were different in association to the phenotypic condition, we found two genera and one family increased in overweight and obese, the genus *Faecalibaterium* sp (P=0.042) (Figure 27B); the family Lachnospiraceae (P=0.018) (Figure 27D), and the genus *Roseburia* sp (P=0.015) (Figure 27I). In addition, the analysis of the operational taxonomic units (OTUS's) data, showed a decrease in the genus *Succinivibrio* sp (P=0.003) (Figure 27A), the genus *Erwinia* sp (P=0.003) (Figure 27E) and the genus *Oscillospira* sp (P=0.024) (Figure 27G); however the abundance of the last genus was lower in overweight with respect to obese phenotype. Besides,

the genus *Blautia* sp (P=0.036) (Figure 27C), the genus *Coprococcus* sp (P=0.023) (Figure 27F), and the family Enterobacteriaceae (P=0.030) (Figure 27H) were clearly increased in overweight phenotype.

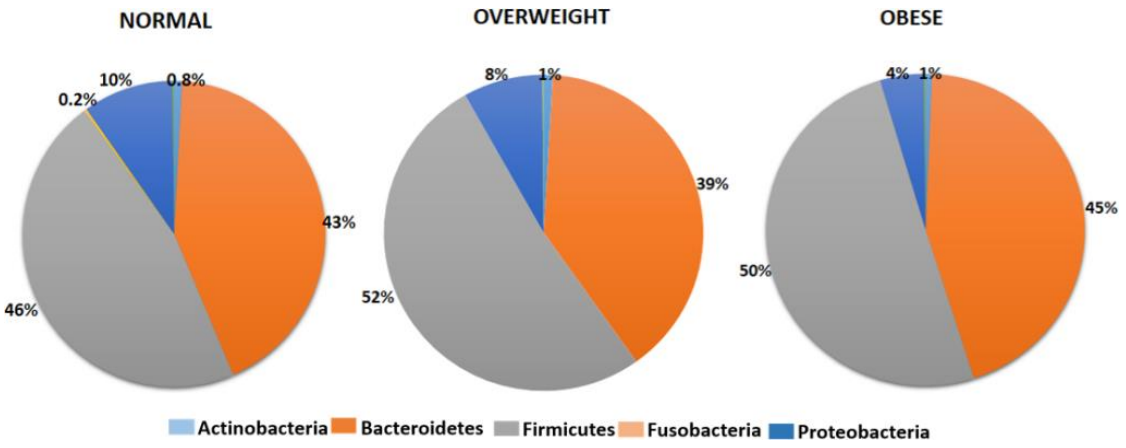


Figure 13. Relative abundance of dominant bacterial phyla in Normal, Overweight and Obese Mexican children

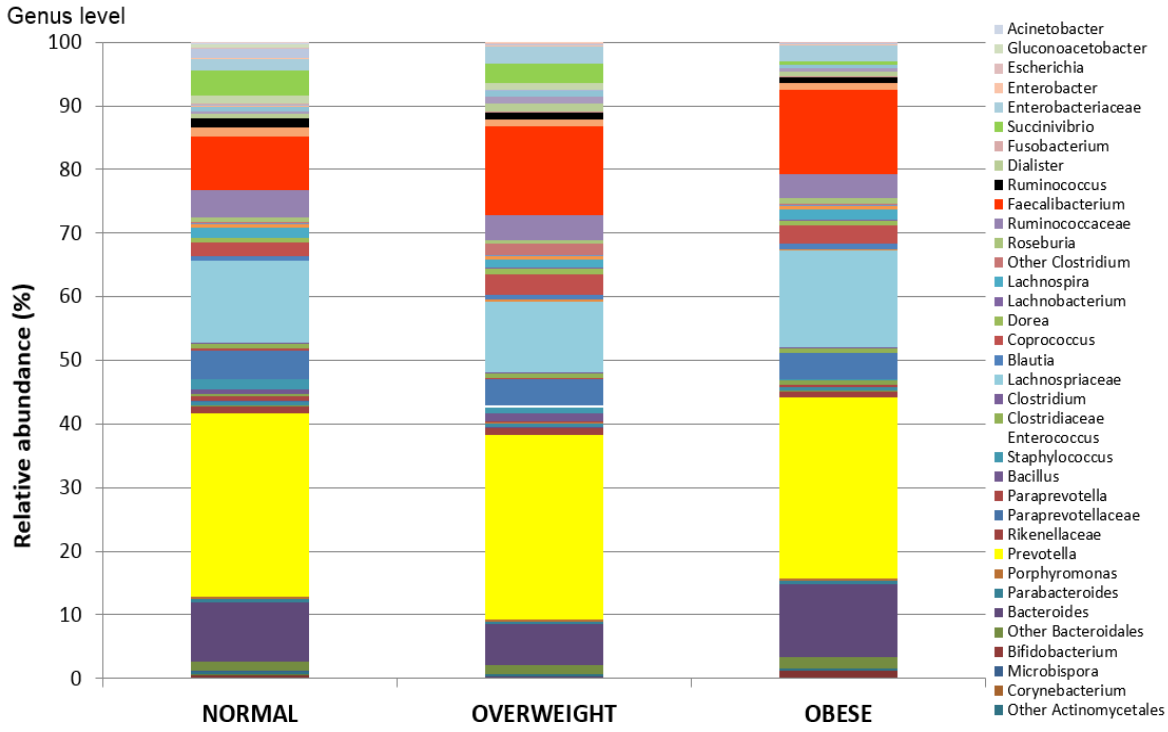
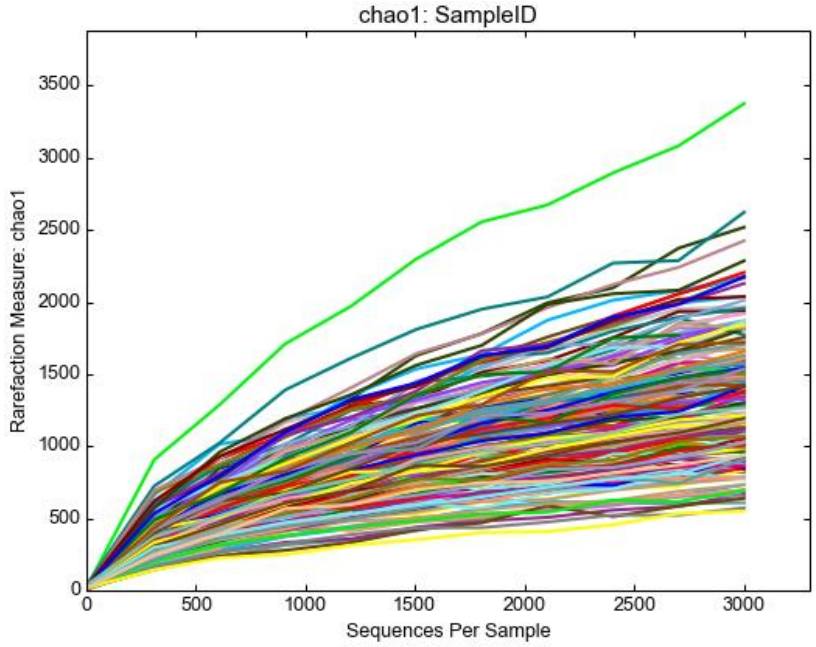


Figure 14**.** Relative abundance of dominant bacterial genus in Normal, Overweight and Obese Mexican children

A)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Rarefaction Measure: chao1
Sequences Per Sample
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500
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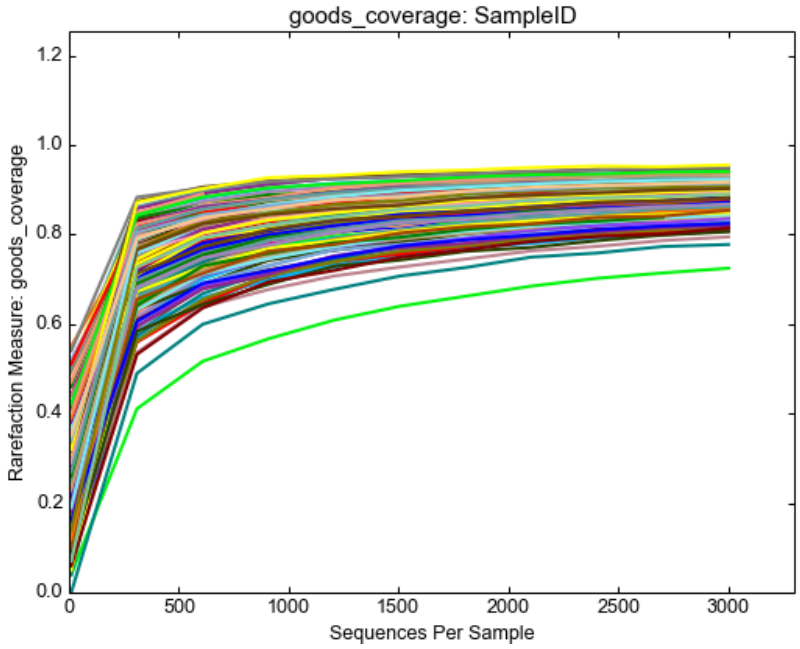
1500
2000
2500
3000
3500

B)


goods_coverage: SampleID
Rarefaction Measure: goods_coverage
Sequences Per Sample
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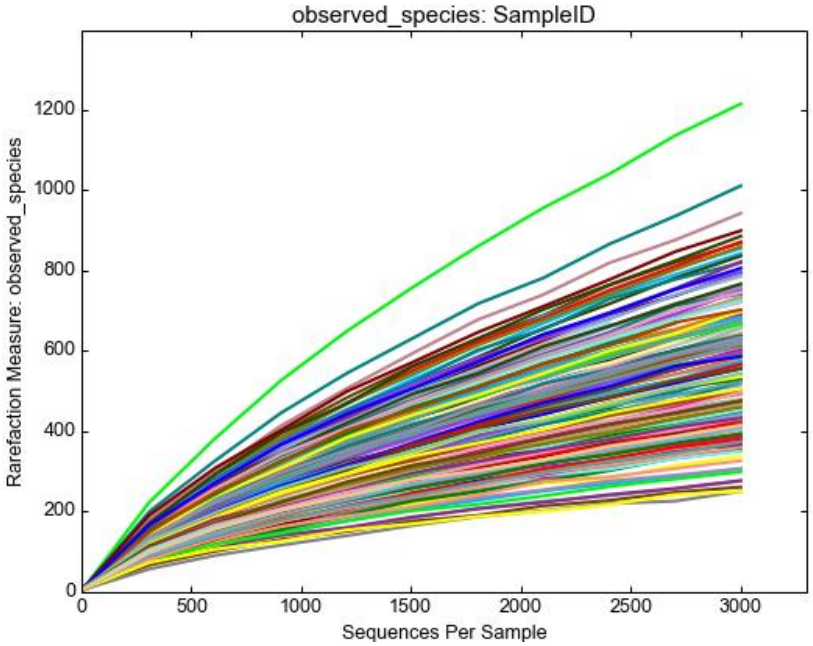
0.2
0.4
0.6
0.8
1.0
1.2
0
500
1000
1500
2000
2500
3000

C)


observed_species: SampleID
Rarefaction Measure: observed_species
Sequences Per Sample
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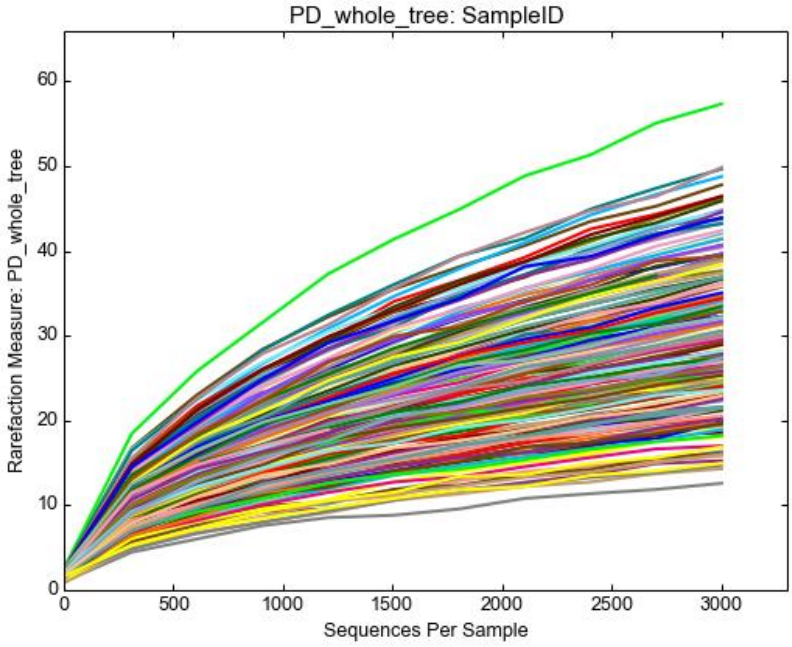
200
400
600
800
1000
1200
0
500
1000
1500
2000
2500
3000

D)


PD_whole_tree: SampleID
Rarefaction Measure: PD_whole_tree
Sequences Per Sample
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10
20
30
40
50
60
0
500
1000
1500
2000
2500
3000

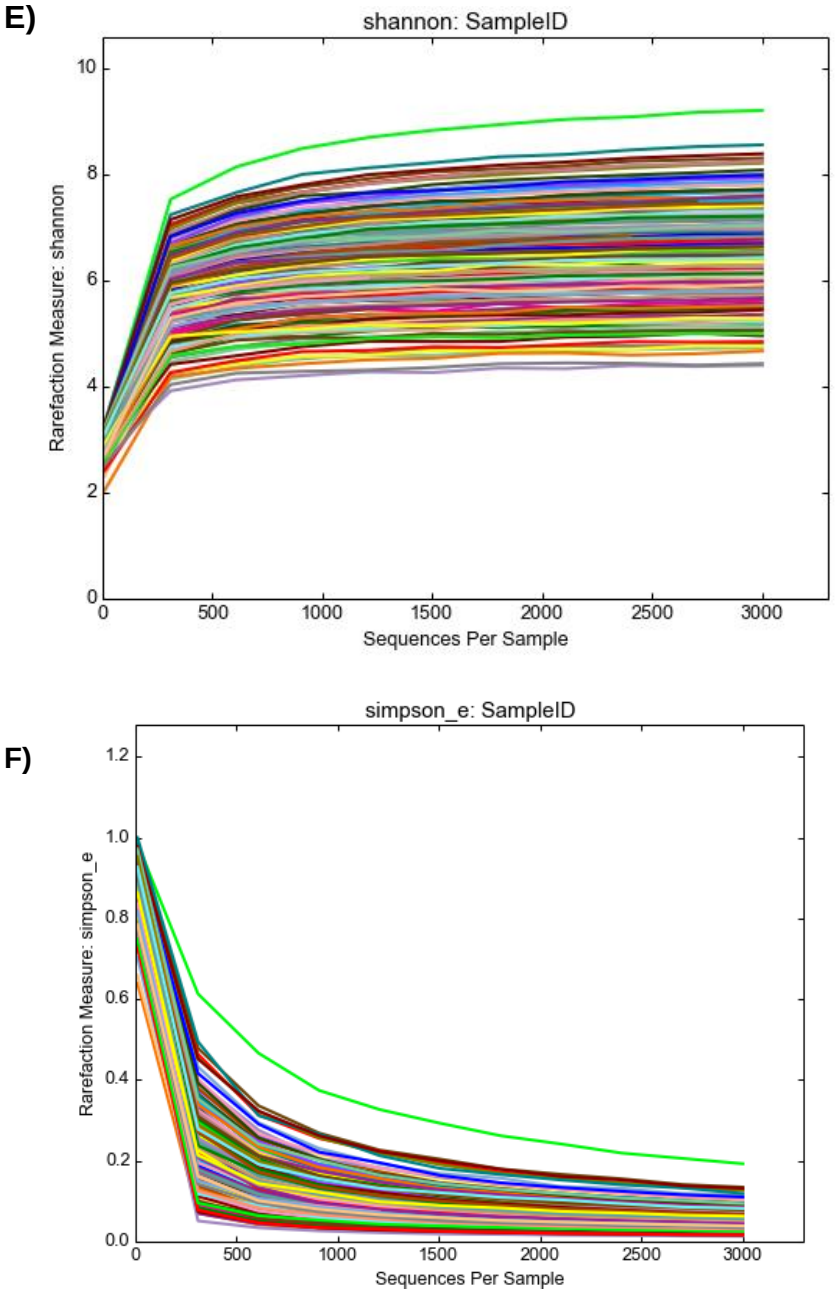

Figure 15. Alpha diversity Rarefaction curves Rarefaction curves generated for 16S rDNA V3 sequences obtained from faecal samples of Normal, Overweight and Obese Mexican Children. The vertical axis displays the diversity of the community, while the horizontal axis displays the number of sequences considered in the diversity calculation. Each color indicates diversity of community of each children. Panel A displays rarefaction curves using the Chao1 index. Panel B represents rarefaction curves using Good coverage index. Panel C displays rarefaction curves of the number of observed species was calculated at a similarity threshold of 97%. Panel D shows the number of phylotypes identified against the number of sequences per sample. Panel E represents the rarefaction curves using the Shannon index. Panel F displays the rarefaction the rarefaction curves using the Simpson index.

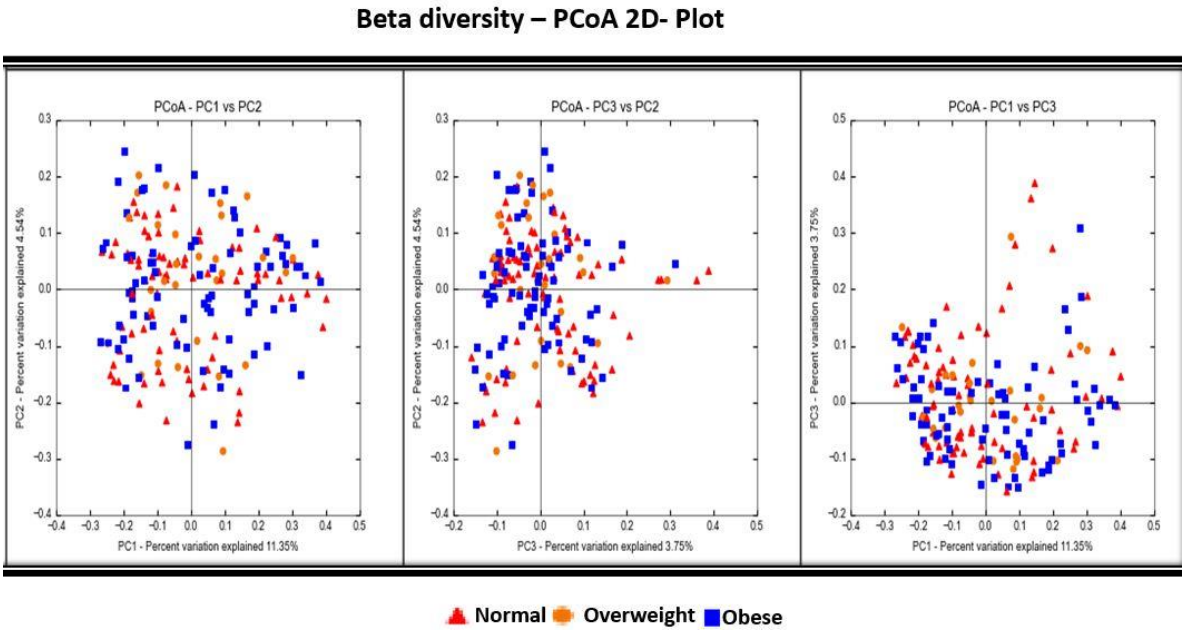

Figure 16. Unweighted UniFrac analyses were used to calculate distances between samples obtained from Normal, Overweight and Obese children and Two-dimensional scatterplots were generated by using principal coordinate analysis (PCoA).

7.8 Activity 8. Microbial diversity analysis for both alpha and beta diversity.

Alpha diversity, or within-sample diversity, was analyzed using an OTU table of Normal weight, Overweight and Obese Mexican children (Figure 15). Species Richness estimator matrix Chao1 revealed that almost all the samples presented more than 2000 OTUs. Observed species matrix showed that most of the samples of Normal weight, overweight and obese were presented more than 800bacterias and samples from obese showed 1200 bacteria. Good's coverage revealed that these libraries represented the majority of bacterial 16S rDNA sequences present in each fecal sample, with values ranging from 75% to 95%. Community diversity index Shannon showed its average ranged from 4.7 – 9.2 in most of the samples. Another community diversity index Simpson revealed that its average ranged from 0.7 - 0.9. From the results of matrices of Goods coverage, Shannon, PD whole tree, chao1, observed species, Simpson showed that those three groups of children did not have significant difference when we compare the alpha diversity. Beta diversity was analyzed through UniFrac

distances. UniFrac distances were used, and calculated through OTU table that lists the abundance of each OTU in a sample and a phylogenetic tree. Since the distance between the normal weight children, overweight and obese children was very little. We did not find a hierarchical cluster among these groups which was visualized through PCoA.

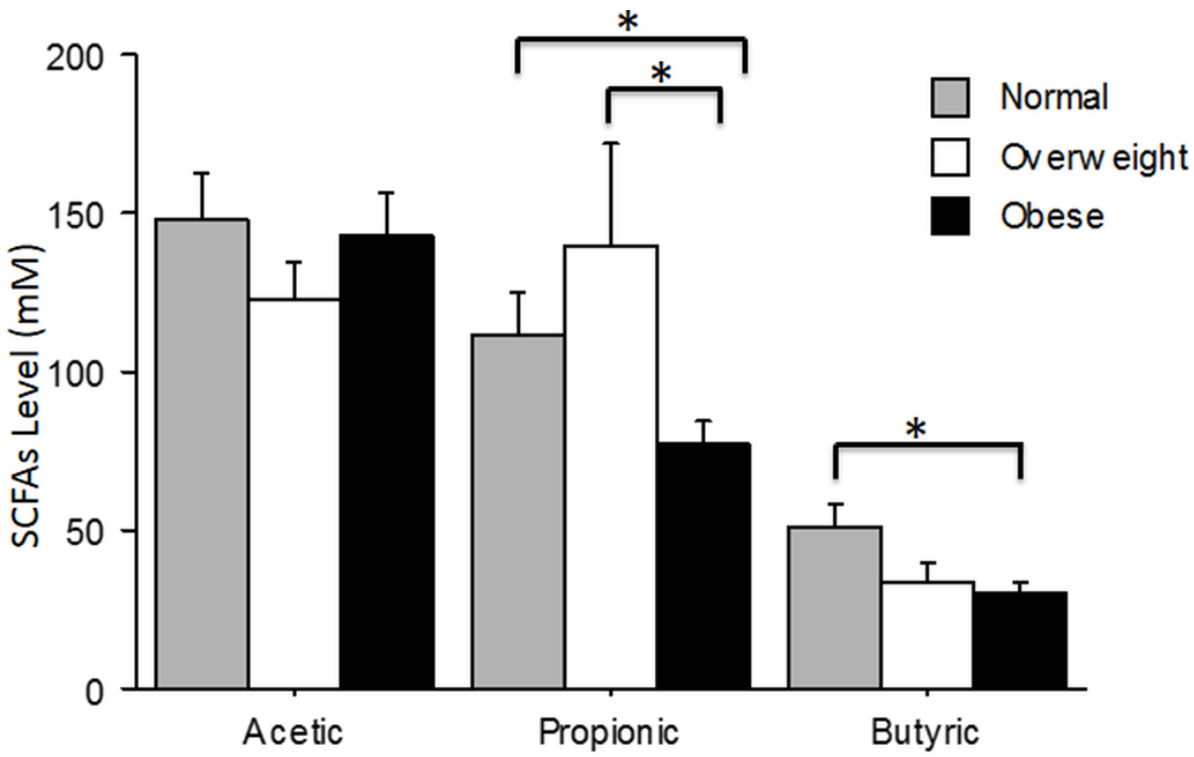

Figure 17. Short chain fatty acid (SCFA) concentration in feces. The short chain fatty acid (SCFA) concentration in feces was measured as described in Materials and methods. Data are for Normal weight, grey color bars; Overweight, white color bars; and Obese, solid bars. Error bars represent mean ± standard error. Asterisks (*), indicates statistically significant differences in data ($P<0.05$). Y-axis, SCFA concentration in mM; X-axis, SCFA and phenotypic categories.

7.9 Activity 9. Data processing using multivariate analysis.

Multiple variables such as BMI, blood glucose, triglycerides, HDL, LDL, cholesterol, SCFAs profiles and relative abundance distal colon microbiota were subjecte to multivariate analysis to find the interrelation of these variables among the normalweight, overweight and obese Mexican children. A principal component analysis (PCA) was carried out in order to determine whether distinct

patterns could be discerned from the data. In the beginning, Principal Component Analysis was done between the following variables, Principal Component Analysis of Triglycerides-Normalized, Butyric-Normalized, Actinomyces, Bifidobacteria, Bacteroides, Prevotella, Blautia, Coprococcus, Faecalibacterium, Oscillospira, Succinivibrio, Enterobacteriaceae and BMI. It showed that in PC1 (Principal Component) *Blautia and Coprococcus* was positively increased among the Mexican children. On the other hand, PC2 showed an increase in *Actinomyces and Bifidobacterium* negatively among the Mexican children (Table 7.). This analysis has been demonstrated through Score plots of components order, loading plot and Score plots of variables which are grouped among normal weight, overweight and obese Mexican children (Figure 18).

Since the previous analysis showed a minimal variance among the variables, in the second PCA few variables like Lactobacillus and Enterobacteriaceae were excluded from the analysis. Among other variables *Blautia and Coprococcus* were positively increased in PC1 in Mexican children and Bacteroides and Prevotella were positvies increased in PC2. Eventhough the variance were improved slightly, but there is no discriminant cluster pattern among the normal weight, overweight and obese children (Table 8) and (Figure 19).

In continuing with PCA, to improve the variance among all other multiple variables with the combinations such as BMI-Normalized, and Triglycerides-Normalized along with variables like *Saccharospirillum, Coprococcus, Faecalibacterium*, *Blautia, Roseburia, Oscillospira and Succinivibrio* were analyzed separately (Table 9-15), It showed a prominent increase in the variance and these variables showed clear cluster between overweight and obese children, in which normal weight children showed a pattern different from overweight and obese children (Figure 20-26).

Table 7. Principal Component Analysis of Triglycerides-Normalized, Butyric-Normalized, *Actinomyces, Bifidobacteria, Bacteroides, Prevotella, Blautia, Coprococcus, Faecalibacterium, Oscillospira, Succinivibrio,* Enterobacteriaceae, BMI-Norm1

Variable	PC1	PC2
Triglycerides-Normalized	-0.110	-0.057
Butyric-Normalized	0.15	-0.166
Actinomyces	-0.144	**-0.641**
Bifidobacterium	0.038	**-0.657**
Bacteroides	-0.039	-0.173
Prevotella	-0.317	-0.391
Blautia	**0.419**	-0.012
Coprococcus	**0.513**	0.033
Faecalibacterium	0.433	-0.135
Oscillospira	0.001	-0.044
Succinivibrio	-0.262	0.262
Enterobacteriaceae	-0.134	0.156
BMI-Norm1	-0.034	-0.236

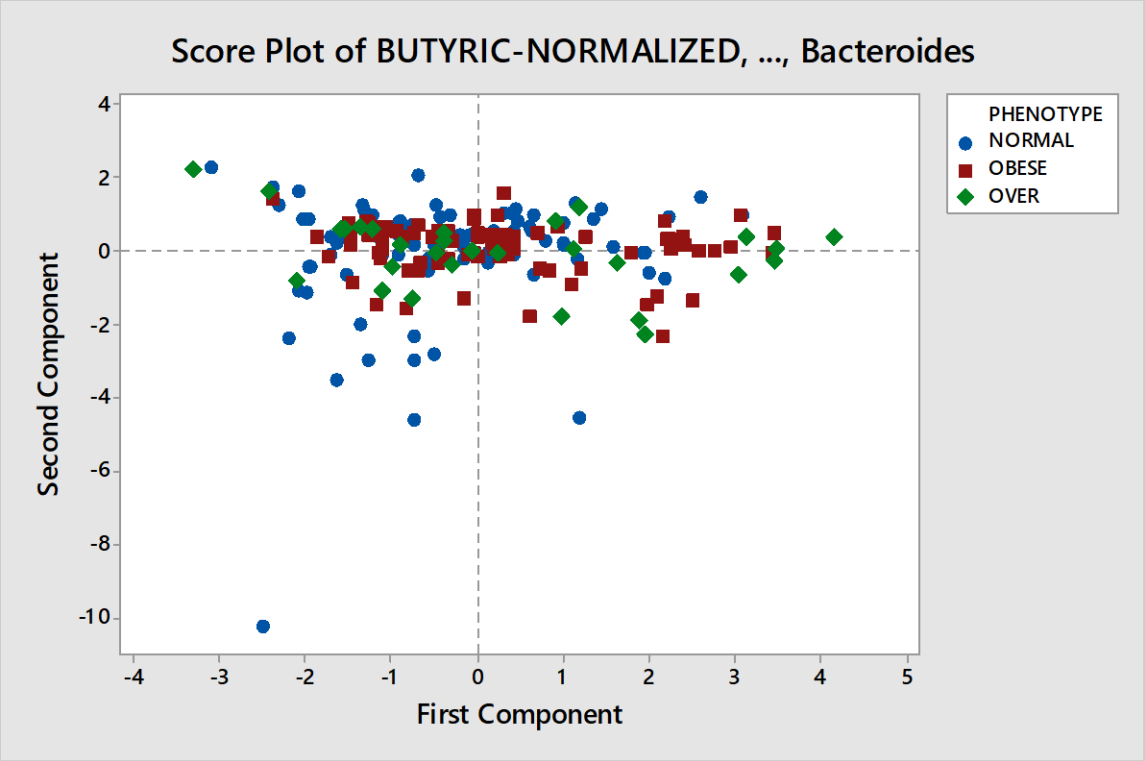

Figure 18. Multivariate- Principal Component Analysis of Triglycerides-Normalized, Butyric-Normalized, *Actinomyces, Bifidobacteria, Bacteroides, Prevotella, Lactobacillus,* Lachnospiraceae*, Blautia, Coprococcus, Faecalibacterium, Oscillospira, Acetobacteriaceae, Succinivibrio,* Enterobacteriaceae and BMI-Norm1

Table 8. Principal Component Analysis of Triglycerides-Normalized, Butyric-Normalized , *Actinomyces, Bifidobacter, Bacteroides, Prevotella,* Lachnospiraceae*, Blautia, Coprococcus, Faecalibacterium, Oscillospira,* Acetobacteriaceae*, Succinivibrio*, BMI-Norm1

Variable	PC1	PC2
Triglycerides-Normalized	-0.110	-0.045
Butyric-Normalized	0.212	-0.194
Bifidobacterium	0.037	-0.655
Blautia	**0.439**	-0.140
Coprococcus	**0.454**	-0.289
Faecalibacterium	0.37	-0.021
Oscillospira	0.083	-0.272
Acetobacteraceae	0.046	0.242
Succinivibrio	-0.268	0.174
BMI-Norm1	-0.025	-0.253

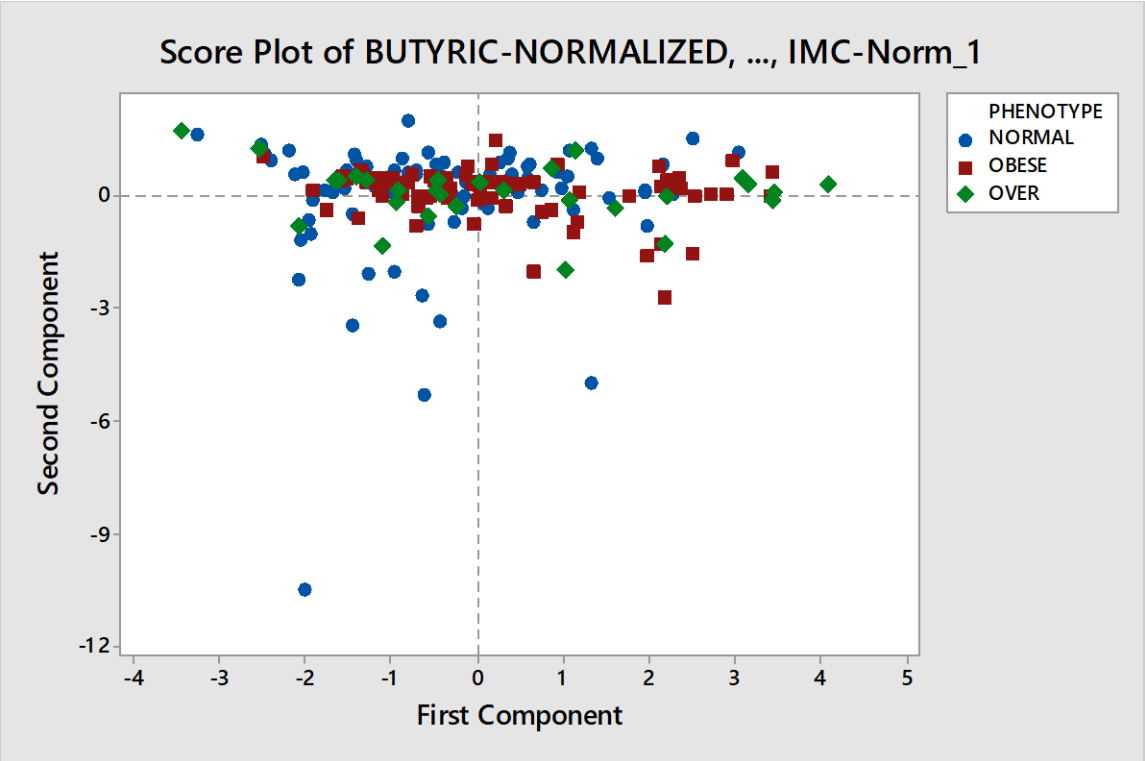

Figure 19. Multivariate- Principal Component Analysis of Triglycerides-Normalized, Butyric-Normalized, *Actinomyces*, *Bifidobacteria, Bacteroides, Prevotella*, Lachnospiraceae, *Blautia, Coprococcus, Faecalibacterium, Oscillospira*, Acetobacteriaceae, *Succinivibrio.*

Table 9. Principal Component Analysis: Saccharospirillum, BMI-Normalized, Triglycerides-Normalized.

Variable	PC1	PC2
Saccharospirillum sp	**0.673**	0.218
BMI- Normalized	**0.673**	0.216
Triglycerides- Normalized	-0.307	**0.952**

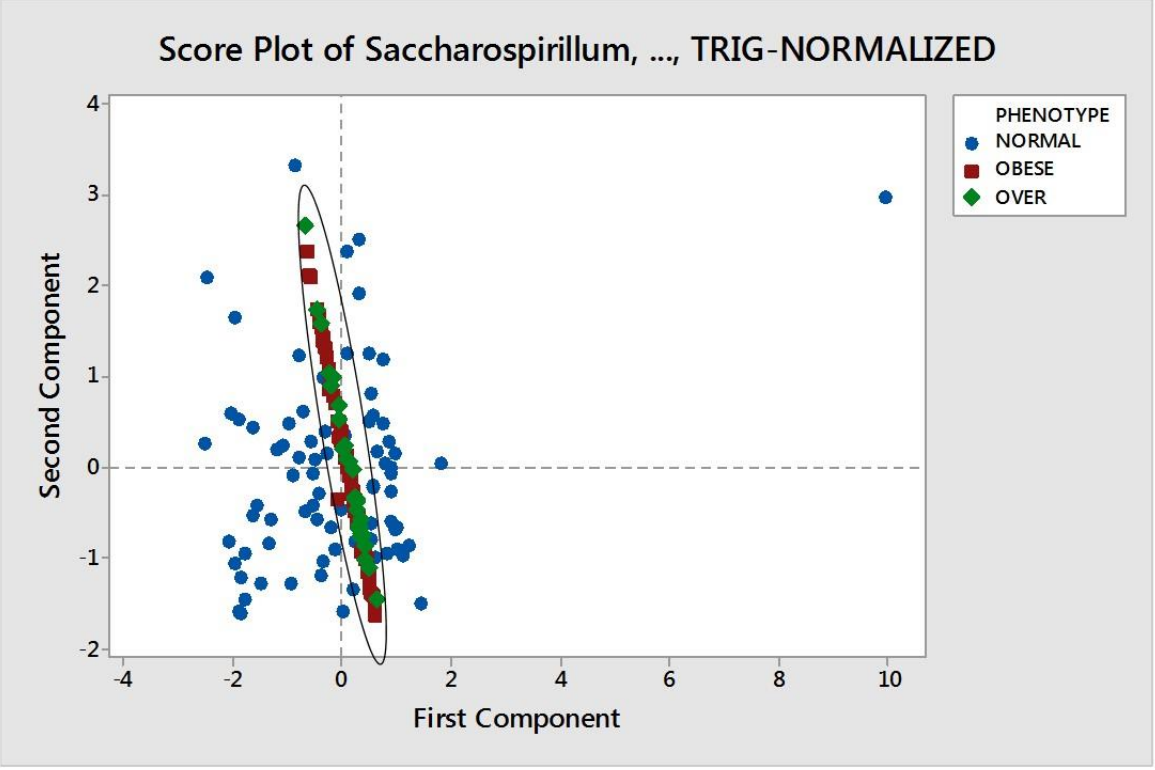

Figure 20. Multivariate **-** Principal Component Analysis**:** Saccharospirillum, BMI-Normalized, Triglycerides-Normalized.

Table 10. Principal Component Analysis of *Coprococcus* sp, BMI-Normalized, Triglycerides-Normalized

Variable	PC1	PC2
Coprococcus sp	**0.688**	-0.281
BMI- Normalized	**0.125**	0.954
Triglycerides- Normalized	-0.715	**0.952**

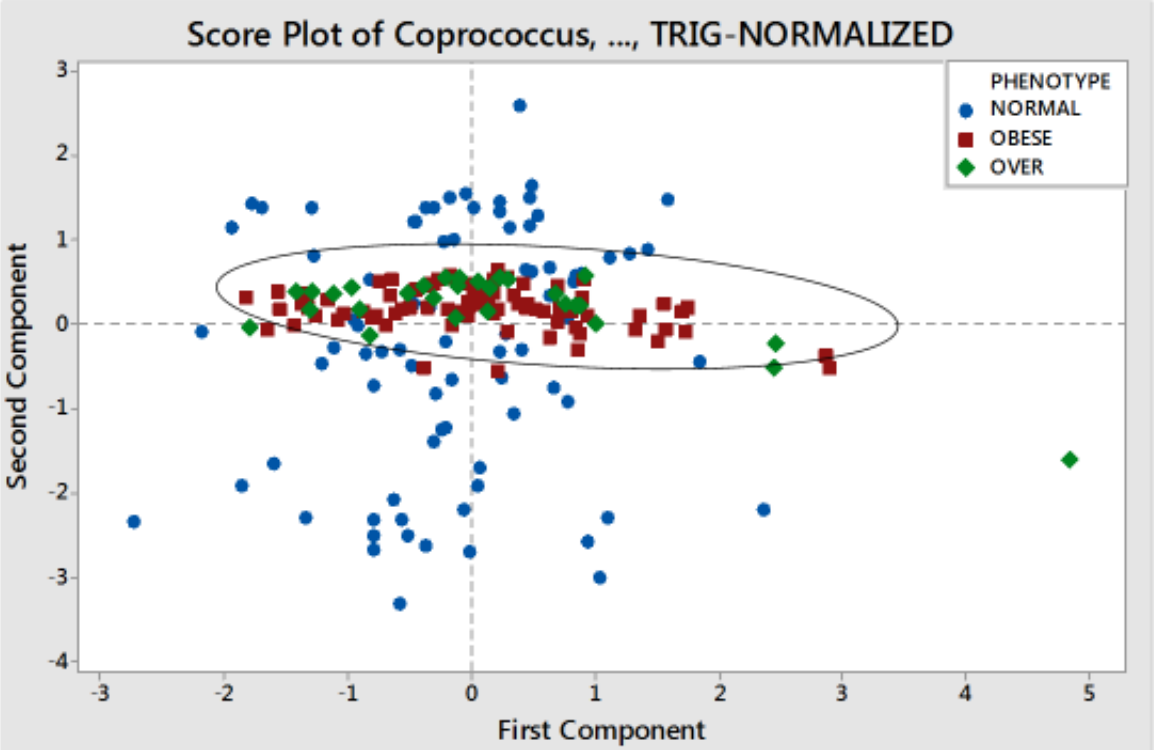

Figure 21. **Multivariate -** Principal Component Analysis of *Coprococcus* sp, BMI-Normalized, Triglycerides-Normalized.

Table 11. Principal Component Analysis: *Faecalibacterium* sp, BMI-Normalized, Triglycerides-Normalized.

Variable	PC1	PC2
BMI- Normalized	0.297	**0.946**
Faecalibacterium sp	**0.688**	-0.117
Triglycerides- Normalized	**-0.663**	0.302

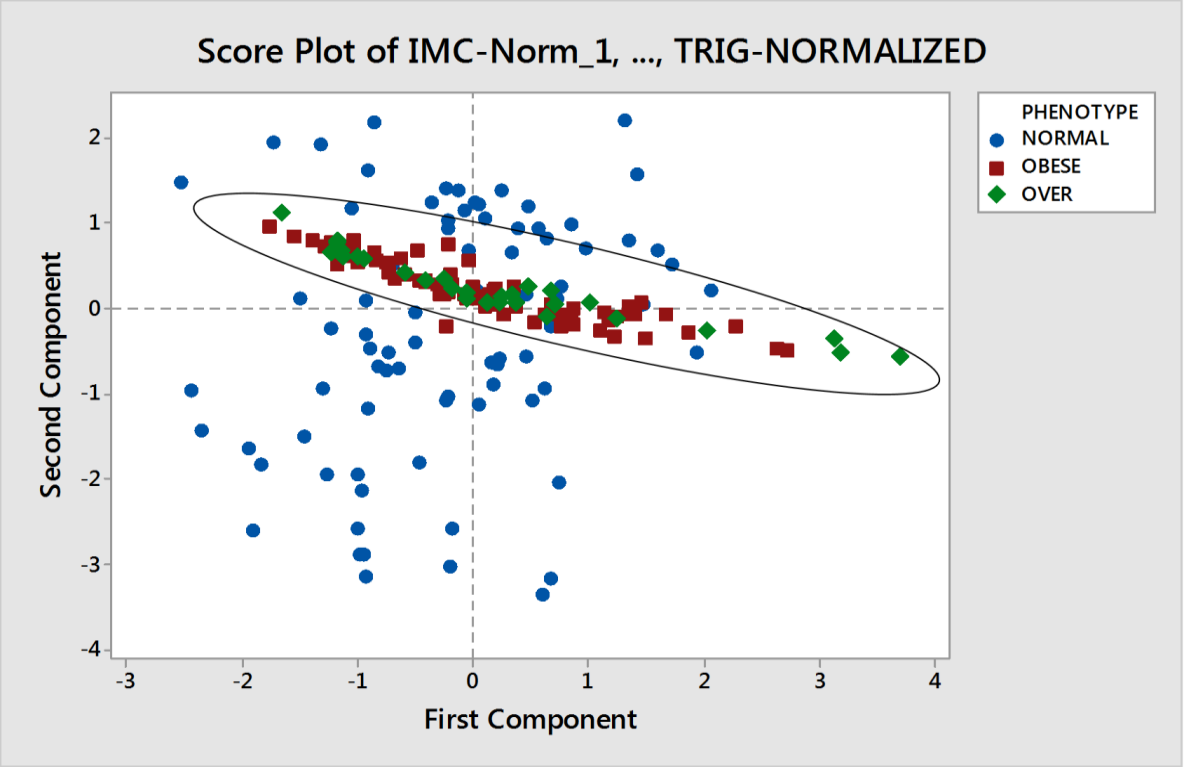

Figure 22. Multivariat**e -** Principal Component Analysis of *Faecalibacterium* sp, BMI-Normalized, Triglycerides-Normalized. A) Screen plot B) Loading plot of variables C) Score plot.

Table 12. Principal Component Analysis: *Blautia* sp, IMC-Normalized, Triglycerides-Normalized.

Variable	PC1	PC2
IMC- Normalized	-0.476	**0.840**
Blautia sp	**0.651**	0.137
Triglycerides- Normalized	**-0.592**	**0.525**

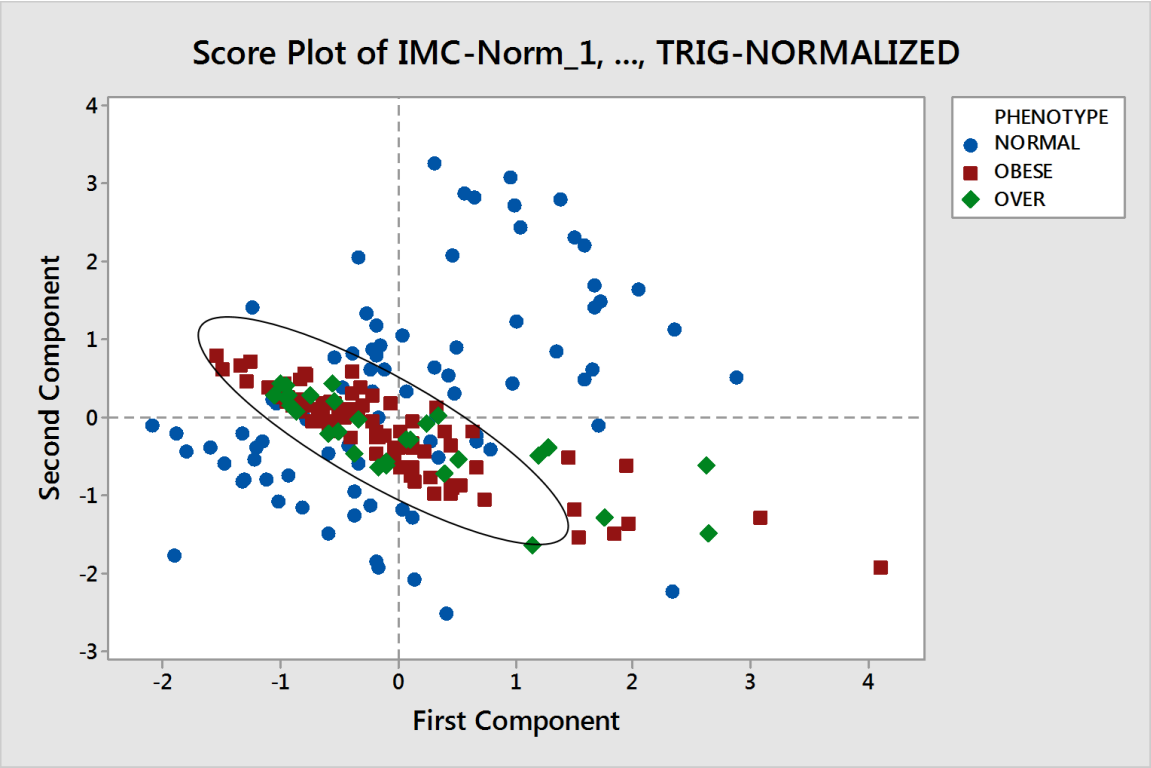

Figure 23. Multivariate **-** Principal Component Analysis**:** *Blautia* sp, BMI-Normalized, Triglycerides-Normalized.

Table 13. Principal Component Analysis: *Roseburia* sp, IMC-Normalized, Triglycerides-Normalized.

Variable	PC1	PC2
IMC- Normalized	0.459	**-0.824**
Roseburia sp	**0.671**	0.075
Triglycerides- Normalized	**-0.583**	**-0.562**

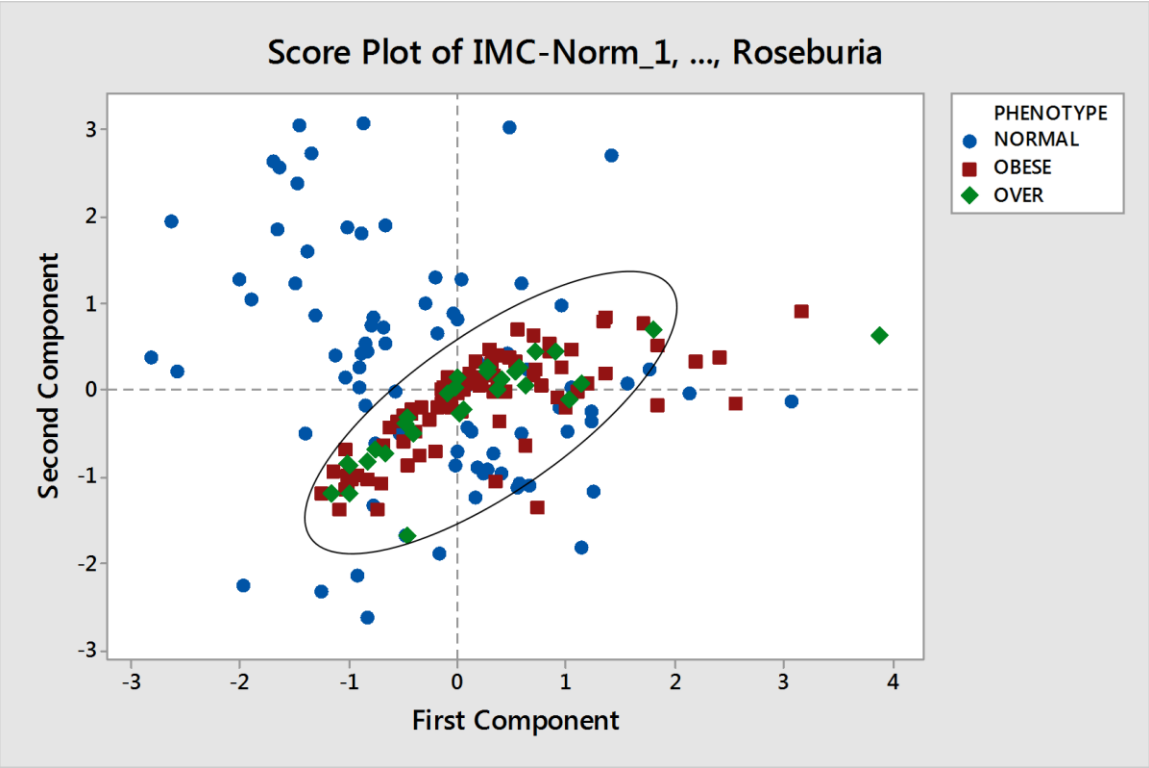

Figure 24. Multivariate **-** Principal Component Analysis of *Roseburia* sp, BMI-Normalized, Triglycerides-Normalized.

Table 14. Principal Component Analysis: *Oscillospira* sp, IMC-Normalized, Butyric acid-Normalized.

Variable	PC1	PC2
IMC- Normalized	-0.275	**-0.955**
Oscillospira sp	**0.669**	-0.276
Butyric acid- Normalized	**0.690**	-0.113

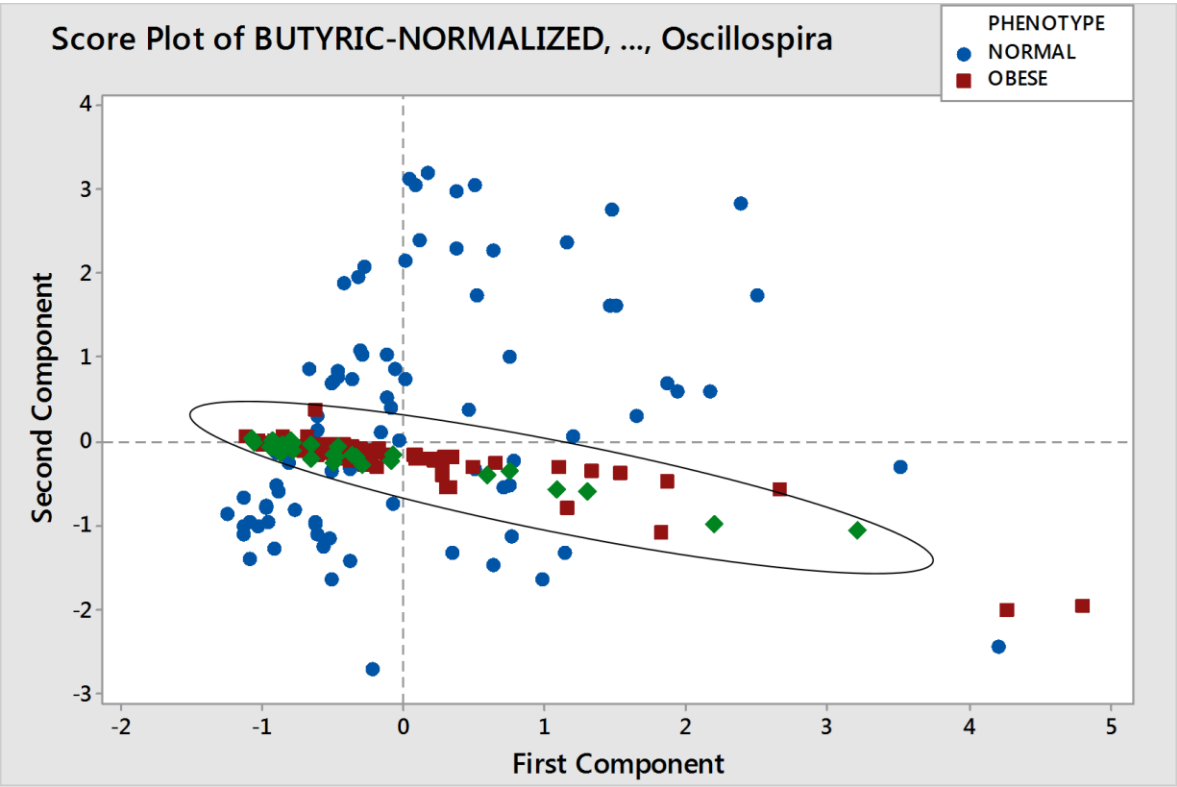

Figure 25. Multivariate **-** Principal Component Analysis of *Oscillospira* sp, BMI-Normalized, Butyric acid-Normalized.

Table 15. Principal Component Analysis: *Succinivibrio* sp, IMC-Normalized, Triglycerides-Normalized.

Variable	PC1	PC2
IMC- Normalized	0.702	0.069
Succinivibrio sp	**-0.149**	**0.988**
Triglycerides- Normalized	**0.697**	**0.142**

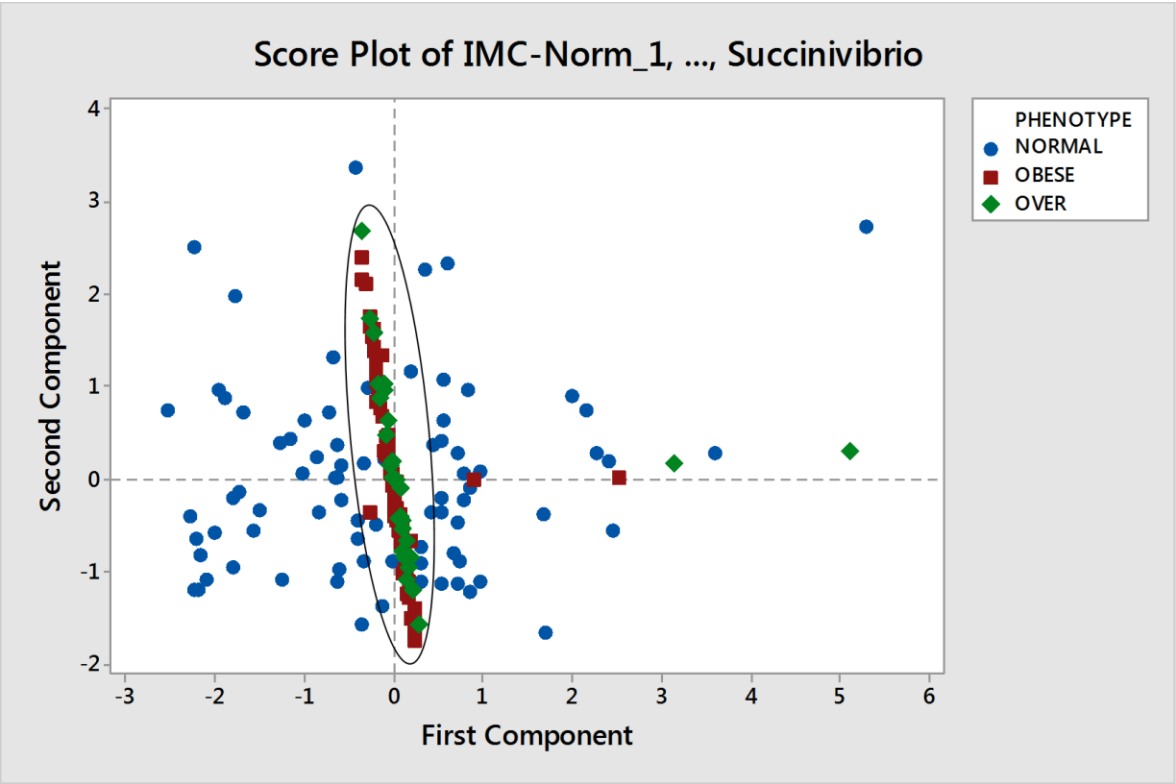

Figure 26. Multivariate - Principal Component Analysis of *Succinivibrio* sp, BMI-Normalized, Triglycerides-Normalized.

8. DISCUSSION

In this work we studied well characterized individuals selected from a cohort studying overweight and obesity in Mexican children, measuring its anthropometric characteristics, its biochemical profile, and the concentration of short chain fatty acids (SCFAs), as well as the bacterial diversity of the microbiota found in stool samples.

In recent years, the impact of colon microbiota in human health has been widely recognized, and current reports mainly focus on the aspect of exploring the association of colon microbiota on obesity development (Samuel *et al*., 2006; Turnbaugh *et al*., 2006). It has been reported that the relative abundance of two phyla Bacteroidetes, and Firmicutes may play a role in the development of obesity and type 2 diabetes (Larsen *et al*., 2010; Turnbaugh *et al*., 2012). These two phyla include members capable of producing SCFAs, from otherwise undigested carbohydrate fibers in the distal colon, thus providing additional energy from diet. Alternatively, instead of focusing on single bacteria, it is believed that some diseases and health disorders are associated to an imbalance in the microbial community, a condition known as dysbiosis, being obesity a well-known example (Turnbaugh *et al*., 2009).

Colonic epithelium receives about 70% of energy from SCFAs, mainly from butyric acid (Scheppach 1994). Propionic acid on the other hand, is a precursor for protein synthesis, gluconeogenesis and liponeogenesis in the liver (Kotzampassi *et al*., 2014; Wolever *et al*., 1991). Acetic acid, another SCFA, is a substrate for cholesterol synthesis (Vogt *et al*., 2003), as well as a suppressor of appetite through a central hypothalamic mechanism (Frost *et al*., 2014). In our work, the overweight and obese children showed altered propionic and butyric concentrations; being the butyrate concentration in feces significantly reduced in obese children, in comparison to normal weight. The decrease in SCFAs concentration observed in feces, might be explained for instance, by a general dysbiosis in the microbial population causing a lower production, or a higher

mucosal absorption. Although we did not observe a significant general dysbiosis in the bacterial population in feces in overweight and obese children (Figure 13.), the abundance of some key members for SCFA production can be compromised. It has been reported that SCFAs levels are predominantly linked to changes in the distal colon bacterial diversity (Duncan *et al*., 2007; Gostner *et al*., 2006). Additionally, an increase in SCFAs absorption, occurring in parallel, could explain the increase in triglyceride levels observed in the overweight and obese children in our study.

The current concept supports that the microbial biome in the gut is somehow stable, and that the chromosomal genetic inheritance in the individual, selects the gut microbial diversity, although environmental factors may fine-tune the diversity (Ley *et al*., 2014). In such sense, it has been reported the human gut microbiome may be altered by long- or short-term diet changes, regardless if it is an herbivorous or carnivorous regimen. Particularly, animal based diet stimulates the gut microbiota to induce the secretion of inflammatory molecules by the host, which could be associated to disorders such as obesity, and metabolic syndrome (David *et al*., 2014).

The next-generation sequencing technology has greatly advanced the studies of human microbiota. The data of bacterial diversity characterized in bacterial ribosomal V3-16S rDNA libraries by massive sequencing in our work, showed the phylum Proteobacteria was relatively more abundant in normal-weight in comparison to overweight, and more abundant in overweight than in obese children. Moreover, the phylum Firmicutes was less abundant in normal-weight than overweight and obese children. It is remarkable to find the phylum Bacteriodetes was decreased in overweight but increased in the obese children in comparison to normal-weight. It cannot be concluded from our data, if the differences in the proportion of the mentioned phyla for each phenotypic category are the result of the low intensity inflammatory condition occurring in the gut of overweight and obese children, or that changes in the bacterial communities, have a role in the development of this condition. It could be also argued that a particular diet regime in the overweight and obese Mexican

individuals, may favor for instance, the disappearance of key members of the phylum Proteobacteria, whose functional activity producing metabolites, is related to maintain a healthy gut. However, we did not find a significant difference in the diet diversity as is indicated by the 7-d recall study performed in this work. Our study is one of the first reported metagenomic characterization of the gut microbial communities in Mexican children; and it could be that the reported abundances may be related to the particular genetic background found in Mexicans.

We did not find a clear general dysbiosis in the bacterial communities, but a closer view of our sequencing data, revealed the abundance of particular bacteria was changed in overweight and obese with respect to the normal controls. In this manner when we look at the genera level, the proteobacteria *Succinivibrio* sp was more abundant in normal weight children. It has been proved that *Succinivibrio* sp has a vital role to regulate the energy produced by SCFAs in swine (Looft *et al*., 2012); one possibility is that a decrease in the abundance of this genus deregulates the SCFAs production in the gut, reducing particularly the production of butyrate. Interestingly, we found more abundance of *Erwinia* sp a well-known phytopathogen in normal-weight compared to overweight and obese children. It has been reported that this bacteria can produce ketoaldonoic acid from sugars (Truesdell *et al*., 1991), which is a precursor to SCFAs. In addition, in the case of genus Oscillospira sp, a metagenomic study made on monozygotic twins, revealed that BMI negatively correlated with the abundance of genus *Oscillospira* sp (Tims *et al.*, 2013), which is in agreement with our results (Fig. 27G). We found this genus is more abundant in lean children with lower BMI index (Table 1).

For the phylum Firmicutes, the most abundant genus in obese children of our study was Fecalibacterium sp, which has a high capacity of energy extraction from undigested carbohydrate fibers in the distal colon; a similar result has been reported in obese Indian children (Balamurugan *et al.*, 2010). A decrease in the abundance of *Roseburia* sp, a saccharolytic, butyric acid producing bacteria, was associated to a reduction in body weight of obese

Spanish adolescents (Sotos et al., 2008). We observed in accordance an increase in the abundance of *Roseburia* sp, in overweight and obese children. It has been reported that the family Lachnospiraceae abundance increased along with increase in bodyweight in mice fed with high fat diet, in comparison to low fat diet (Ravussin *et al*., 2012). This result is in agreement with our findings in overweight and obese Mexican children. In another work, the family Lachnospiraceae colonizing germfree ob/ob mice, induced elevation of blood glucose, increase in liver and mesenteric adipose weight, strongly suggesting this bacteria has a role in development of obesity and diabetes (Kameyama *et al*., 2014).

In overweight children, we found significant increase in the abundance of Blautia sp, Coprococcus sp and the family Enterobacteriaceae. The family Enterobacteriaceae, includes gram negative bacteria whose Lipid A portion of lipopolysaccharide induces pro-inflammatory activity in the host (Zhao 2013); *Blautia* sp was reported to induce high fat diet obesity in gnotobiotic rat model (Becker *et al*., 2011), and *Coprococcus* sp, is reported to increases its abundance in rats, under high cholesterol diet (Lee *et al*., 2015). The increase in the abundance of these two genus and one family of bacteria, might favor the sustained low intensity inflammatory condition of the gut, an important trait of obesity occurring in overweight and obese children. The increase of *Coprococcus* sp, may also be the explanation of the rise in Propionic acid production found in feces obtained from overweight children (Figure 17); it has been reported this genus produces abundant propionic acid in human gut (Reichardt *et al*., 2014). Although *Coprococcus* sp is still abundant in obese (Figure 27F), the decrease in propionic acid production may be the consequence of the proliferation of another bacteria in the gut which transforms this acid to a different metabolite.

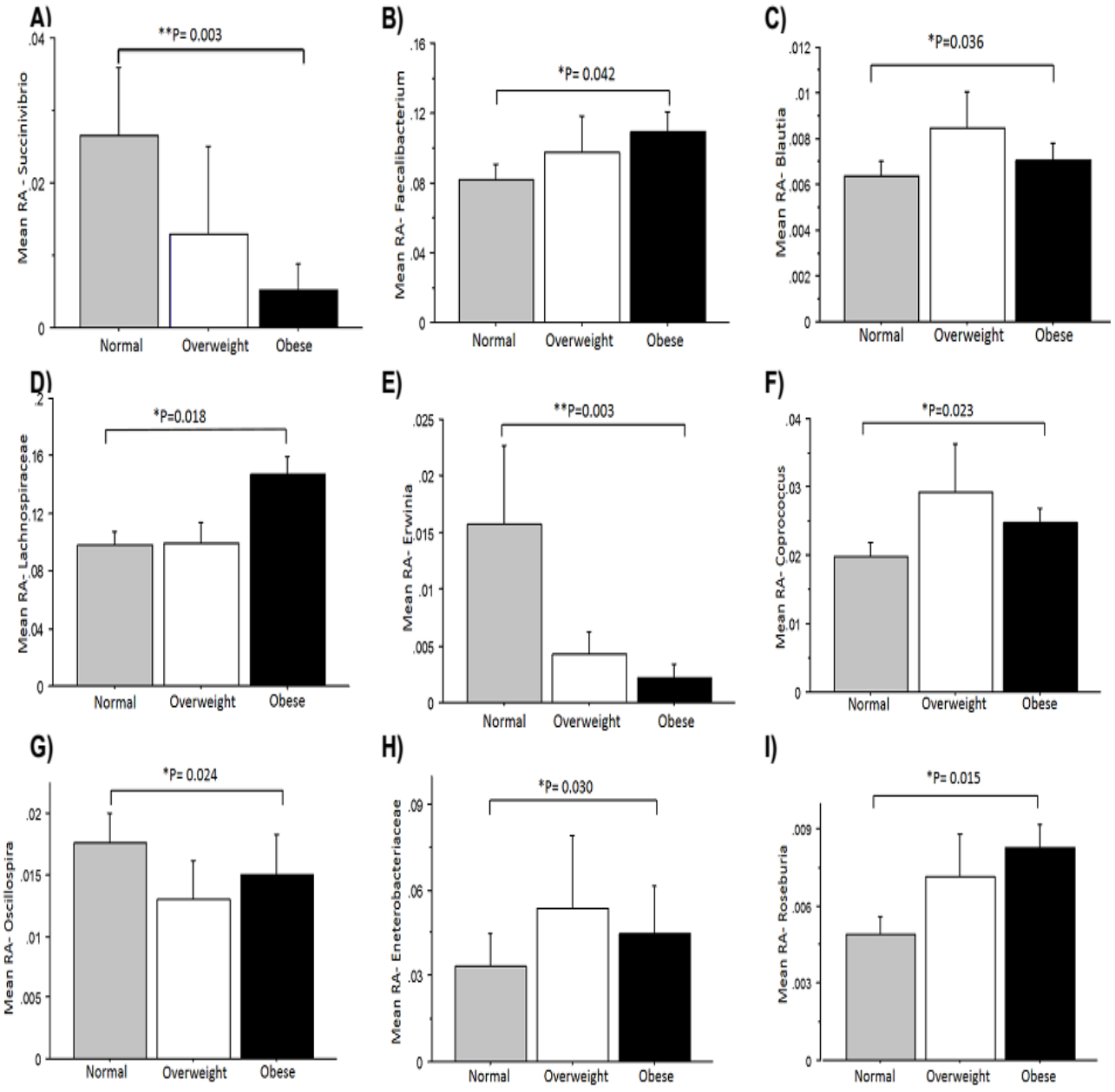

Figure 27. Relative abundance of particular bacteria in Normal, Overweight and Obese Mexican children. Histograms charts show the relative abundance of nine major bacterial genera, found in feces from the normal-weight (n = 81), overweight (n = 29) and obese (n = 80) Mexican children of the study. Metagenomic analysis was made as described in Materials and methods. Data are for Normal-weight, grey color bars; Overweight, white color bars; and Obese, solid bars. Error bars represent mean ± standard error. *P<0.05, **P< 0.01. Y-axis, indicates relative abundance (RA) of each operative taxonomical unit; X-axis, indicates the phenotypic categories. A) genus *Succinivibrio* sp; B) genus *Faecalibaterium* sp; C) genus Blautia sp; D) family Lachnospiraceae; E) genus Erwinia sp; F) genus *Coprococcus* sp; G) genus *Oscillospira* sp; H) family Enterobacteriaceae ; I) genus *Roseburia* sp.

From this study we conclude that in Mexican obese children, the distal colon microbiota has higher abundance of *Fecalibacterium* sp, Lachnospiraceae and *Roseburia* sp from the Firmicutes phylum. It is believed that these bacteria extract energy from undigested fiber, affecting the energy balance. Thus it is plausible that this effect in the energy balance, along with other factors such as the chromosomal inheritance in children, the environment, and the diet, will cooperate to develop obesity. On the other hand, in normal-weight children, there are commensal bacteria such as *Oscillospira* sp and *Succinivibrio* sp helping to control the energy balance.

A study on the role of gut microbiota in obesity of preschool children in Sweden revealed that the gut microbiota varied in overweight and obese children, compared to normal group with significant increase in the family Enterobacteriaceae and significant decrease in *Desulfovibrio* sp and *Akkermansia* sp (Karlsson *et al*., 2012). Another study correlating the abundance of Bacteroidetes and Firmicutes in association to the obesity in school children of Khazakhstan, unveiled a significant decrease in Bacteroidetes in stool of obese Khazakh girls, but no significant differences was found for Firmicutes (Xu *et al.,* 2012). In Egypt, it has been reported that for normal and obese children, as well as adults, the frequency of both phyla Bacteroidetes and Firmicutes significantly increased in the obese group (Abdallah *et al*., 2011). The particular features found in the bacterial diversity of Mexican children associated to obesity, might be due to the particular genetics of the chromosomal background found in Mexicans, which is clearly different to the chromosomal genetic background of other world populations (Martínez-Marignac *et al*., 2007; Silva-Zolezzi *et al*., 2009). In future, the drawing of definite conclusions on the importance of various bacterial groups associated to obesity, should take into account parameters such as diet, genetic background, environment, and overall fitness, in order to understand the definite role of colon microbiota.

9. CONCLUSIONS

- Lower level of unabsorbed butyric acid and Propionic acid were found in feces of obese Mexican children compared to normal weight and overweight children.
- A prominent increase in the level of triglycerides in overweight and obese children compared to normal weight children, this may be due to higher mucosal absorbtion of SCFAs in obese children.
- In the Mexican obese children, the distal colon microbiota has higher abundance of *Fecalibacterium* sp, Lachnospiraceae and *Roseburia* sp from the Firmicutes phylum. It is believed these bacteria extract high extent of energy from undigested fiber, affecting positively the energy balance.
- On the other hand, in normal-weight children, there are commensal bacteria such as *Oscillospira sp* and *Succinivibrio sp* helping to regulate the energy balance.
- This imbalance in particular bacteria in the obese children in addition to genetic and environmental factors predisposes to Obesity.

10. Publication

This thesis is based on the following original article, In addition, some unpublished data is presented.

Murugesan S, Ulloa-Martínez M, Martínez-Rojano H, Galván-Rodríguez FM, Miranda-Brito C, Romano MC, Piña-Escobedo A, Pizano-Zárate ML, Hoyo-Vadillo C, García-Mena J. (2015). 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 Mar 12. DOI 10.1007/s10096-015-2355-4.

Eur J Clin Microbiol Infect Dis
DOI 10.1007/s10096-015-2355-4

ARTICLE

Study of the diversity and short-chain fatty acids production by the bacterial community in overweight and obese Mexican children

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