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Characterization of gut microbiota from a population with growth hormone receptor deficiency (GHRD) from the El Oro and Loja Provinces

Tesis en torno a una hipótesis

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Characterization of gut microbiota from a population with growth hormone receptor deficiency (GHRD) from the El Oro and Loja Provinces

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DEDICATORIA

Para Andrés, gracias por ser mi apoyo constante, por tu amor y resiliencia.

Para Amelia, mi luz, te amo.

Para mis padres, gracias por su tiempo, amor y apoyo.

A mi padre, gracias por ser el constante puntal en mi carrera y en mi vida. Con tu infinita humanidad y don de gente me has enseñado a llegar a las personas de maneras que solo un gran ser humano lo puede hacer.

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RESUMEN

La ausencia de los efectos contrarreguladores de la GH sobre el metabolismo de los carbohidratos, así como sus implicaciones resultantes, permite categorizar a los sujetos con GHRD (Laron Syndrome -LS-) como un modelo distintivo de sensibilidad incrementada a la insulina. Notablemente, en la literatura no solo que no existen reportes sobre la composición del microbioma fecal (GM) en estos sujetos, sino que, además, las descripciones sobre la composición del GM y su relación con las acciones de la insulina en sujetos con obesidad sindrómica son contradictorias (14). En consecuencia, tanto la caracterización del GM y el establecimiento de su correlación con las características fenotípicas y metabólicas del LS, ayudara a identificar los posibles vínculos e interacciones entre la GM y sus peculiaridades, con el fenotipo metabólico de estos sujetos.

Con estos objetivos en mente, diseñamos un estudio descriptivo transversal y comparamos un grupo de sujetos con LS debido a GHRD con sus respectivos controles familiares y/o comunitarios que presentaron obesidad o sobrepeso y fueron cotejados por edad y sexo. Encontramos que los pacientes con LS, de menor tamaño en general, registraban menor peso y talla que sus controles; sin embargo, su índice de masa corporal (IMC) fue comparable al de sus controles de tamaño normal.

Sorprendentemente, al comparar el GM de los grupos, se observaron diferencias en la beta diversidad así como mayor abundancia relativa del filo Bacteroidetes en el LS. Esta es una paradoja clínica, puesto que, a pesar de su marcado exceso porcentual de grasa corporal, el patrón de su GM corresponde más bien a individuos de peso normal o reducido. Por el

contrario, y desde una perspectiva clínica común, los controles exhibieron mayor cantidad de elementos del filo Firimicutes, caracteristico de sujetos con obesidad, especialmente central.

Esta es la primera caracterización del GM en sujetos con LS debido a GHRD; sin embargo, el presente es tan solo un estudio pionero. Es indispensable realizar estudios genómicos, proteómicos y metabolómicos que nos permitan tener una perspectiva más específica para dilucidar las extremadamente complejas relaciones existentes entre el GM y su activa dinámica con los péptidos hormonales tales como insulina, glucagón, amilina, GIP, GLP1 y otros, que se encargan de la regulación del metabolismo de los glúcidos en los seres humanos.

Palabras clave: deficiencia del receptor de la hormona del crecimiento, Síndrome de Laron, microbioma intestinal, obesidad, resistencia a la insulina, hormona del crecimiento, factor de crecimiento similar a la insulina 1, vías de señalización

ABSTRACT

The absence of counter-regulatory effects of GH on carbohydrate metabolism and the resulting implications, allow for a categorization of subjects with GHRD (Laron Syndrome-LS-) as a distinctive model of increased insulin sensitivity. It should be noted that the composition of the fecal microbiome (GM) in these subjects has not been reported and the available descriptions of GM and its relationship with insulin actions in syndromic obesities are contradictory (14). In consequence, the characterization and establishment of the possible correlation between phenotypic and metabolic characteristics in LS, will help to identify possible links and interactions between the GM and the metabolic phenotype of these subjects.

With these objectives in mind, we have designed a descriptive cross-sectional study in which we compared a group of LS due to GHRD with age-sex matched community members? O members within their close community? or related family control subjects who present obesity or overweight. Overall, we found that LS subjects were smaller and registered lower weight and height than the control subjects. However, they had a comparable BMI with that of the normal stature control group.

Surprisingly, when the GM of both groups was compared, we observed differences in beta diversity between groups and a high relative abundance of Bacteroidetes phylum in the LS group. This constitutes a clinical paradox because this GM pattern is usually associated with individuals of normal or low weight but it was observed in the LS subjects which have higher

body fat percentage. On the contrary and from a common clinical perspective, controls exhibit a higher abundance of Firimicutes phylum associated with central obesity.

This is the first characterization of GM in subjects with LS due to GHRD. Nevertheless, this is a pioneer study and it is of keen importance to perform genomic, proteomic and metabolomic studies that will allow to hone in a wider perspective to further comprehend the complex relationship between the GM dynamics and hormonal peptides such as insulin, glucagon, amylin, GIP, GLP1 which are in charge of the glucide metabolism regulation in human beings.

Key words: growth hormone receptor deficiency, Laron syndrome, gut microbiome, obesity, insulin resistance, growth hormone, insulin like growth factor 1, signaling pathways.

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INTRODUCCION

In the southern provinces of Ecuador, El Oro and Loja, resides the largest cohort of individuals affected by growth hormone receptor deficit (GHRD) in the world. This rare recessive autosomic affection encodes for a purine substitution at position 3 of codon 180/exon 6 of the growth hormone receptor (GHR), as a result, the extracellular portion of the GHR is non-functional, this leads to a general and peripheric insensibility of all tissues to the actions of the growth hormone (GH). In consequence, the clinical phenotypic expression is a dramatic short stature (-5.3 to -12 standar deviation score [SD]), accompanied by an increase of total and percentual fat content, inevitably leading to the development of obesity. However, despite the high prevalence of this pathology among the GHRD population, a paradoxically co-existence of higher insulin sensitivity has been documented, that accordingly correlates with lower incidence of chronic diseases such as type 2 diabetes mellitus (T2DM) and cancer (1-8).

These metabolic occurrences can be attributed to the lack of counter regulation of GH in the carbohydrate metabolism, and the presence of a highly efficient insulin. These hormonal determinations along with low levels of leptin, high levels of adiponectin and notorious modifications in the metabolism of amino acids like proline, hydroxyproline and metabolites such as hydroxybutyric acid have been documented in the Ecuadorian GHRD cohort, highlighting the possible implications of these peptides and metabolites in the actions of

insulin. These findings also imply the possible connections between body fat excess and the development of chronic pathologies, that have yet to bet studied. (11-13)

In a different but related manner, over a decade of human microbiome studies has put into evidence that gut disbiosis can be related with the pathogenesis of metabolic diseases. This has been shown in several studies, where subjects affected with non-communicable chronic diseases when compared with healthy populations have changes in the composition of taxa, genes, and microbial metabolites of the gut microbiome (GM) (8). Such is the case of patients with T2DM, obesity or insulin resistance, in whom, some of these GM modifications are considered a metabolic hallmark some which present specific variations in bacterial filums and microbial abundances (9-14). Nevertheless, there are only a few descriptions of the relationship between the specific action of peptides, such as insulin in the GM during the development of this pathologies. This may be due to the difficulty that possess the study of a single factor within the human metabolism. Also, it is known that the GM can be influenced by a myriad of environmental factors, including the type of sample handling, and analysis used when studying the GM (19).

Recently in the literature, through the description of the GM in patients with syndromic obesity and diminished insulin sensitivity, such as in Prader-Wili syndrome (PWS), a relation between the actions of the peptide hormone insulin and the GM has tried to be stablished (57-60). Some of the findings in the GM of these subjects when compared with obese subjects, have been associated to metabolic health, such as phylogenic diversity and increases in some bacterial

genera. These patterns have also been described in populations without metabolic disturbances and normal weight, thus, these findings may stablish the relationship between insulin actions, as well as other metabolic markers and the GM (49-54).

Thus, the characterization of the GM in GHRD subjects, a natural model of insulin sensitivity, will allow the observation of the microbial composition under a unique environment, the absence of the counter regulatory actions of GH over the carbohydrate metabolism. Enabling the correlation between the phenotypic characteristics of the syndrome and the GM composition and, the comparison to that obtained from their heterozygote family (WT/ss180), or with community controls (WT/WT).

BACKGROUND

1. LS DUE TO GROWTH HORMONE RECEPTOR DEFICIENCY (GHRD)

The first descriptions of growth hormone receptor deficiency (GHRD) made in 1966-1968 by Zvi Laron *et al*., depicted subjects with severe short stature, obesity, mental retardation, and characteristic facial features. The cause for these findings was linked to an exon deletion in the growth hormone receptor (GHR) gene, which leads to a generalized insensitivity to growth hormone (GH) (1). Since then, other mutations have been characterized along with important changes in the phenotype and metabolomes of these subjects. Such is the case of the Ecuadorian Laron syndrome cohort (LS-E), first characterized in 1988 by Guevara-Aguirre et al., as the largest cohort of individuals affected by GHRD mainly clustered in the southern provinces of Loja and the neighboring province of El Oro (2,3).

Regarding the origin of the mutation in the GHR gene that characterizes the LS-E, a single Jewish subject with Moroccan ancestry has been identified, supporting the hypothesis of a founder effect that gave rise to this population (4,5) in which, almost all individuals are homozygous for the mutation characterized by Berg et al. in 1992 (6). A splice site mutation that encodes an adenine for guanine substitution, at position 3 of codon 180 of exon 6 of the growth hormone receptor (GHR) gene (ss180), renders a nonfunctional extracellular domain of the GHR leading to the general GH insensitivity (4,7).

Interestingly, and aside from age or clinical phenotype, in the Ecuadorian mutation (E180) fluctuating levels of growth hormone binding protein (GHBP or extracellular domain of GHR) ranges from 79 -746 picomoles/liter (pmol/L) have been documented (8). Also, in accordance with GH insensitivity, high levels of GH (>10 – 200 ng/ml), extremely low levels of insulin-like growth factor 1 (IGF-1), and low levels of IGF binding protein 3, (IGFBP-3) have been quantified (7,8). The result of this marked disruption in the GH-IGF-1 axis is a characteristic phenotype that becomes evident at birth. In the prenatal stage, no intrauterine growth restriction has been reported and normal or slightly low weight and adequate length for the gestational age are seen at birth. Also, distinctive features that characterize the syndrome can be noticed at birth, such as frontal prominence, depressed nasal bridge, shallow orbits, sparse hair, and hypoplastic fingernails (2,3,4,7,8).

Further, during infancy, other hallmark features of the syndrome become more evident, such as high-pitched voice, blue sclera, diminished vertical dimensions of the face, small hands, hypo-muscularity, increase in body fat content, and dramatic growth delay. When compared with their peers, these patients have significant variations in height mean standard deviations (SD), between -6.8 to -9.6 SD until the end of puberty when they reach final heights with SD between -12 to -5.3. In addition, an accumulation of total and percentual body fat is evident in all adults, leading to the onset of clinical obesity (2,3,4,7,8).

The chief characteristic phenotype features described above, are shared both by the LS-E and the Israeli cohorts. Nevertheless, there are unique phenotypical and metabolic expressions

particular to the specific molecular defect in each cohort. Thus, in the Ecuadorian cohort with the ss180 mutation, there have not been any cases of intellectual disability in comparison to originally reported cases in the Israeli cohort (9). Results of magnetic resonance imaging (MRI) and cognitive tests performed on these subjects show normal size, structure, and function of the brain of LS-E subjects when compared with their controls (10). The cause of these phenomena has been pinpointed to the low levels of IGF-1, inherent to the syndrome and, with very efficient insulin actions (11).

In previous metabolomic reports and when compared with age and sex-matched controls, subjects from the LS-E cohort, aside from low serum levels of insulin, also exhibit low plasma glucose, very low-density lipoprotein (V-LDL), and triglyceride (TG) levels. These results convey the presence of insulin sensitivity (IS) as well as diminished insulin resistance (IR) due to the absence of GH counter-regulation in the carbohydrate (CHO) metabolism (12). These effects have been mainly evidenced in the efficient clearance of nutrients from plasma, and the net increase of fat mass (12). The assessment of IR and IS have been registered in the homeostatic minimal models (HMM) elsewhere (13).

The above-described phenomena are associated with the potent and unopposed anabolic effects of insulin which are evidenced in the increased lipogenesis in the adipose tissue. Increasing plasma TG breakdown and blockage to the release of glycerol and free fatty acids (FAA) from the fat cells promotes the accumulation of TG and the consequent adipose hyperplasia. This explains the excessive accumulation of body fat and the ubiquitous incidence

of obesity in this population (14). In this regard interestingly, when obesity-related adipokines are measured, low levels of leptin and high quantities of adiponectin have been reported. This is associated with a heightened IS, low inflammation levels as well as a decreased cardiovascular risk (15). In a related matter, the efficient TG breakdown described above allows for normal to low plasma levels measurements which is accompanied by elevated levels of LDL, HDL, total cholesterol, and apolipoprotein (Apo) A and B (12,13,14). These highlight the possible implications of these peptides and metabolites previously detailed in the action of insulin and the possible connections between body fat excess and the development of chronic pathologies that have yet to be studied (12,13,14).

Additionally, in regard to nutrient plasma clearance, despite low levels of insulin, GHRD subjects have better glucose management after postprandial oral glucose or meal challenges (12). The same is true for branch chain amino acids (BCAA), 2-hydroxybutyric acid, and free fatty acid (FAA) levels, supporting the existence of a heightened IS state in skeletal muscle as well as in liver tissue (12,13). Unlike the GHRD Israeli population, these findings are associated with a lower incidence of IR type II diabetes mellitus (TIIDM) in the Ecuadorian cohort in contrast with a 6% prevalence in their relatives (13). Lastly, in relation to the redox state of the cell, low levels of proline and hydroxyproline have been reported suggesting a lower impact of reactive oxygen species in these subjects (15).

The previous events in the LS-E cohort have been documented and reported for over 34 years (13). Low incidences of TIIDM, cardiovascular and malignant diseases have been evidenced

(13,16). In regards to the lesser metabolic compromise exhibited by the LS-E subjects, it has been previously stated that differences in the degree of obesity, as well as the latter timing for nutritional transitions, can play a role in the lack of progress towards the so-called modern diseases of civilization (16). Nevertheless, economic shortcomings have dispersed this population, especially the youngest, towards more populated and economically important cities of the country which has largely impacted their diet. The westernization of the way of living and eating, as stated in previous reviews of this syndrome, poses an important metabolic impact to this cohort of unique individuals (12,13,14,16).

Finally, epidemiological data gathered from 1988 to 2011 (16), suggests that when compared with family members, these individuals do not have a relevant cancer incidence as illustrated by only one case of malignancy which has been documented in the cohort. This is attributed to the low activation of the IGF-1 pathway that ultimately yields less DNA damage from oxidative stress, lower mutagenic events in the DNA, and increases apoptosis of damaged cells. This DNA protection is related to molecular events that involve the activation of FoxO transcription, the downregulation of protein kinase B, the expression of mTOR genes, and up-regulation of the superoxide dismutase gene, all involved in the RAS/RAF/MAPK and PI3K/AKT/mTOR pathways (16).

In summary, GHRD Ecuadorian subjects present a unique metabolic environment that has highlighted the keen roles that insulin, GH, and IGF-1 play in the development of metabolic and malignant diseases. Thus, this specific internal milieu may help to further comprehend the

influence of other components linked to the development of these derangements, such as the gut microbiome.

Most reports about GM and its modulation of human physiology, have studied this relationship from the perspective of the impact that metabolites such as short chain fatty acids (SCFA), inherently arising from the GM, exert on the human host. However, SCFA are a true exogenous influence for the host. From a complementary perspective, our study was designed to explore the modulatory influence that the human endogenous factors inherently have. These endogenous determinants, such as hormones, peptides, and metabolic substrates such as glucose, triglycerides and others that might interplay with the afore mentioned exogenous GM influence, to finally determine the dynamic features that eventually shape not only the specific composition of the GM but also, its major influence in health and disease in humans.

2. MICROBIOME

a. THE MICROBIOME AND THE HOLOBIONT

Historically, microbes as singular entities have been identified as the causative agents of disease in humans as stated in the treatise of G. Castoro in the 16th century. Since then, several pioneers through the 17th century helped consolidate this knowledge in the widely accepted germ theory and settle the basis for the field of microbiology and public health (17). However, alongside this irrevocable truth, A. Leeuwenhoek and many other scientists during this period

and through the 19th century, homed in the presence of microbial communities in the human body that were not related to disease (17, 18).

These observations were mainly recorded in the gastrointestinal tract of healthy adults and children (17, 18). Nevertheless, the relevance of these findings would not become evident until 1885 when the anaerobic culture technique was developed. This milestone allowed the description of fecal microbes that were part of larger and more complex bacterial communities (18). This idea shifted the postulate from the pathogenic bacteria to a more ecological conception based on the observations of S. Winogradski. The definition of bacterial communities had as key points the multi-species composition and the interactions microorganisms established with their environments (19).

This concept was further refined in 1988, with the introduction of the term microbiome. The most accepted definition was made by Whipps and collaborators: "a microbial community, in a reasonably well-defined habitat which has distinct physiochemical properties, as their theater of activity" (19). To better comprehend this delineation, microbial assemblages can be described as all living members in a determined environment, bacteria, archaea, fungi, protists and algae. To determine their presence, their structural elements such as nucleic acids, peptides, polysaccharides, or lipids must be detected, quantified, and classified. Also, their dynamic behaviors must be recorded by the determination of their metabolites. All these are considered essential for the interaction within the assemblage and with a given environment (19).

Since the original microbiome definition, other internal components have been unveiled: viruses, phages, and genetic mobile elements. The functions and roles they might play are yet to be fully understood (19). In any case, all these elements have been described within a given environment that has "distinct physiochemical properties". In humans, this environment is set by the immune and endocrine systems with which the microbiota has established complex two-way interaction networks. This has generated new insights on the host-microbe relationship, their evolution, and their impact on human health (20, 21).

In 1991 Lynn Margulis described the "holobiont": humans as superorganisms that have coevolved and have symbiotic relations with their microbiota (19,20). Accordingly, this co-evolution implies that the microbiota has been subject to the same evolutionary pressures as humans and has had to rapidly adapt to gain fitness to thrive in new environments and also adapt to dietary changes. This is known as microbiome dynamics which acknowledges a core and a transient microbiota. The first is defined as a resident microbiota, constantly associated with a given host genotype or specific environment, while the latter is a transient state of the microbiota, influenced by environmental factors (21). Thus, the ongoing evolution of microbial communities through an individual's life, from birth to senescence, has been outlined. Nevertheless, there are more intertwining interactions to be discovered, as the holobiont has many age-dependent physiological processes and age-related non-communicable diseases that have been associated with gut dysbiosis, although no causative links have yet been found (21).

In a related matter, even though the microbiota composition and its dynamics may seem influenced only by environmental and host hormonal/immune factors, genetic influences have also been described. Phylogenetic signals detected in the primordial microbiota, vertically transferred from the matrilineal line through generations, have been identified and have also been associated with an impact on the offspring phenotype. These may contribute to understanding how microbial communities are conditioned to respond to environmental shifts and their roll in the development of health disruptions (21).

At present, research on the holobiont has generated 48TB of data obtained through the Human Microbiome Project since 2008. Through novel sequencing technologies, over 11000 samples from 5 different body sites have been analyzed and the gut has been importantly linked to the development of chronic non-communicable diseases (22).

b. **GUT MICROBIOTA**

i. **COMPOSITION, AND DIVERSITY**

To understand the role of the gut microbiome (GM) during healthy and diseased periods, it is important to understand its composition and functionality. Given that more than 70% of the microbial species in the human GM have not been cultured (29), the documentation of the phylogenetic identity of microbes is still the main and most reliable methodology available. The detection of microbial genetic material with novel third-generation sequencing technologies, such as sequencing analysis of bacterial 16S rRNA gene amplicon (16Sseq) and shotgun

metagenomic sequencing (MGS) have helped to delineate the GM structure and functionality. Nevertheless, there is much “dark matter” remaining in the GM (26,27).

Regarding sequencing technologies, the discovery of DNA structure was the platform that established the basis for three generations of DNA sequencing techniques, following the first one developed by Sanger and collaborators in 1970. These techniques have enabled the sequencing of thousands of human and bacterial genes including the human genome project that has sequenced 20,000 genes since 2001 (19), and the Human microbiome project (HMP) with approximately 33 million bacterial genes sequenced since 2009 (25). These two projects alongside METAgenomics of the human intestinal tract (MetaHIT) have generated comprehensive catalogs of microbial genes as the one reported by Li, J et al (28), with 9’87,896 non-redundant gut microbial genes. These catalogs include core genes, common functions, and population-specific microbiota compositions (28). Still, this cannot be universally applicable, even though it generates great metadata to refer to.

The use of these techniques has enabled the delineation of the taxonomic composition of the GM by quantifying the abundance of distinct species and its diversity (26). With the extended use of 16Sseq when studying GM, it has been possible to identify hyper-variable sequences of the conserved bacterial 16S rRNA. These sequences are then grouped by similarity into taxonomic levels from phylum to genus into operational taxonomic units (OTUS) representing one species-level sequence cluster or phylotype. This has allowed the identification of over 100 phylotypes in the GM classified into 6 main phyla: Firmicutes, Bacteroidetes, Proteobacteria,

Actinobacteria, Fusobacteria, and Verrucomicrobia (26). The predominating phylum, which is the gram-positive Firmicutes, has revealed more than 200 different genera of which Clostridium is the most abundant. While the gram-negative Bacteroidetes are mainly represented by Bacteroides and Prevotella genera. Although less abundant, the genera Bifidobacterium from the phylum Actinobacteria has been described as prevalent in GM (35).

Throughout the characterization of the taxa present in the GM, it has also been possible to determine the relative abundance and diversity of the existing communities. Diversity can be studied as the distribution of microbes within or between communities, the first is known as alpha diversity and it refers to the number of bacteria or bacterial richness in the sample and their distribution or evenness. The study of different microbial compositions among samples or bacterial communities is known as beta diversity (26). The study of diversity is important because GM is characterized by a high dynamism that results in large diversity fluctuations, even though a constant state of microbiota has been described during adulthood years and is associated with health (24,25,26).

This last has been described as the core microbiota, community type, or enterotype, in which variations of Bacteroides (enterotype I), Prevotella (enterotype II) or Ruminococcus (enterotype III) determine the three main GM compositions. that have been documented in individuals from different geographical locations, ages, and healthy-diseased stages (34,35).

Nevertheless, taxonomic profiles are not as similar between individuals as microbial genes. (31,32) So bacterial metabolic functions can be considered a more unifying feature for a core GM among individuals also, these metabolic pathways are common throughout microbial and human populations and can help to determine the localization of the bacteria in the gut. As some bacteria can adhere to the mucosal layers of the intestine and are prone to interactions with the host immune system, others prevail in the lumen where they need to establish adequate metabolic pathways for nutrient processing (36, 37).

These metabolic pathways have been identified through bioinformatic tools for 16srRNA sequencing such as PICRUST and Tax4Fun which enable the assignment of metabolic functions to bacterial genes when contrasted with previously annotated genomes. Nevertheless, whole metagenomic shotgun sequencing (WMGS) is a better method for the identification of the encoded functions of the assemblages (32,33)

Fluctuations of taxonomic groups along with changes in their metabolic pathways have been attributed to the influence of intrinsic and extrinsic factors during different time points in human life (24). Some of the most important are early life events such as route of delivery, breastfeeding vs commercial formula, use of antibiotics, sex, siblings among others, and stimuli could even pre-date post-natal life as has been evidenced by the presence of bacterial DNA in utero milieu (23,24). Also, environmental influences such as diet, geographical location, living arrangements (rural or urban), prescription drugs, and metabolic and immune host status also exert determinist influences on the way the community ensembles. Nevertheless, the influence

that these factors exert in shaping the community composition is estimated to be 20%. (26). Thus, stochastic ecological processes such as dispersal of bacterial species from local sites, selection of the fittest under different intestinal environments, drift of the lesser taxa and diversification events such as horizontal gene transfer, can lead to adaptation of the taxa and are associated with life-long impact on the microbiome (24,25).

When describing the development of GM, its plasticity must be considered because it presents significant variations which start at birth and continue through infancy. These variations are characterized at first by a low diversity-dominated by Actinobacteria and Proteobacteria and then diversity increases and approximately at 2-2.5 years of age it resembles that of an adult with a predominance of Bacteroidetes, Firmicutes, and Actinobacteria. More than *12* phyla, with 2172 bacterial species have been identified in the GM, of which 286 are strictly anaerobic and can be classified in the 6 phyla previously mentioned. More than 98% of the identified species belong to the Bacteroidetes and Firmicutes phylum whose ratio and variations are associated with the development of chronic diseases (25, 30,37). Further, to be able to establish a relationship between the GM and the role it may play in the development of diseases, its metabolic functions must be considered. It is to be noted that to date, approximately 89% of the GM annotated genes have not yet been assigned any function (26,34).

The use of sequencing techniques has made it possible to identify microbial metabolic pathways, some of which are common to more than one bacterial species due to the

redundancy of metabolic pathways, necessary for host-associated microbial life. This implies the existence of “core” or “housekeeping” pathways that bacterium need to thrive within the gut environment and are also called minimal gut genomes (34). These pathways involve ATP synthesis, glycolysis, nucleotide charging, and ribosomal and translation machinery (26). Further, common functions that all bacterial species must possess to contribute to the environment homeostasis are referred to as minimal gut metagenome (34) which refer to a few, but consistent pathways associated with low abundance taxa regarding biosynthesis of the polyamine’s precursor spermidine, methionine degradation and production of hydrogen sulfide, uptake pathways for pectin, sorbitol and capacity to ferment mannose, fructose, cellulose and sucrose (26,34). Some of these pathways have been linked to the enterotypes previously detailed where glycolysis and pentose phosphate pathways have been strongly associated with enterotype I, whereas the other two enterotypes are more specialized in mucin degradation from the intestinal mucosal layer (35).

When comparing 16Sseq to WMGS for the characterization of functional pathways in the GM, the latter annotates genes through the ensemble of the whole genome. During a series of steps, this method allows the analysis and comparison of fragmented sequences obtained from total extracted DNA to existing annotated gene databases that help establish phylogeny and eventually, functionality. This is mainly achieved after the assemblage of the metagenome, through scaffolds and comparisons based on DNA similarity using kmer frequency, GC content, or homology. The identification of genes relies on the length of the reads as well as the closeness between species previously categorized, thus enabling the assessment of microbiome

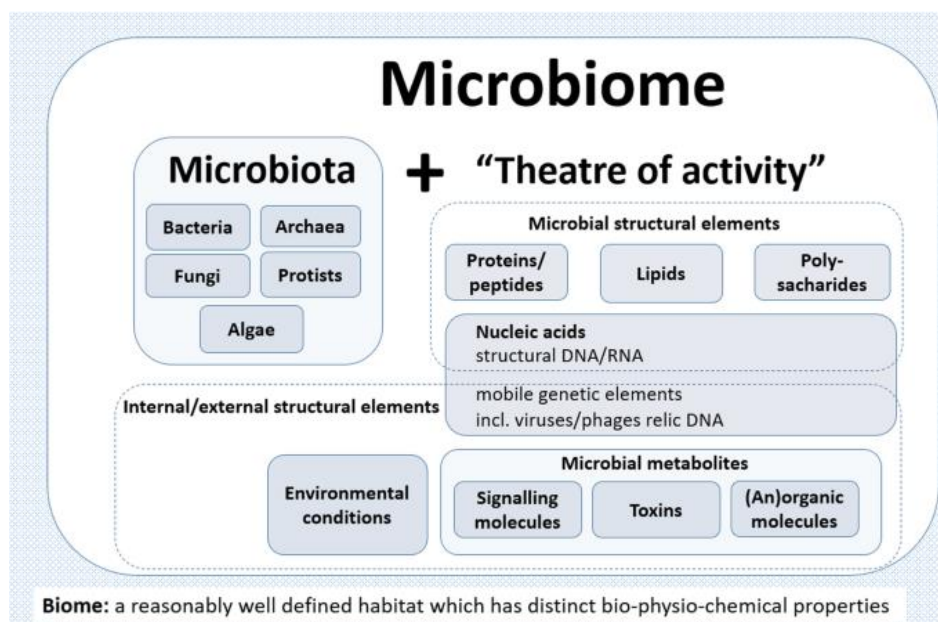
functionality (33). This functional annotation is made with the help of homology-based datasets, such as BLAST, and ortholog databases like KEGG. The creation of diverse bioinformatic pipelines has allowed the study of GM from a functional point of view, allowing associations between taxa and clinical metadata in health-disease states (33).

Despite extensive research on GM composition and functionality, there is not yet a unanimous concept of a "healthy" microbiome. For this purpose, the International Life Science Institute (ILSI), has considered four caveats when defining a healthy GM (24,42).

"1) a healthy microbiome cannot be defined by a single idealized community composition, 2) a healthy microbiome is more resistant and resilient to disruption, 3) certain microbial distributions may increase susceptibility to infection and disease, and 4) it is unknown if dysbiosis, an imbalance in the types of microorganisms present in a given microbiota, is a cause or consequence of disease." (42)

These conclusions highlight the importance of thoroughly defining the study populations and ecological factors that may influence the microbial composition. Only through the documentation of GM population variations through time, it will be possible to identify reliable biomarkers that will aid in the search for links between GM, host health, and disease status (24).

Figure 1. Components of the microbiome



https://media.springernature.com/lw685/springer-static/image/art%3A10.1186%2Fs40168-020-00875-0/MediaObjects/40168_2020_875_Fig2_HTML.png?as=webp

ii. **GUT MICROBIOTA METABOLOME**

The identification of microbial metabolic activity has generated breakthrough knowledge regarding the biochemical influences that GM can exert over its hosts' metabolic environment through several molecules. Short-chain fatty acids (SCFAs) have been proposed to be the most relevant-and are obtained in the gut from insoluble CHO processing. It has also been suggested that gut microbial communities can also influence the host's physiological processes through other metabolic activities such as nutrient obtention and metabolism, synthesis of amino acids and vitamins, and contribute to the maintenance of gut anatomical integrity, immune system maturation as well as interactions with the neuroendocrine axis (44).

The host-microbe metabolome interactions have been mainly studied through fecal analysis, where microbial metabolites can be identified and correlated with the alfa diversity, the host plasmatic and urinary metabolites, and fecal biomarkers (44). This type of sampling is considered the standard methodology when studying GM, and unlike intestinal biopsies, the noninvasiveness allows better patient compliance. Nevertheless, the quality of the sample can be altered by the steps prior to its analysis. The lack of standardization on processes of sample acquisition, handling, and storage, can compromise microbial composition and diversity (24,30).

The GM metabolic activity has allowed to classify the GM as an endocrine organ that locally produces metabolites that target distinct organs and tissues and in turn, can be impacted by host-derived metabolites (45). In this sense, the main functions of the GM which are food processing and energy harvest will be further reviewed to hone-in the associations of the GM with the development of metabolic diseases.

iii. **GLYCAN PROCCESSING**

As a major interface in the human body, the gastrointestinal system (GIS) is constantly exposed to a large variety of non-digestible substrates such as fiber polysaccharides, fermentable (carbohydrate) CHO, and non-digestible plant components. These substrates which are also called microbiota-accessible carbohydrates (MACs), are the main energy source of the GM and can be degraded by microbes through several carbohydrate-active enzymes (CAZymes) coded

by microbial genes to produce SCFAs. Glycoside hydrolases (GHs) and polysaccharide lyases (PLs) have been detected in the most abundant phyla of GM. Nevertheless, the Bacteroides genus has been shown to possess a greater diversity of CAZymes, and is known as a glycan generalist given the larger number of polysaccharides that they can process (36).

Bacteroides have starch utilization systems (SUS), encoded in polysaccharide utilization loci (PULs) that allows for greater codification of CAZymes. First described in *B.* Thetaiotaomicron, one PUL can codify for extracellular and periplasmic GHs as well as PLs targeting glycosidic linkages thus aiming at specific polysaccharide or glycans that will later be degraded and imported to the cytoplasm by enzymes conforming the system (36, 40, 41). As for how Bacteriodes determine which CAZyme and system to use, it has been proposed that gene regulation and positive induction and prioritization are determined by the kind of substrates present and thereby facilitating the coexistence of species with common pathways (36,40,41).

Further, this phylum lacks transporting systems such as ATP-binding cassette transporters (ABC), phosphotransferase systems (PTS) transporters, or major facilitators superfamily (MFS) transporters for the import of mono and oligosaccharides described in Firmicutes (36). Unlike the Sus-like complexes present in various proteins, these systems can be combined in one large polypeptide, thus when lacking specific actions for CHO processing, Firmicutes may require the cooperation of other starch-degrading organisms, such as Bacteroidetes to process it completely. This has been described in the butyrate-producing species *F. prausnitzii* which possesses PTS systems and an ABC transporter that enables the processing of fructose, glucose,

glucosamine as well as fructooligosaccharides, maltose, galactose, N-acetyl, and neuraminic acid but not polysaccharides (36).

Cooperation between phylum metabolic activities is known as cross-feeding and it has been evidenced in several interactions between bacterial species and bacterial-host interactions. An example of this is the utilization of CHO fermentation intermediate and end products which is initially, generated by Firmicutes and can be used by other surrounding bacteria for their metabolic purposes. The consumption of intermediate products such as fumarate, succinate, and lactate by bacteria and consequently by its host, can be associated with overall health. In this sense, reports of the presence of these intermediate products in human feces have been linked with dysbiosis and compromised metabolic health (46). Also, other cross-feeding activities can be evidenced after the breakdown of polysaccharides by Bacteroides, which allows surrounding *Firmicutes* to utilize monosaccharides thus generating mutualistic relationships that help maintain the bacterial community. These relationships have aided in the identification of housekeeping metabolic pathways as well as keystone organisms that contribute to the maintenance of the gut environment through unique metabolic capabilities (36).

Further, dietary glycans are not only the main source of CHO in the GM but highly glycosylated intestinal mucins MUC2 which are secreted by goblet cells, also provide O-glycans rich in N-acetylglucosamine, galactose, and N-acetyl-galactosamine. These are used as energy sources and binding sites for different bacterial species, especially in the colon where the mucin layer is

thickest (25). Also, it has been shown that glycosylation patterns of mucins impact the relative abundance of bacterial groups, and the way communities are shaped (25).

In a healthy human colon mucosa, the predominant colonizing bacteria are those in charge of sulfate-reducing, acetogenic and methanogenic pathways (38) from which the most abundant mucin-degrading bacteria are *A. muciniphila, Bacteroides thetaiotaomicron, Bifidobacterium bifidum, Bacteroides fragilis, Ruminococcus gnavus* and *Ruminococcus torques* (40). Bacteroides have been largely studied for their ability to degrade mucin and it has been reported that some can use intestinal glycan sources interchangeably. This capacity is as previously stated, related to the number of genes in the PULS coding for GH enzymes such as α-L-fucosidase, endo-β-N-acetylglucosaminidase, endo-β-galactosidase and α-mannosidase (38). This capacity to consume mucin also allows for a greater colonization of this genus in deeper anatomical layers of the intestine, in crypts, where the availability of their nutrients of choice is more abundant (25,38,39). As for the species that do not possess any mucolytic activity, their capability to adhere to the intestinal mucosal layer allows them to a position near the species that generate monosaccharides from the mucosal glycans and use this for their metabolism (36).

Also, bacterial species such as *Rumminococcus* in the Firmicutes phylum and *Bifidobacterium* in the Actinobacteria phylum, have been identified as mucin degraders despite their lower capacity for mucosal glycan degradation. As for the Verrucomicrobia phylum, its main representative *Akkermansia muciniphila,* has been identified as the key supporter of mucosal integrity and its abundance has been ubiquitously described in healthy human populations in

the mucosal intestinal layers as well as in feces. Its variations have been associated with the development of several diseases such as diabetes mellitus, hypertension, dyslipidemia, and obesity, among others (38,39). This mucin degrading specialist utilizes mucin as carbon and nitrogen sources and helps to increase the mucus layer by various beneficial molecular mechanisms which, contribute in great measure to the hosts' metabolic health (39).

In summary, the GM impacts the host's metabolome through the fermentation of oligosaccharides (fructooligosacharides, glucooligosacharides) and polysaccharides (inulin) that mainly results in the production of SCFAs by Firmicutes phylum. Butyrate (C4) and acetate (C2) are two of the three SCFA detected in human feces produced by Firmicutes, while Bacteroidetes generate propionate (C3). The production of these metabolites by different phyla in GM has shown to share approximately 300 pathways. Nevertheless, when specific microbial genes are analyzed, two paths for butyrate and three for propionate production in all phyla can be detected (46).

iv. SCFAs AND HOST INFLUENCE

Besides glucose and endogenous triglycerides, SCFAs are also an important source of energy in humans and they may contribute as much as 10% of the daily energetic requirements of the host. SCFA might also modulate lipid, glucose, and cholesterol metabolism through Fatty Acid Receptors (FFAR) or G-protein-coupled receptors (GPR). These receptors are present in several immune, epithelial, and endocrine cells and organs. Furthermore, it has been documented that SCFA, acting through post-receptor and signaling pathways, are involved in intestinal and

energetic homeostasis, immune function, and hypothalamic appetite regulation and CNS ludic systems (45, 46,48).

In the context of intestinal homeostasis, SCFAs have been shown to contribute to the maintenance of intestinal tight junction proteins and the epithelial integrity associated with less insulin resistance (51). Also, anti-inflammatory effects through GPPCR109a signaling in macrophages and dendritic cells have been attributed to the action of SCFAs in the intestine. Butyrate and propionate have been shown to induce stronger effects by down-regulating cytokine and chemokine gene expression in these immune cells. Lastly, butyrate has been associated with the alteration of the regulatory activities of the transcription factor Fox03 involved in cell cycle regulation, energy metabolism, oxidative stress, apoptosis, immunity, and inflammation (48).

As to host metabolic activities, the signaling through GPCR41/FFAR3 and GPCR43/FFAR2 located in enteroendocrine, pancreatic cells, and adipocytes, has unveiled their involvement in the regulation of appetite, lipid, insulin, and glucose metabolism. In this sense, butyrate and propionate through the recognition of both receptors, have been associated with the secretion of the incretin glucagon-like peptide 1 (GLP-1), grhelin, insulin, and the release of peptide YY (PYY) and the consequent regulation of appetite (45, 46,48,52).

Regarding energetic homeostasis, in the intestine, these molecules for up to 70% of the energetic source after oxidation. It has been reported that colonocytes present a higher affinity

for butyrate over the other two SCFAs and can oxidize this metabolite into ketone bodies and CO_2 (46,48,50). Similarly, acetate contributes to hepatic gluconeogenesis through portal circulation where it is used for the synthesis of cholesterol, long-chain fatty acids, glutamine, and glutamate synthesis (50). Also, butyrate and acetate have been described to take part in IGN by signaling pathways through the FFAR3 and activating the necessary enzymes through CAMP-dependent mechanisms or act as fuel sources, to contribute to glucose homeostasis as seen with propionate (44,45).

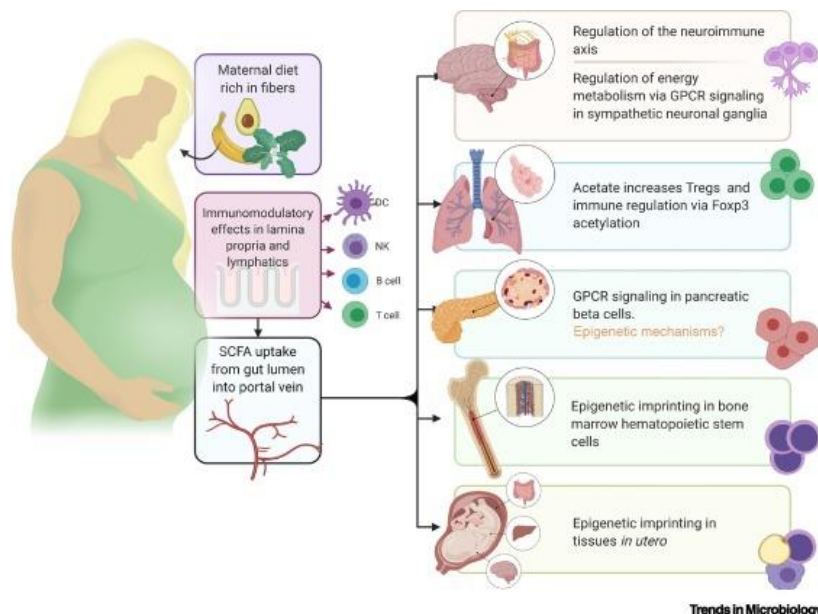
Also, SCFAs can activate free fatty acid (FFA) oxidation in the liver and muscles while inhibiting de novo synthesis and lipolysis in the liver. These actions also involve a higher activation of the AMPK activity in the mentioned tissues resulting in a net reduction of FFA in plasma (50). Regarding adipocyte lipidic metabolism via FFAR2, acetate and propionate have been shown to inhibit lipolysis mainly through the inactivation of hormone-sensitive lipase (HSL) through a decrease in the activity of protein kinase A (PKA). Also, Agus et al. (50) concludes that through the activation of this receptor, SCFAs have two main effects in adipocytes, namely, insulin signaling suppression by not allowing the phosphorylation of AKT and thus inhibiting fat storage in adipose tissue and promoting a better metabolism of glucose and lipids. In addition, an increase in leptin expression can be attributed to reduced adiposity (50).

SCFAs can also act as substrates in the liver through the generation of de novo lipogenesis that in turn generates triglycerides that will store as fat in adipocytes. Also, choline metabolism contributes to fat storage through the synthesis of very low-density lipoproteins and activation

of the bile acid farnesoid X receptor that can modulate the lipid and glucose metabolism through the actions of the fibroblast growth factor (FGF)-19 (53).

The efficient production and utilization of SCFA by the GM and its host have been evidenced by the absence of intermediate fermentation products of CHO in healthy human feces as opposed to some inflammatory or metabolic diseases where SCFA and intermediate products can be quantified. The presence of these products in feces can be considered as biomarkers for diseases although their presence is also subject to other variables such as intestinal transit time and type of diet which greatly influence the composition and metabolism of the GM (46). This statement highlights the crucial role that dietary CHO availability has in GM's composition and functionality, and it also puts into perspective the need to further investigate dietary composition in GM research (36, 46).

Figure 2. Effects of SCFAs and tissues



van der Hee, B., & Wells, J. M. (2021). Microbial Regulation of Host Physiology by Short-chain Fatty Acids. *Trends in Microbiology*. https://doi.org/10.1016/j.tim.2021.02.001

3. GM IN OBESITY, INSULIN SENSITIVITY AND SECRETION, AND INSULIN RESISTANCE

Obesity has been considered a global epidemic since 1997 by the World Health Organization (WHO) and is categorized as an adiposity-based chronic disease (ABCD) as proposed by the European Association for the Study of Obesity (EASO) (61). The development and recurrence of this disease are also determined by environmental, cultural, and physical activity. Nevertheless, its underlying metabolic derangements are not yet fully understood although its pathophysiology has been linked with adipocyte malfunction and hormonal dysregulations including peripheric insulin resistance (61). Further, these explanations do not suffice to clarify the development of obesity and its consequences and in consequence, other factors such as

diet and gut microbiome have been considered to possibly intervene in the development of this disease. (44).

As for the role of GM, energy extraction from dietary indigestible fibers has been appointed as the main mechanism involved in the development of obesity. In consequence, descriptions of changes in bacterial diversity involving the ratio Firmicutes/Bacteroidetes (F/B) have been considered and linked with body mass index (BMI) variations (51,53,54). In early GM studies, increases in Bacteroidetes and decreases in Firmicutes were linked with lean phenotype and the inversion of this ratio with obesity. Nevertheless, when links are searched between metabolic diseases and variations of the F/B ratio, results are controversial. This could be attributed to human population heterogeneity (sex, weight), and environment, among the most important variables. (53,54).

The inconsistency in results when the ratio is used has been attributed to differences caused by using diverse metagenomic sequencing technologies when analyzing samples. It may be that by centering the analysis at the phylum level rather than at lesser taxonomic levels, key species that could be involved in the development of these metabolic pathologies could be overlooked. Also, the lack of standardization of the microbial content in the samples may be an additional factor that impacts the Firmicutes/Bacteroidetes ratio (53).

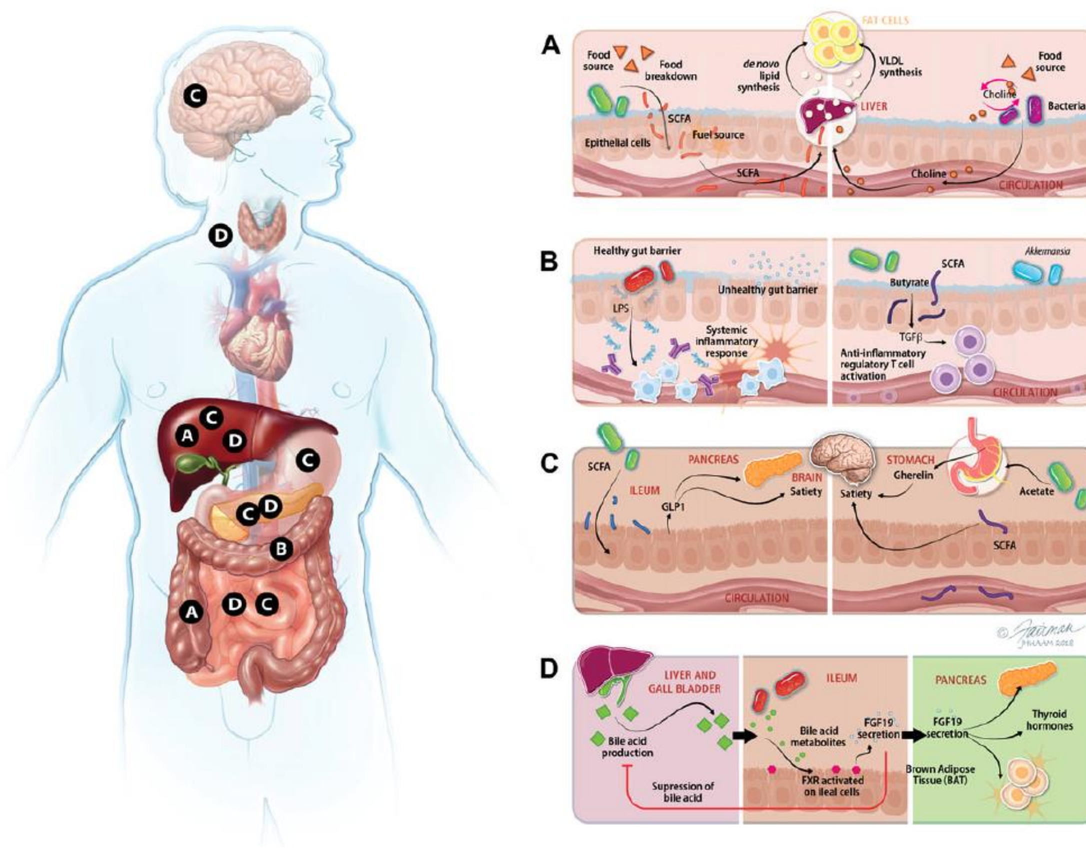
In any case, the remarks on the differences of bacterial diversity between lean and obese subjects is still relevant and has shown to be diminished in obesity and overweight. Alpha

diversity has not shown associations with obese states or BMI, unlike beta diversity. Also, when analyzing other taxonomic metrics, it has been described that a higher relative abundance of family and species in the Firmicutes phyla such as: *Blautia hydrogenotorophica*, *Coprococcus catus*, *Eubacterium ventriosum*, *Ruminococcus bromii*, *Ruminococcus obeum, Veillonella, Bulleidia,* and *Oribacterium* species, and decrease in the *Christensenellaceae* family are associated with obesity. In contrast, in lean individuals, species from both phyla*, Firmicutes* and *Bacteroidetes are* present; *Bacteroides faecichinchillae*, *Bacteroides thetaiotaomicron* and Firmicutes *Blautia wexlerae*, *Clostridium bolteae*, and *Flavonifractor plautii* (54).

Despite a correlation between lower taxonomic levels and elevated or normal BMI, bacterial gene count or richness and measures of bacterial-derived metabolites may be better when analyzing the GM. Low counts of bacterial genes have a correlation with unhealthy phenotypes including chronic diseases with a common metabolic pathway such as insulin resistance, dyslipidemia, and inflammation (53).

The presence of SCFAs-generating bacteria in the GM highlights the metabolic functions that these metabolites exert in their host. Thus, its actions must be carefully considered and analyzed in the context of metabolic derangements. As has been previously detailed, these metabolites influence endocrine pathways through actions on their receptors such as insulin, glucose, appetite and adipocyte metabolism (Figure) (44).

Figure 3. GM mechanisms which impact host metabolism and produce obesity



(A) energy metabolism, (B) gut barrier health, (C) insulin resistance, and (D) bile acid metabolism. Lee, C. J., Sears, C. L., & Maruthur, N. (2019). Gut microbiome and its role in obesity and insulin resistance. *Annals of the New York Academy of Sciences*, *1461*(1), 37–52. https://doi.org/10.1111/nyas.14107

4. **SHORT STATURE, INSULIN SECRETION AND THE GM**

GH and IGF-1 are cornerstone hormones in human growth and nutrition. However, their post-receptor downstream pathways are shared with other hormones and metabolites implicated in intestinal permeability, macronutrient absorption, and immune functions. Therefore, the influence these hormones exert over intestinal homeostasis and the GM has raised questions

on the existence of a bi-directional connection in which GM-derived metabolites play an important role in growth, adiposity, and hunger (55).

Regarding bone development, it has been observed that through SCFA's action on the growth hormone secretagogue receptor (GHS-R), GH secretion can be reduced. Also, propionate has the strongest inhibitory action on GH secretion via the cAMP/PKA/CREB pathway. Although these measurements have been linked with short stature and lesser bone development, it is important to keep in mind the pulsatile nature of GH secretion (55). Also, serum modifications of IGF-1 by the GM have been associated with enhanced bone resorption and decreased formation. Nevertheless, these results have been contradictory because GM influences immune functions and can also contribute to bone density (55, 56). In any case, dysbiosis associated with metabolic, nutritional, and inflammatory diseases that cause secondary growth failure has been characterized by the reduction of microbial richness, evenness, diversity, and the consequently reduced production of SCFAs thus reflecting the associations between GM and linear growth (55,56).

To further involve the GM with the GH/IGF-1 axis, derived SCFAs can increase ghrelin secretion leading to increased adipogenesis, regulation of gluconeogenesis, intestinal motility, and bone formation also via GHS-R. Lastly, the correlation between GM and ghrelin has been associated in murine models with the presence of *Bacteriodes* and *Prevotella* underlining the important effects that GM by-products can have on their host. This has been evidenced in early descriptions of GH/IGF-1 axis microbial mimetics like somatostatin produced by E. coli and B.

subtilis, and by descriptions of viral IGF-1-like peptides (VILPs) in the *Iridoviridae* family that can bind with higher affinity to the IGF-1 and insulin receptor eliciting the same downstream pathways that human hormones do, yet more studies in human models are needed (55).

5. PRADER-WILLY SYNDROME AND METABOLIC HEALTH

Prader-Willy syndrome (PWS) is a complex genetic disorder and the most common cause of syndromic obesity. It is accompanied by short stature, behavioral abnormalities, and sleep-awake cycle disruptions. It is caused by defects on chromosome 15 and has an estimated incidence between 1/10000 and 1/45000 in live births (57). Despite their high adiposity, these subjects exhibit insulin sensitivity, increased glucose metabolism, high adiponectin, and decreased inflammation and fibrosis. Consequently, the description of the GM in these subjects can generate new insights into the contribution that intestinal microbes can have on some of the most important metabolic pathways involved in the development of chronic diseases (58).

In this matter, only a few observational studies have characterized the GM in these patients while comparing the results with those obtained from obese and familiar controls. In the study by Olson et al (58), the authors described that the GM of the PWS subjects differed from obese controls and had a higher prevalence of taxa associated with insulin sensitivity and mucosal integrity such as *Akkermansia* and *Desulfovibrio.* The authors found a low abundance of the obese-related *Dorea* genus in the PWS subjects as opposed to the obese controls (58). Nevertheless, the GM was similar in richness and composition to those of their family members

which emphasizes the influence that shared environments and diets have in the shaping of microbial communities (58).

Nevertheless, results are conflicting considering that other studies on PWS subjects have not found differences in the GM when compared to age-matched controls with normal or higher weight (59). Interestingly, Dahl et al (60) have described GM's unique profiles in PWS subjects when comparing it with individuals from different geographical locations and ages linking the GM to the unique metabolic environment this complex syndrome possesses (60).

METHODOLOGY AND INVESTIGATION DESIGN

1. STUDY DESIGN, SUBJECTS, AND ETHICAL APPROVAL

This study is a descriptive cross-sectional study, which aims to characterize the gut microbiome of GHRD subjects (ss180/ss180) and that of controls, either a family member (WT/ss180) heterozygous or community control (WT/WT) and related it to the phenotypic and metabolic characteristics of each group.

Both cohorts of patients are originary from the southern Ecuador provinces El Oro and Loja. A cohort of 20 patients was examined in several towns from El Oro province: Piñas, Balsas, Santa Rosa, El Guayabo, eight subjects were visited in the city of Loja from January 31 to February 5,

2022. Also, ten subjects from these provinces living in Quito, were also assessed on February 22nd, 2002.

Subjects with previously documented LS (ss180/ss180) were located at their home addresses or by cellphone call, and according to the inclusion criteria for this study, sex, and age-matched control had to be chosen, a family member WT/ss180 heterozygous or community control WT/WT could be considered. Also, voluntary participation, a minimum age of 18 years was considered. Exclusion criteria specified antibiotic intake a month prior to the visit, as well as acute gastrointestinal disorders prior to, or at the time of the visit. Lastly, subjects with evidence of diabetes mellitus type 2, were excluded from this study.

Informed consent from 38 subjects were obtained prior to intervention, this document details for participation and a description of the procedures to obtain biological samples were described, as approved by the Human Research Ethics Committee, CEISH-USFQ (2021-125M).

Anthropometric and biological samples were obtained from a group of thirty-eight (N=38) age- and sex-matched subjects, comprised of eighteen (n=18) LS subjects and twenty (n=20) controls. Subjects were either WT/ss180 heterozygous or WT/WT; all of whom complied with the inclusion criteria at the time of recruitment. Of the 38 enrolled subjects, one LS and one control were excluded due to abnormalities in fasting glucose and HbA1c. Two controls were excluded because of insufficient fecal sample.

2. CLNICAL AND LABORATORY ASSESMENTS

Anthropometric and vital signs measurements were made using standard equipment (tensiometer, pulse oximeter, floor stadiometer, and a calibrated digital scale); body composition was determined with BMI calculus, as well as waist (cm) and hip (cm) measures to obtain the waist to hip ratio. Also, skin folds (mm) were measured with a caliper and included tricipital, bicipital, subscapular, and suprailiac folds; lastly, neck, armpit, groin, and arm folds were examined to document acantosis nigiricans.

Regarding the biological samples, due to geographical distances, with a portable point of care testing (POCT), HbA1c analyzer A1C EZ 2.0 (BioHermes, Wuxi, China) on site, HbA1c values were obtained from 1 drop of capillary blood in patients in a fasting state. Also, a tube of blood was obtained by venipuncture in either arm fold to determine by routine laboratory techniques: pre-prandial blood glucose, lipid profile, liver enzymes aspartate transaminase (AST), alanine transaminase (ALT), and uric acid. After subjects were given a standard meal and 2-hour post-prandial glucose was measured in capillary blood with an Accu-Check Diabetes monitor on site.

Fecal material for microbiota analysis was collected in OMR-200 OMNIGENE-GUT ® vials by the subjects complying with the manufacturer's instructions (59). All samples were given to the principal investigator and kept at room temperature until transfer to the laboratory facilities in the School of Medicine at Universidad San Francisco de Quito, where they were stored at room temperature until DNA extraction (60).

3. DNA EXTRACTION

For DNA extraction the DNeasy® Blood & Tissue kit from QIAGEN was used, following the tissue protocol for sample processing as detailed in the manufacturer´s instructions (61). A volume between 50 – 60 uL of the stool samples was obtained directly from the collection tube and placed into a microcentrifuge tube with a capacity of 1,5 uL; then 180 uL of ATL buffer was added to the sample and homogenized in the FastPrep® Instrument for 30 seconds at a set speed of 6. Then 10 uL of proteinase K was added, and mixed trough vortexing for 30 seconds, then all samples were incubated at 56°C for 60 minutes. Prior to the next steps all samples were vortexed for 15 seconds and, 200 uL AL buffer was added and mixed toughly through vortexing. After, 200 uL of ethanol was added and vortexed again. Then, a DNeasy Mini spin column was placed in a 2 uL clean collection tube and centrifuge at 10000 rpm for 1 minute, the flow-through and collection tube were discarded. The column was then placed on a second 1,5 uL clean tube and 500 uL of Buffer AW1 was added, the tube was centrifuge at 10000 rpm for 1 minute, after the flow-through and collection tube were discarded. A third collection tube was used to centrifuge the spin column after adding 500 uL of Buffer AW2 at 13500 rpm for 5 minutes, the flow-through and collection tube were discarded. Lastly, the spin column was transferred to a new 1,5 uL microcentrifuge tube and the DNA was eluded by adding 100 uL of Buffer AE and centrifuged at 10000 for 1 minute to bring DNA into the tube. The spin column was then discarded and from the 100 uL supernatant obtained, 50 uL aliquots were placed into a 1,5 uL clean microcentrifuge tube.

All DNA samples obtained were measured for quantity (absorbance at 260 nm), and quality (absorbance ratio 260/230) with a Take3 micro-volume plates for an EPOCH (BioTech, VT, USA) spectrophotomer. After, 34 aliquots were lyophilized for shipment to UNC Chapell-Hill to be sequenced, and the other 34 aliquots stored at -20°.

4. GENOMIC LIBRARIES AND SEQUENCING

The quantified and lyophilized DNA of 36 samples was sent to the Genomics Facility in University of North Carolina at Chapel Hill in the United States. Libraries were prepared with Kapa DNA on MANTIS, and WGS sequencing was preformed using NextSeq 2000 technology, for paired end reads. This yielded a total of 151 bp 2D paired end reads, 63 GB total.

5. DATA ANALYSIS

R version 4.2.1 was used for metadata analysis with the gtsummary package, version 1.6.1 .(67) Descriptive and inferential statistics, Wilkonson test for categorial and numeric variables were calculated, p value ≤ 0.05 for statistical significance was contemplated.

RESULTS

1. SUBJECT CHARACTERIZATION

a. DEMOGRAPHIC DATA

Eighteen subjects (12 females, 6 men) with GHRD from the Ecuadorian cohort where selected, all homozygous for the Ecuadorian mutation ss180/ss180. The mean age of the GHRD group was of 42 +/- 15 years (y). Also, eighteen subjects (12 females, 6 men) were selected as controls, either heterozygous WT/ss180 heterozygous or community controls WT/WT. With a mean age of 42 +/- 10 yo.

Table 1. Demographic information of the studied subjects

	STUDY GROUP		
Variable	**Control, N = 18[1]**	**GHRD, N = 18[1]**	**p-value[2]**
GENDER			NS
F	12 (67%)	12 (67%)	
M	6 (33%)	6 (33%)	
AGE	42 (15)	42 (15)	NS
AGE GROUP			NS
Middle age	11 (61%)	8 (44%)	
Young	7 (39%)	10 (56%)	

[1]n (%); Mean (SD)

b. ANTHROPOMETRY

GHRD were significantly shorter and weighed less than their respective normal controls, accordingly, expected BMI in the GHRD group was higher (30) than in the control group (27), although it did not reach statistical significance, higher BMI were expected. And can be contrasted with the higher hip to waist ratio in the GHRD group, denoting a predominance of central obesity in this group (0.70 vs 0.64).

As to body fat evaluation, controls reported higher measures of total skin folds mean and bicipital, tricipital, subscapular, and suprailiac folds than in GHRDs, nevertheless it was not significant. Also, presence of acanthosis nigricans in skinfolds, neck, armpit, and thighs was documented, in the control group more AN was registered, 10 subjects vs 3 GHRD, and the most affected zones where armpits and thighs.

Regarding blood pressure measurements, GHRD group register higher systolic values than control group (114 mmHg vs 110 mmHg, respectively), and similar diastolic values (75 mmHg for each group). Of interest, two GHRD and two controls presented high blood pressure measures (≥ 130/90 mm/Hg). Lastly, the pulse rate was lower in the control group than in the GHRD group, yet none of these measurements reach statistical significance.

Table 2. Anthropometric variables

	STUDY GROUP		
Variables	**Control, N = 18[1]**	**GHRD, N = 18[1]**	**p-value[2]**
Weight (Kg)	69 (15)	45 (9)	< 0.001
Height (cm)	158 (8)	120 (9)	< 0.001

BMI (Kg/m^2)	27 (5)	30 (7)	NS
Normal	9 (50%)	3 (22%)	NS
Overweight	9 (50%)	14 (78%)	
Waist-to-hip ratio (cm)	0.64 (0.43)	0.62 (0.41)	NS
Waist (cm)	64 (42)	61.9 (42)	NS
Hip (cm)	72 (48)	73 (47)	NS
Skin folds	87 (34)	75 (35)	NS
BLOOD PRESSURE (mmHg)			NS
Systolic pressure (mmHg)	114 (13)	110 (14)	NS
Diastolic pressure (mmHg)	75 (6)	75 (6)	NS
Heart rate (pulse/min)	82 (13)	86 (15)	NS

[1] Mean (SD) or Frequency (%)

[2]Wilconxon rank sum test; Fisher´s exact test

Table 2A. Subclassification of the subjects according to the anthropometric variables

	STUDY GROUP		
Variables	**Control, N = 18[1]**	**GHRD, N = 18[1]**	**p-value[2]**
Weigth			
Normal	9 (50%)	3 (22%)	NS
Overweight	9 (50%)	14 (78%)	
Waist-to-hip			
Elevated	3 (16.6%)	0 (0%)	NS
Normal	10 (56%)	10 (56%)	
No	5 (28%)	5 (28%)	
Skin folds			NS
Average	6 (33%)	2 (11%)	
Below average	3 (17%)	4 (22%)	
Excellent	8 (44%)	10 (56%)	
Good	1 (5.6%)	1 (5.6%)	
No	0 (0%)	1 (5.6%)	
Skin folds			NS
Average	6 (33%)	2 (11%)	

Below average	3 (17%)	4 (22%)	
Excellent	8 (44%)	10 (56%)	
Good	1 (5.6%)	1 (5.6%)	
No	0 (0%)	1 (5.6%)	
Acanthosis nigricans			NS
Absence	8 (44%)	15 (83%)	
Partial	5 (28%)	2 (11%)	
Presence	5 (28%)	1 (5.6%)	
Blood pressure			NS
Elevated	4 (22%)	2 (11%)	
High	0 (0%)	2 (11%)	
Normal	14 (78%)	14 (78%)	

[1] Frequency (%)
[2] Fisher´s exact test

c. **BIOCHEMICAL TESTS**

Fasting glucose levels were lower in GHRD group than in the controls (84 mg/dl vs 108 mg/dl), and showed a better post prandial glucose manage, showing lower pos prandial values than controls (119 mg/dl vs 128 mg/dl). Among the study groups, a total of four subjects (2 controls, 2 GHRD) presented diabetic pre and post-prandial glucose levels (≥ 136 mg/dl, ≥ 200 mg/dl, respectively), this was contrasted with HbA1c levels and three subjects (2 controls, 1 GHRD) exhibit high HbA1c levels (≥ 6.4%), documenting the diabetic state.

Regarding the lipidic profile, fasting measures of total cholesterol, HDL, LDL, and triglycerides did not reach statistical significance, although total cholesterol, HDL, LDL level were higher in the GHRD group, while triglyceride levels were lower in GHRDs. In the hepatic profile TGO showed little variance among groups, yet TGP was higher in the control group. Also, uric acid levels were lower in GHRD group than in the control group.

Table 3. Biochemical **parameters in the studied subjects**

	STUDY GROUP		
Variables	**Control, N = 18[1]**	**GHRD, N = 18[1]**	**p-value[2]**
Glucose pre-prandial (mg/dl)	108 (53)	84 (37)	NS
Glucose post-prandial (mg/dl)	128 (79)	119 (70)	NS
HbA1c	5.6 (0.99)	5.10 (1.54)	NS
Uric acid (mg/dl)	4.8 (1.07)	4.4 (1.6)	NS
Total cholesterol (mg/dl)	195 (38)	206 (79)	NS
Triglycerides (mg/dl)	151 (79)	155 (107)	NS
LDL (mg/dl)	115 (33)	121 (54)	NS
HDL (mg/dl)	50 (11)	51.7 (16)	NS
TGO (U/l)	27 (12)	27 (13)	NS
TGP (U/l)	32 (16)	29 (18)	NS

[1] Mean (SD)
[2]Wilconxon rank sum test; Wilconxon rank sum exact test

2. MICROBIOME ANALYSIS

Sequences were imported to the computational tool for profiling the composition of microbial communities from metagenomic shotgun sequencing data, MetaPhlan4 (62). Alignment of sequence reads was done with BowTie2 tool (63), obtaining a total of 4,196,380,664 reads with a mean per sample of 116,566,129.55. Also, tables with taxa counts and 2596 amplicon sequencing variants (ASVs) were generated for the 36 samples. Then the ASVs were classified with the taxonomy table generated by MetaPhlan4 and merged in one OUT table with BowTie2 prior to be imported to the software Quantitative Insights into Microbial Ecology (QIIME2) version 2022.2.8 (64) and MicrobiomeAnalyst (65, 66) for analysis.

For statistical analysis R version, 4.2.1 was used, packages: phyloseq, tidyverse, bioformat, and vegan were used to analyze sample diversity. Alpha diversity differences between groups were

determined by Shannon, Simpson, Chao1 and Pielou indexes. Kruskall-Wallis test for all samples was used for analysis, a p value of ≤ 0.05 was considered significant, as well as for a q value $\leq$ 0.05 for pairwise comparisons. For beta diversity analysis between study groups, PERMANOVA analysis was used with Bray-Curtis and Jaccard indices, a p value <0.05 was considered significant.

a. **ALPHA DIVERSITY**

Alpha diversity was evaluated using four indices, community richness by Chao1, diversity by Shannon and Simpson, and evenness by Pielou index. For demographic (age, sex, place of origin) and anthropometric variables (height, weight, BMI, blood pressure, pulse rate, skin fold, acanthosis nigricans, waist, hip), there were no significant differences between the study groups in diversity indices (Shannon, Simpson $p \geq 0.05$). Chao1 analyses to estimate the number of different bacterial ASVs in a sample, reached statistical significance when the biochemical parameter HbA1c was assessed by Kruskal-Wallis test, obtaining a p-value = 0.003 between study groups (GHRD vs controls). The statistical test was also used for paired samples analysis between samples in the HbA1c groups, obtaining a significant q value= 0.03, for the pairwise analysis between groups with normal and pre-diabetic HbA1c values.

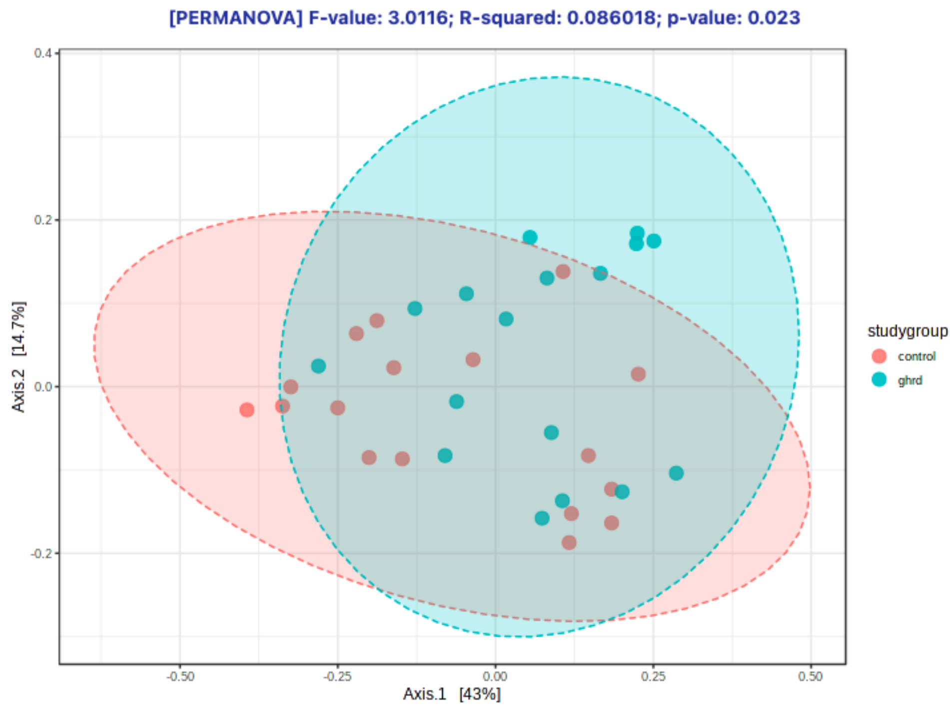
When the sample evenness was assessed by Pielou index, statistical significance was reached when analyzing HDL between study groups (p-value= 0.02), and between different HDL groups. When Kruskal-Wallis tests are performed pairwise between groups with better and poor HDL values a significant q value= 0.03 is reported (indexed figure X).

In summary, fecal microbiota richness, abundance, and diversity do not seem to be impacted by the demographic or anthropometric variables of both study groups. Nevertheless, biochemical variables such as HbA1c and HDL normal do appear to influence fecal microbiota, by denoting a higher bacterial abundance and richness independent of the study group.

b. BETA DIVERSITY

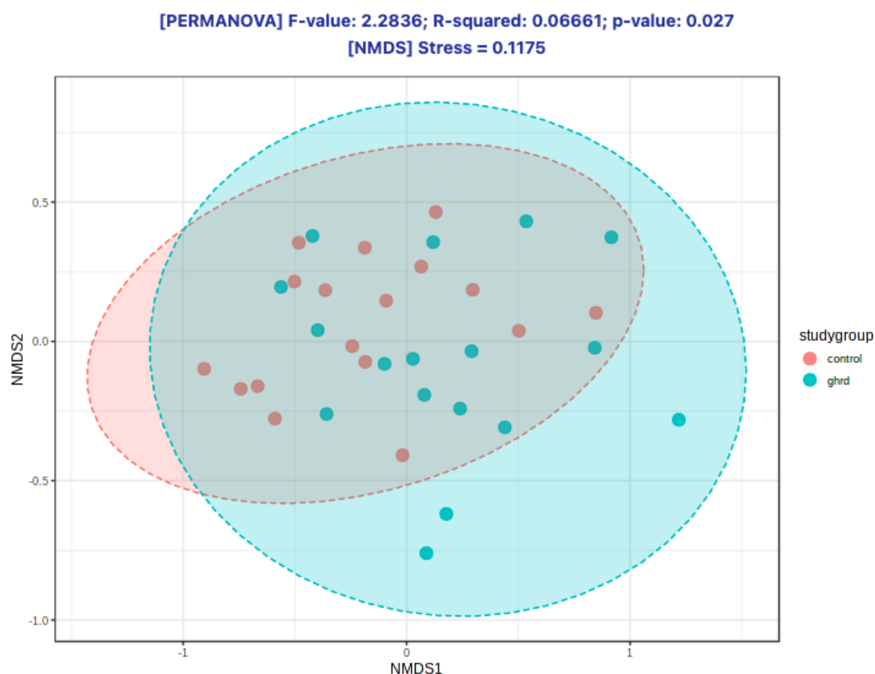
Beta diversity metrics, Bray-Curtis, and Jaccard index were used to assess composition, and with PERMANOVA analyses between study groups (control and GHRD), the two indices reached statistical significance. This demonstrates that the microbiotas of GHRD subjects are different from that of the controls. None of the other twenty-tree studied variables had an impact on fecal microbiome.

Figure 4. Bray-Curtis index between study groups



Bray Curtis dissimilarity showed in NMDS plots at the species level, displaying every sample as a dot, colored accordingly to the study group they belong. (GHRD in green, control in red). Samples from both groups can be seen grouping in the middle of the two axes, denoting high similarity between groups and low intra and inter-variability between samples (F-value of 3.0116), with a significant effect between groups. (p-value: 0.023)

Figure 6. Jaccard index between study groups



Jaccard index shown in NMDS plots at species level, displaying every sample as a dot, colored accordingly to the study group they belong. (GHRD in green, control in red). Samples from both groups, can be seen grouping in the middle of the two axis, denoting low intra and inter-variability between samples (F-value of 3.0116), with a significant effect between groups. (p-value: 0.023)

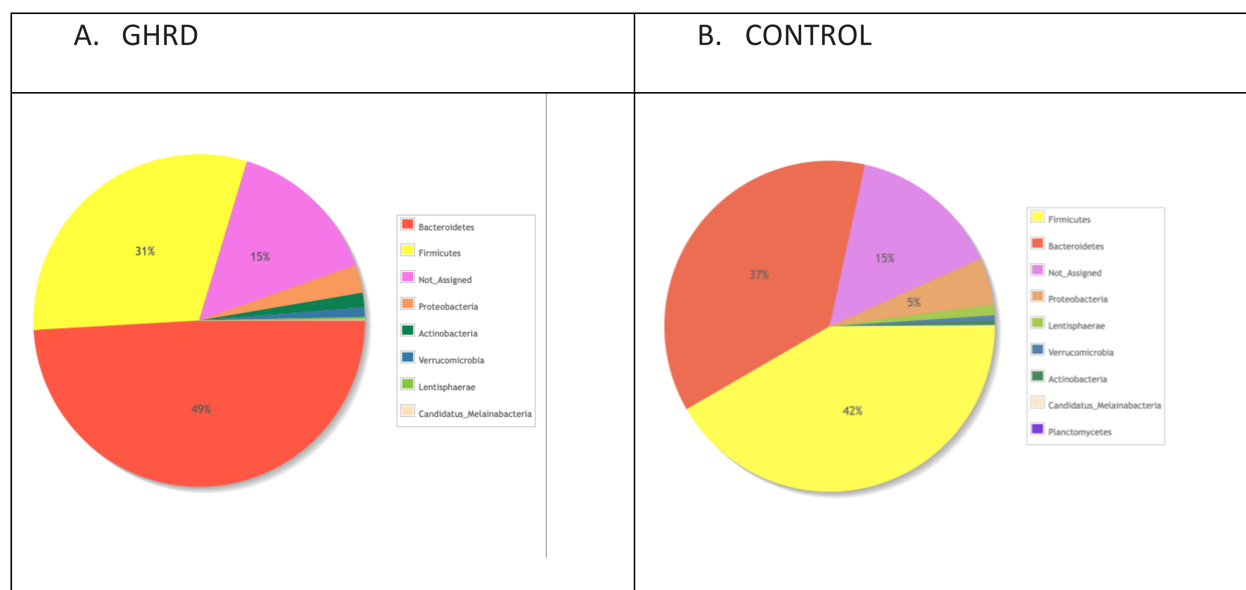
c. RELATIVE ABUNDANCE

To assess the microbial composition between study groups, relative abundance at the phylum level was obtained (supplementary table 1), and nine predominant phyla were present, grouped by higher ≥ 10 taxa count. Bacteroidetes (Bacteria), Firmicutes (Bacteria), and other non-classified bacteria (taxa count ≤ 10) constituted most phyla across study groups. Also, differences in taxa abundance were identified between GHRDs and controls, Firmicutes and

Bacteroidetes were more abundant in the control group was more even (F:42%; B:37%), than in the GHRD group (F:31%; B:49%). Whereas the non-classified bacteria abundance accounted for 15% in both groups.

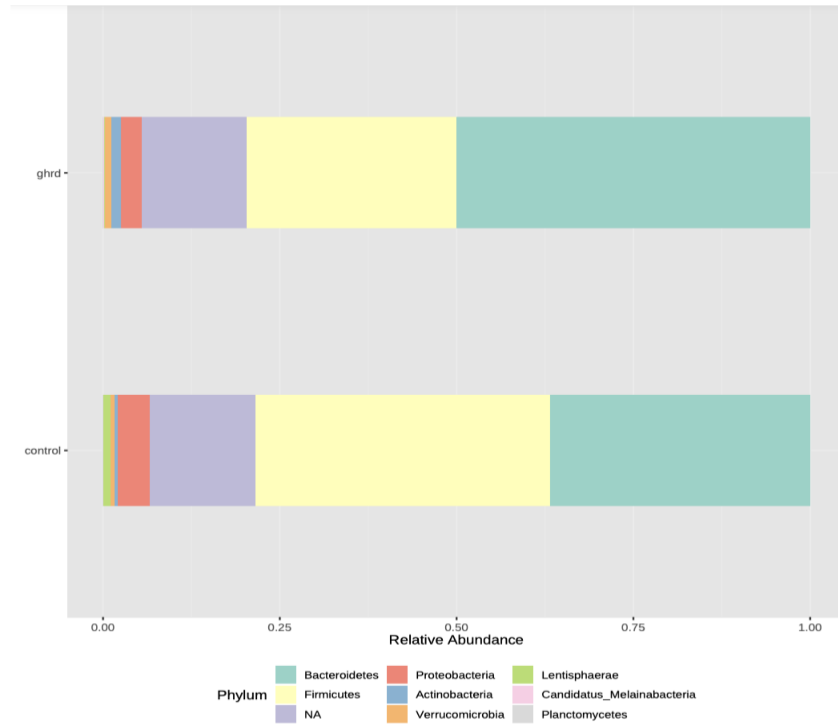
Also, in the control group higher abundances of Proteobacteria (C:5%; GHRD:2%), and Lentisphaerae (C: 2%; GHRD: 1 %) where registered, while in the GHRD group Actinobacteria (C:4%; GHRD:13%), Verrucomicrobia (C:4,7%; GHRD:8,4%), Candidatus Melainabacteria (C: 1%; GHRD: 8%) were documented.

Figure 7. Relative abundances between study groups



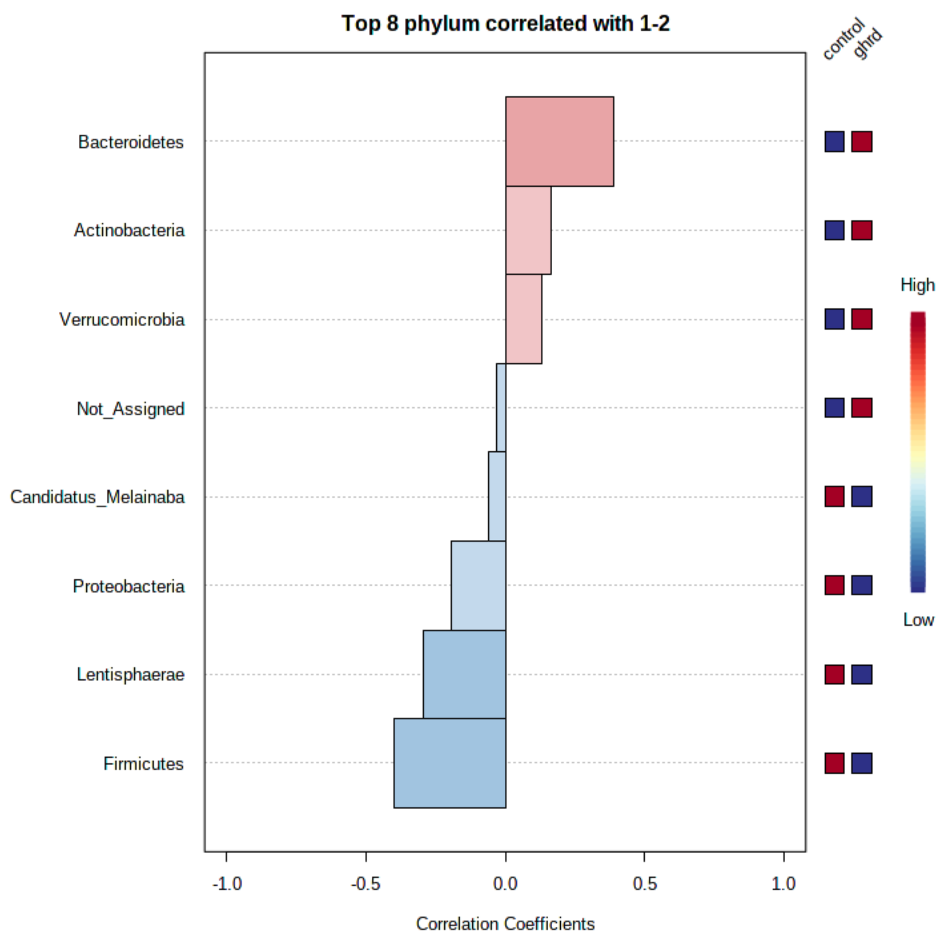
Pie charts displaying the abundance profiles of the two study groups at phylum level. Left pie chart A represents GHRD subjects, B represents control group.

Figure 8. Relative abundances between study groups



Stacked bar of microbial abundance at phylum level between study groups. Nine phyla are present grouped by higher ≥ 10 taxa count.

Figure 9. Patter plot between study groups



"The Pearson *r* correlation, showing the top 25 features correlated with the taxa of interest. The 8 phyla were ranked by their correlation. The blue color represents negative correlations, whereas the red color represents positive correlations. The deeper color (blue or red) means the stronger correlation. The mini heatmap on the right side of the plot shows the high or low abundance in two groups." (66)

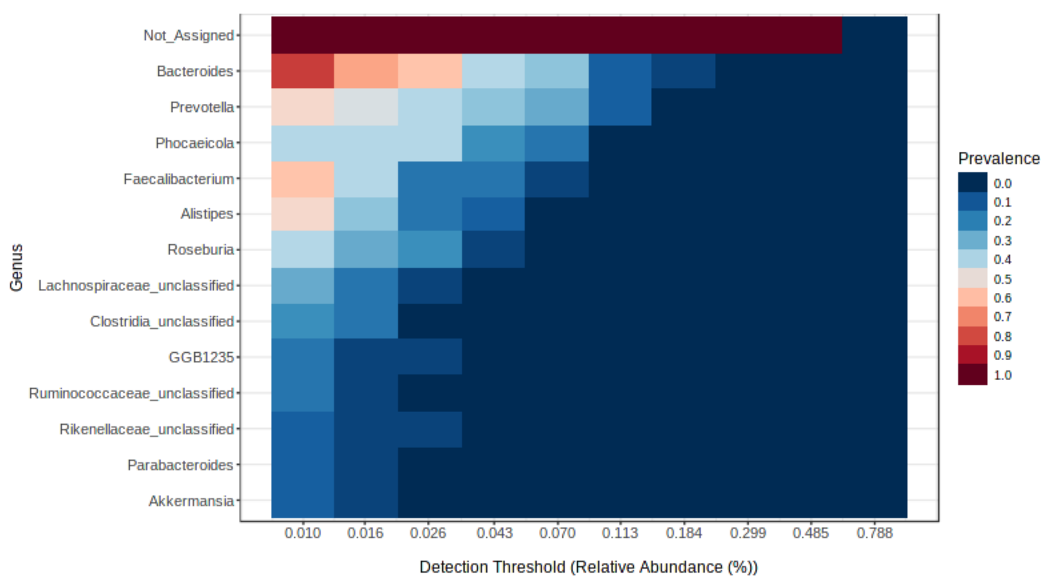
In the analysis, 8 phyla of bacteria correlated with each study group are shown. Bacteroides, Actinobacteria and Verrucomicrobia were positively correlated with the GHRD group. Whereas

the phyla negatively correlated with the control group were Firmicutes, Lentisphaerae and Proteobacteria, in relation to the relative abundances previously described.

d. CORE MICROBIOME

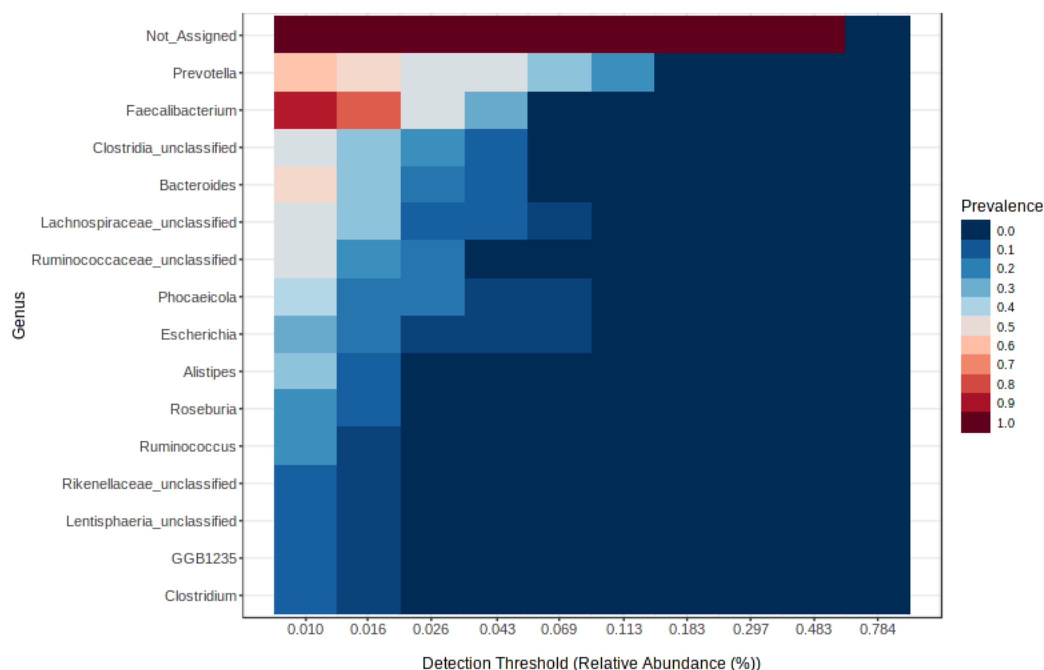
A core microbiome analysis was performed at specie level for study groups, were the specie had sample prevalence of 20% and a relative abundance of 0.01. This analysis showed similar core microbiomes between study groups. Species grouped as non-assigned showed to have a high prevalence in both groups, also *Faecalibacterm prasnitzzi* (Firmicutes) and *Prevotella copri* clade C (Bacteroidetes), even though in lower abundances, are common species to GHRD and control groups.

Figure 10. Core taxa for GHRD group



Heat map by bacterial specie for GHRD group. Non assigned species are the most abundant, followed by Bacteroidetes, Prevotella, Faecalibacterium, Alistipes

Figure 11. Core taxa for the control group



Heat map by bacterial specie for the control group. Non-assigned species are the most abundant, followed by Faecalibacterium, Prevotella, and Bacteroides.

DISCUSSION

We present two study groups comprised of 18 subjects with GHRD (ss180/ss180), and 18 controls either WT/ss180 or WT/WT. Genetic characterization of the GHRD subjects have been previously described elsewhere (16). Demographic and anthropometric characteristics are illustrated in Tables 1 and 2 respectively. The mean age (SD) at data collection time was 42

years (SD 15) for both groups, and the predominant age groups were young (≥ 18 – 39 years) and middle-aged adults (≥ 40 – 59 years). Also, regarding geographical location, in our study groups, 28 patients were intervened in El Oro and Loja provinces, and ten subjects in Quito.

GM analysis through different life stages has reported a predominance of Actinobacteria, Bacteroidetes, and Firmicutes during adulthood, nonetheless more than 98% of the GM is composed of Bacteroidetes and Firmicutes and can be influenced greatly by several factors, diet, geographical locations, living arrangements, health status among others (23, 24, 25). In accordance, with our study groups, GM showed the predominance of the three above-mentioned phyla, although in different proportions (Figure 8). Also, geographical locations did not seem to influence the GM, as all our subjects were intervened in cities with high altitudes, ≥ 2000 mts above sea level.

Concerning anthropometric variables, in line with GHRD syndrome descriptions (1-16), subjects were shorter and weighted less than their respective controls (Table 2). Consequently, due to their syndromic short stature and elevated weight, their BMI was higher than in controls. The high frequency of obesity in the GHRD group was also determined by the high waist-hip ratio in GHRD when compared with controls (0.70 vs 0.64 respectively), demonstrating the tendency of central adiposity in GHRDs. Also, when body fat was evaluated, controls reported higher measures of total skin folds mean than in GHRDs, nevertheless, this fat distribution could be age dependent. As to blood pressure measurements (Table 2), the control group register higher

values overall, non the less two GHRD and two controls presented high blood pressure measures (≥ 130/90 mm/Hg).

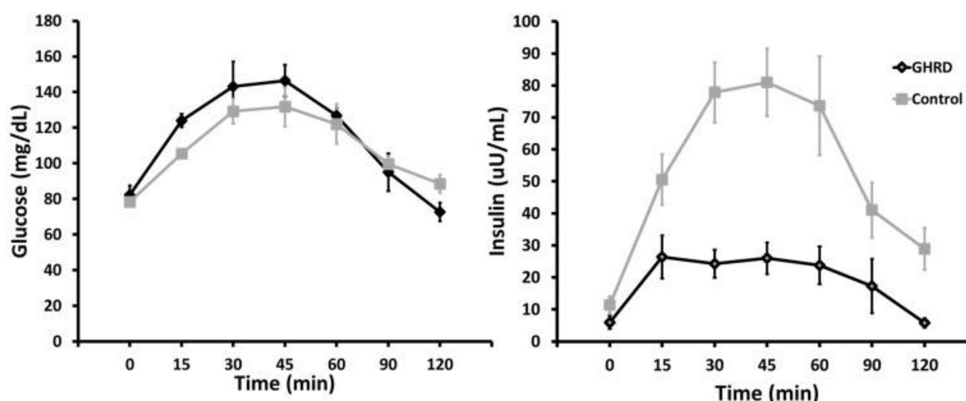
Another anthropometric variable related to obesity and the underlying metabolic disturbances was the presence of acanthosis nigricans. Skinfolds, neck, armpit, and thighs were examined in each study subject and found more AN in the control group. Ten controls vs three GHRD, presented AN in all the examined sites, and the most affected zones were armpits and thighs. AN is associated with high insulin levels, and the activation of IGF-1 in keratinocytes that lead to fibroblast proliferation, as well as epidermal keratinocytes stimulation (15). Even though we did not measure insulin, this parameter can offer a simple and not expensive way to approach insulin resistance in obese patients.

Also, fasting glucose levels were lower in GHRD group than in the controls (84 mg/dl vs 108 mg/dl), and showed better post-prandial glucose management seen in lower pos prandial values than controls (119 mg/dl vs 128 mg/dl). Among the study groups, a total of four subjects (2 controls, 2 GHRD) presented diabetic pre and post-prandial glucose levels (≥ 136 mg/dl, ≥ 200 mg/dl, respectively), this was contrasted with HbA1c levels, and three subjects (2 controls, 1 GHRD) exhibit high HbA1c levels (≥ 6.4%), documenting the diabetic state. The better management of glucose in the GHRD group has been previously documented (1-16), and it relates whit efficient insulin levels.

About other biochemical parameters measured in this study (Table 3), total cholesterol, HDL, LDL, and triglycerides levels were taken in a fasting state. We found a lipidic profile in the GHRD group characterized by high TG, total cholesterol, HDL, and LDL levels when compared with the controls, in accordance with the generalized obesity in both groups.

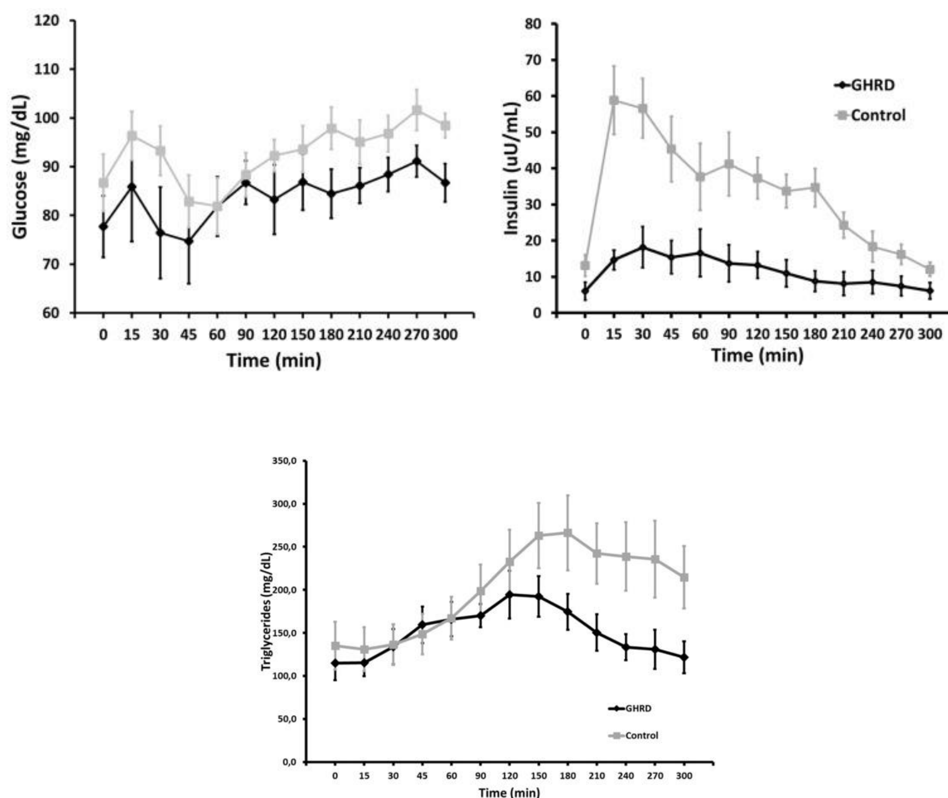
In line with the above stated, in a previous study Guevara-Aguirre et al (15), measured glucose values in fasting state and 2-hour post oral glucose test in GHRDs and controls (Figure 12), and found similar glucose patterns as the one presented in this study. Also, in the report, a 5-hour monitorization of insulin, TG and glucose levels was made after the ingestion of a standard meal (figure 13). Interestingly, low levels of these metabolites in GHRDs when compared with the control group, were observed during the challenge. Denoting a better insulin response, glucose, and TG management in the GHRD group. These results could have been reproduced in this present study, nevertheless we did not measure insulin levels and did not monitor our patients for a longer period. In conclusion, we believe that a two-hour challenge may be insufficient to appreciate significant variations in glucose and TG levels between study groups.

Figure 12. Two-hour glucose responses in GHRD and control groups



Guevara-Aguirre, J., Rosenbloom, A. L., Balasubramanian, P., Teran, E., Guevara-Aguirre, M., Guevara, C., Procel, P., Alfaras, I., De Cabo, R., Di Biase, S., Narvaez, L., Saavedra, J., & Longo, V. D. (2015). GH Receptor Deficiency in Ecuadorian Adults Is Associated With Obesity and Enhanced Insulin Sensitivity. *The Journal of Clinical Endocrinology & Metabolism*, *100*(7), 2589–2596. https://doi.org/10.1210/jc.2015-1678. Taken with permission from fist Autor, JGA.

Figure 13. 5-hour glucose, insulin, and TG responses in GHRD and control groups, after a standard meal challenge



Guevara-Aguirre, J., Rosenbloom, A. L., Balasubramanian, P., Teran, E., Guevara-Aguirre, M., Guevara, C., Procel, P., Alfaras, I., De Cabo, R., Di Biase, S., Narvaez, L., Saavedra, J., & Longo, V.

D. (2015). GH Receptor Deficiency in Ecuadorian Adults Is Associated With Obesity and Enhanced Insulin Sensitivity. *The Journal of Clinical Endocrinology & Metabolism*, *100*(7), 2589–2596. https://doi.org/10.1210/jc.2015-1678. Taken with permission from fist Autor, JGA.

In any case, GM characterization studies have tried to establish a relationship between GM and the possible role it may play in obesity, diabetic and insulin-resistant states. Nevertheless, no links of causation have been found. Correlations with the F/B ratio and the bacterial capacity of energy extraction and SCFA production from food sources have been proposed and will be further reviewed, as these metabolites interplay with adipose, insulin, glucose, and liver metabolism through specific receptors (36, 44, 45, 46,48, 50, 51, 52). SCFA are the main exogenous metabolites associated with intestinal health balance, as well as overall metabolic health, and depend on the F/B ratio, widely associated with metabolic disturbances (33-35). Nevertheless, the International Life Science Institute (24, 42) has proposed that a healthy GM cannot only be defined by the presence/absence of certain bacterial groups, as changes in these can associate with health or pathology, although not stablish causality. But a possible hallmark of a healthy GM may be associated with the resilient capacity of its members, and for this purpose, our study lacks the control of our study groups over periods of time (30).

Also, low uric acid was registered in the GHRD group as reported elsewhere (1-15). Lower concentrations of uric acid have been related with a higher purines pool and lower DNA breakdown (73). These events have been previously registered in GHRD serum in-vitro tests, leading to alterations in the PI3K/AKT/mTOR pathways, and associating with a lower incidence of cancer in this population (16). Nevertheless, examination of bacterial metabolic pathways and the possible links with the above stated are needed.

In regard with hepatic enzymes, TGO and TGP were measured. TGO showed little variance among the groups, yet TGP was higher in the control group, no clinical implications were found in both study groups. Studies of GM and its possible inferences in liver disease have proposed that, the presence of high Bacteroidetes and lower Prevotella abundances are related with non-alcoholic fatty liver disease (74), consequently linked with obese phenotypes, nevertheless no other hepatic tests were performed in our population.

In summary, both study groups have been characterized as obese by anthropometric variables, yet despite this generalized obesity among GHRDs, this population exhibits better glucose levels and glucose management after a two-hour meal. In accordance with previously reported findings (1-16), this population exhibits a higher insulin sensitivity when compared with age, sex and BMI matched controls. This can also be documented by lower presence of AN in this group, in contrast with a more generalized AN in the control group, denoting possible high levels of insulin, yet un efficient, as the two-hour post-prandial glucose measures reflected. The mentioned findings are of great interest when analyzing the GM, as variations of taxonomic levels in different populations worldwide studies have correlate with metabolic disturbances as the ones discussed.

Regarding the GM analysis in the study groups, we found that fecal microbiome of subjects with GHRD differs in bacterial composition from that of the control group, by beta diversity metrics. However, no significant differences in alfa diversity metrics, richness, and diversity between

groups were observed. This highlights the similarity between bacterial phyla shared between groups, yet a significant dissimilarity in bacterial distribution (26). In the literature, alpha diversity reported discrepant associations with obese states or BMI, showing great variance when associated with different environmental factors. Unlike beta diversity, that has showed consistent associations with these anthropometric variables, as high F/B ratio (54).

Differences in bacterial abundances at the phylum level were observed, in the control group a higher predominance of Firmicutes and low Bacteroidetes was more evident (high F/B ratio). In contrast, the GHRDs had higher abundances of Bacteroidetes and low Firmicutes (low F/B ratio), these results highlight a possible association between the healthier phenotype of the GHRD group and the F/B ratio. Even though our study groups were defined as overweight or obese, the F/B ratio between both groups show a discrepancy, as classically a high F/B ratio is associated with lean phenotypes and low ratios with obesity and other chronic disturbances.

Nevertheless, controversial findings between populations have challenged the use of this ratio, as mainly environmental factors, such as diet have been considered to influence greatly fluctuations of the ratio. Exposure to high-fat diets has been associated with increases in Firmicutes, due to their capacity for energy extraction from foods, whereas high-fiber diets are related to an increase of Bacteroidetes, thus associating it with lean phenotypes (72). Our study did not consider nutritional variables, yet we have previously stated that these populations have been exposed to a shift in dietary patterns, drifting from a “classic” diet to a more westernize variety of foods (14).

In any case, regarding this phylum capacity to produce SCFA, it would be of great interest to measure these GM derived metabolites, as fecal biomarkers and as part of their metabolic pathways. This would allow us to better comprehend the influence these exogenous metabolites can exert in the host lipidic, insulin, glucose, and cholesterol metabolism and hormonal pathways.

In any case, regarding other F/B ratio discrepancies, the metabolic endotoxemia hypothesis has been proposed as a contributing factor for the development of obesity and states that, the chronic exposure to gram-negative bacteria LPS, contributes to a chronic inflammatory state and obesity development. Nevertheless, this phylum high abundance does not correlate with F/B ratio associated with leanness. It may be that endotoxic activity of this phylum is lower than other gram-negative bacteria, such as Proteobacteria and the exchange rate of the GM does not allow LPS to reach high concentrations (72). In any case, concerning Proteobacteria, this phylum was common for both study groups and more abundant in the GHRD group, even though it has been associated with dysbiosis, metabolic diseases, and low bacterial diversity GM, it is considered the most unstable of the GM bacteria, as environmental factors can influence greatly its abundance (69).

Other important phylum related to gut health, albeit its low abundances in both groups, the mucin degraders Actinobacteria, and Verrucomicrobia were found to be more abundant in the GHRD group. Both phyla have been associated with gut barrier homeostasis manutention and

supporters of intestine mucosal integrity. Actinobacteria also contribute with host health by impacting host metabolic pathways through SCFA production. Lastly, Verrucomicrobia phylum is considered a true hallmark of intestinal health among populations, for its highly specialized mucus production. In both study groups, when the presence or absence of this phylum is related to the F/B ratio previously detailed, it can be said that it associates with its respective metabolic milieu.

Finally, the presence of other gram-negative phylum, Lentisphaerae was mainly identified in the control group, these bacteria are known for their saccharolytic capacities and the production of mucus, even though its implication in the GM has not been established yet. This is also true for Candidatus Melainabacteria, a non-photosynthetic cyanobacterium-like, found in both groups, its presence in environmental surfaces and water has been known, yet in the human fecal microbiome descriptions have been recently made and its influence on human health has yet to be determined (71).

In summary, the inclusion of other environmental variables, and measurements over periods of time could help better define the fluctuation of these phyla and GM in both study groups. Nevertheless, several studies have stated that GM analysis at lower taxonomic levels may have a better correlation with metabolic diseases, as the examination at the phylum level may overview some bacterial species with a stronger correlation with these pathologies. In our study groups, at the genus level, core taxa analysis was carried and a profile with a high

predominance of Bacteroides (G-), Prevotella (G-), Facalibacterium (G+), was evident in both groups. This correlates with the bacterial abundance findings previously detailed.

This study has characterized the GM of Ecuadorian subjects, as well as a control group of obese subjects, and found a bacterial profile with a predominance of Bacteroides, Actinobacteria, and Verrucomicrobia positively correlated with the GHRD group, all with mucolytic capacity. Also, in the control group, we found a higher predominance of Firmicutes, Lentisphaerae, and Proteobacteria that negatively correlated with this group. Nevertheless, genomic, and metabolic studies are needed for a better characterization of these phyla and can help highlight the associations with the study groups phenotypes.

Lastly, we couldn't find any significance or correlation between the GM and biochemical parameters measured, it maybe is that a more sensitive analysis must be performed over longer periods of time to correlate with any changes in the GM. In any case, to our knowledge, this is a pioneer study of GM in LS due to GHRD.

Of interest, the findings in the GHRD group can contrast with those obtained with the descriptions of the GM in PWS subjects (58,60). Olson et al. described similarities in richness between PWS subjects and aged-matched controls, yet characteristically a high predominance mucin degrader in PWS subjects. This was evident in our population as well, and it underlines the influences shared environments exert in the study groups, as well as the hormonal milieu of subjects with syndromic obesity and short stature.

SUPLEMENTARY MATERIAL

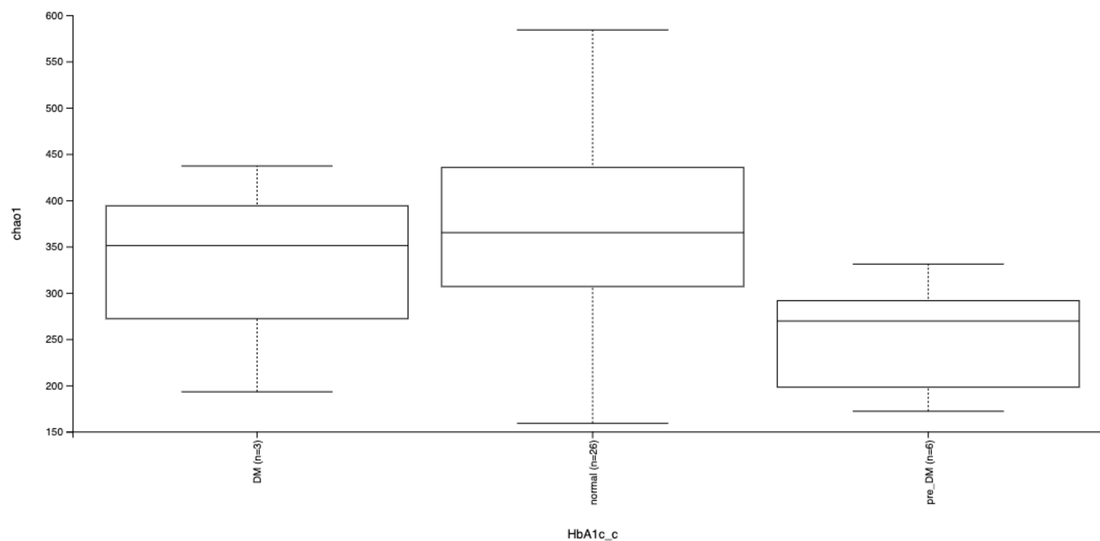
Table 4. Relative abundances of bacteria at phylum level

PHYLUM ABUNDANCE

	CONTROL	GHRD
Actinobacteria	0.00466643578313454	0.0133443626207109
Bacteroidetes	0.367992354087685	0.500753985198524
Candidatus_Melainabacteria	0.00109023870669877	0.000814449024844301
Firmicutes	0.416398536763761	0.29588057956201
Lentisphaerae	0.010442455061347	0.00225624623841503
Planctomycetes	1.08711520268107e-05	0.000487180549574578
Proteobacteria	0.0459475051715013	0.0297924744092601
Verrucomicrobia	0.00473395213320403	0.00842789255594801
NA	0.148717651140641	0.148242829840714

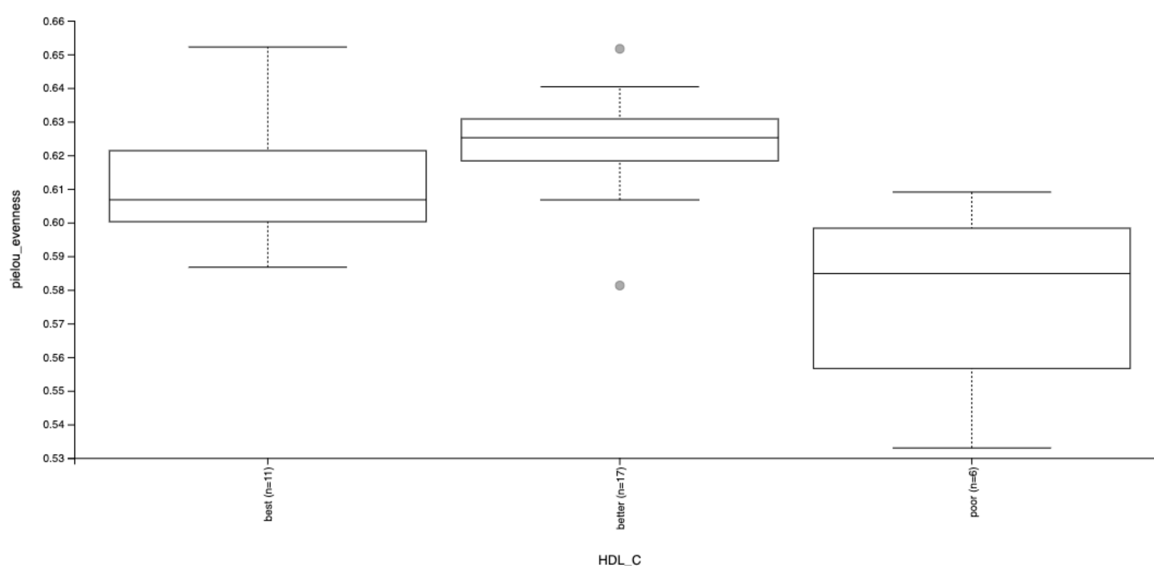
Generated with MicrobiomeAnalyst

Figure 14. Chao1 index for HbA1c



Box plot showing tree different groups based on HbA1c levels (DM (diabetes mellitus), normal, pre-DM). Kruskal-Wallis test for all groups: H value: 6.50, p-value: 0.03. For the normal HA1c group a higher richness of 450 OTUs can be observed vs 260 – 280 of the other groups.

Figure 15. Pielou index for HDL



Box plot showing tree different groups based on HDL levels (best, better, poor). Kruskal-Wallis test for all groups: H value: 612.00, p-value: 0.02. The group with better values has higher species diversity and richness than the group with poor HDL values.

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