GUT MICROBIOTA: A CONNECTION BETWEEN OBESITY AND CARDIOVASCULAR HEALTH IN CHILDREN

Mireia Alcázar López

UNIVERSITAT ROVIRA i VIRGILI

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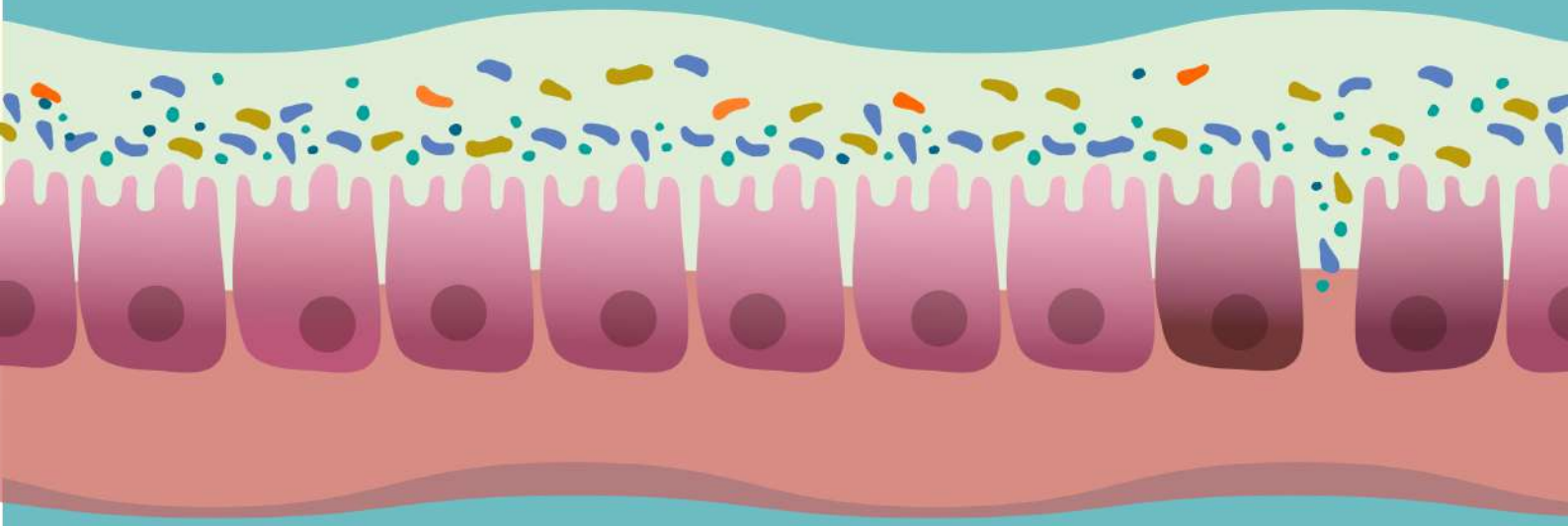

Doctoral thesis
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UNIVERSITAT
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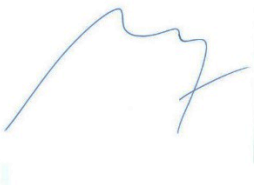
FEM COSTAR QUE aquest treball, titulat "Gut microbiota: a connection between obesity and cardiovascular health in children", que presenta la Mireia Alcázar López per l'obtenció del títol de Doctor, ha estat realitzat sota la nostra direcció al Departament de Medicina i Cirurgia d'aquesta Universitat.

Reus, 22 d'Agost, 2022

Directors Tesi doctoral



Dr. Joaquín Escribano Dra. Verónica Luque Dr. Ricardo Closa

Agraïments

M'agradaria donar les gràcies a totes les persones que han col·laborat d'una manera o d'una altra en aquesta tesi.

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Abbreviation list

AMPK - 5' AMP-Activated Protein Kinase

BCAA – Branched-Chain Amino Acid

BCFA – Branched-Chain Fatty Acids

BMI – Body Mass Index

BMI-R – Body Mass Index Responders

DBP – Diastolic Blood Pressure

FFAR – Free Fatty Acid Receptor

GLP-1 – Glucagon-Like Peptide 1

GLUT4 - Glucose Transporter Type 4

GPR - G-Protein Coupled Receptor

FFA – Free Fatty Acids

HDAC - Histone Deacetylases

HDLc – HDL Cholesterol

HOMA- IR – Homeostatic Model Assessment

IBD – Inflammatory Bowel Disease

IL-10 – Interleukin 10

IL-1B – Interleukin 1B

IL-6 – Interleukine 6

IPA – 3-Indlepropionic Acid

LDLc- Low-Density Lipoprotein Cholesterol

LPS – Lipopolysaccharides

MetS – Metabolic Syndrome

MetScore-R – Metabolic Risk Score Responders

MHO - Metabolically Healthy Obesity

mTORC1 - Mammalian Target of Rapamycin Complex 1

MUO – metabolically unhealthy obesity

NF-KB - Nuclear factor kappa-light-chain-enhancer of activated B cells

NPY – Neuropeptide Y

OUT – Operative Taxonomic Unit

PCoA - Principal Coordinates Analysis

PCR – Reactive Protein C

PYY – Peptide YY

SAT – Subcutaneous Adipose Tissue

SBP – Systolic Blood Pressure

SCFA – Short Chain Fatty Acids

T2D – Type Two Diabetes

TG – Triglycerides

TLRs – Toll-Like Receptor

TMA – Trimethylamine

TMAO - Trimethylamine N-Oxide

TNFα – Tumour Necrosis Factor α

Figures index

Summary

Childhood obesity is a significant public health problem associated with the development of cardiometabolic alterations. Due to its effects on adulthood, most of the efforts are currently focused on preventing and treating childhood obesity and its comorbidities. The gut microbiota is gaining particular interest due to its role in several human body functions.

The development of obesity and its comorbidities such as type 2 diabetes, hypertension or dyslipidaemia has been associated with the composition and diversity of the gut microbiota. Moreover, it has been reported that the composition of the gut microbiota before starting an intervention may predict the efficacy of the such intervention. Most of the evidence is centred on animal models and adult populations; however, evidence is scarce in children.

Within this thesis, the gut microbiota of children with obesity has been identified and characterized. A specific gut microbiota profile characterized by lower *Akkermansia* and *Christensenellaceae* abundances and higher abundance of *Bacteroides* have been associated with higher cardiovascular risk. Higher abundances of *Faecalibacterium* and *Eubacterium coprostanoligenes* and lower *Bacteroides* may predict a better response to a multi-component (diet and physical activity) intervention.

Knowledge gathered within this doctoral thesis may be helpful to be applied in further intervention studies aiming to test tailored interventions to modify gut microbiota profiles in children with obesity.

Justification

The treatment and prevention of obesity and its comorbidities is nowadays a significant challenge in health care. The accumulation of fat that takes place during obesity development causes cardiovascular alterations such as insulin resistance, hypertension, or dyslipidaemia. However, despite their high-fat accumulation, a subgroup of individuals with obesity do not develop any alteration. Thus, understanding the mechanisms associated with the development of these comorbidities may allow improving its prevention and treatment.

Gut microbiota, and its interplay with several physiological pathways, has gained special interest during the last decades. Different works have related specific gut microbiota composition with the development of obesity and its comorbidities.

To date, most of the works linking obesity and its comorbidities through changes in gut microbiota have been performed in animal models and adult human populations. It is known that obesity and cardiovascular disease in adulthood are frequently predicted by obesity in childhood. Besides, the high prevalence of childhood obesity annunciates a rise in adult obesity prevalence and cardiovascular disease.

Detecting specific mechanisms that lead to the development of comorbidities at an early age could bring a window of opportunity to promote tailored obesity treatments. The potential role of gut microbiota in the development of obesity-related comorbidities is still to be elucidated.

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Introduction

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1. Childhood obesity

Obesity has become a major public health problem affecting adults and children in recent decades. Since 1975 the worldwide obesity prevalence has nearly tripled. The increase observed in children and adolescents is especially noteworthy. As is shown in Figure 1, less than 1% of the children presented obesity in 1975, while in 2016, more than 124 million children suffered from it (6% of girls and 8% of boys) (1).

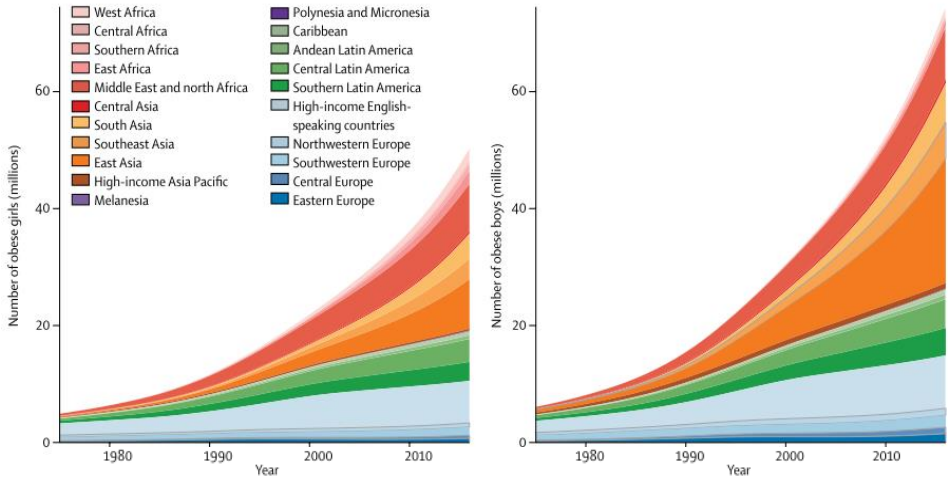

Figure 1. **Trends of obesity from 1975 to 2016.** Representation of the increasing trend of obesity over the past's years. This data includes a population aged between 5 and 19 years over the world. Image extracted from the NCD Risk factor collaboration work (1)

Childhood obesity is the consequence of an interaction between genetic, dietary factors, such as energy intake or the quality of the diet, physical activity and environmental factors, such as family characteristics (2,3), parenting styles (4) or school and social policies (5). By itself, obesity represents several abnormalities and impaired functions in the human body. It can be manifested as altered glucose tolerance, dyslipidaemia, hypertension, early atherogenesis or even orthopaedic complications. All these factors may trigger a reduction

in a child's quality of life and an impaired social life (6). In the long term, childhood obesity is considered a significant risk factor associated with the development of adult overweight and obesity (7,8). Several authors have published that maintaining an excessive weight from childhood to adolescence represents a higher risk of obesity and its associated metabolic disorders in adulthood (9,10). In this sense, childhood obesity must be considered a modifiable factor in obesity and cardiovascular disease prevention strategies.

1.1 Childhood obesity and cardiometabolic health

Several hypotheses have been proposed to understand the underlying mechanisms leading to metabolic alterations in paediatric obesity; however, the pathophysiologic mechanisms have not yet been established.

The fat accumulation that occurs as part of obesity development, especially abdominal adiposity, is associated with changes in the adipose tissue. Some associated changes may be disruption of its normal function, dysregulation of hormonal production and release of inflammatory cytokines (11–13). This process triggers a chronic low-grade inflammatory response linked to insulin resistance, lipid abnormalities, hypertension, non-alcoholic fatty liver disease and metabolic syndrome in adulthood and childhood (14). However, some people with obesity seem to be protected from the development of cardiometabolic effects related to high-fat accumulation. Around 47% of patients with obesity would develop metabolic syndrome; however, it has been described that around the 10% of patients with obesity would not develop any cardiometabolic alteration (15,16). This observation emerges as new concept known as “metabolically healthy obesity” (MHO) in adults and children.

1.2 Metabolically healthy obesity

The MHO phenotype emerged in the early nineties when some authors introduced the "benign obesity phenotype" concept in the adult population (17,18). It was used to describe obesity, that despite the fat accumulation, was characterized by the absence of insulin resistance, diabetes, dyslipidaemia, hypertension, cardiovascular disease, or metabolic syndrome (MetS) and, therefore, less risk of morbidity and mortality (18,19).

As in adulthood, in childhood, a subgroup of youth with obesity also shows less predisposition to cardiometabolic alterations (20,21). Despite the high levels of fat accumulation, it has been observed that children with MHO present a better metabolic profile than those children with "metabolically unhealthy obesity" (MUO) (22). However, some authors describe it as a transitory state of comorbidities development prelude (23).

There are no universal diagnostic criteria for MHO in adults or children. Gordon et al. (15) reviewed more than 30 definitions used in different adult populations observing that most of the authors used the classical metabolic syndrome components to determine the presence of healthy obesity. These components were systolic and diastolic blood pressure (SBP; DBP), plasma triglycerides (TG), HDL cholesterol (HDLc) concentration, fasting blood glucose, and waist circumference. Most of the studies considered that MHO participants were those who, besides the presence of obesity, presented less than two metabolic syndrome (MetS) components, including the waist or less than one if the waist circumference was excluded. Other parameters such as Homeostatic Model Assessment (HOMA-IR), low-density lipoprotein cholesterol (LDLc), or C-reactive protein (PCR) have also been used in the definition.

Definitions regarding the paediatric population are even more diverse. There are different criteria to define obesity in children, and their differences make it challenging to standardize the MHO definition (24–26). The most common criteria for the MHO definition in different works is the absence of an alteration in any cardiometabolic risk factor (TG, HDLc, blood pressure, insulin resistance), apart from elevated body mass index (BMI). However, other authors follow, as seen in adults, other criteria such as the presence of ≤1 cardiometabolic risk factor excluding waist circumference or the presence of ≤ 2 of them if waist circumference is considered. As there were non-standardized criteria to identify children with MHO, in 2018, Damanhoury et al. (27) published a consensus-based definition. The agreement concluded that MHO in children should reflect the absence of any cardiometabolic alteration, including dyslipidaemia, hypertension, or glucose intolerance. The experts agreed that there is a need to develop a universal definition of MHO for children. They established cut-off values for HDLc, TG, SBP and DBP. However, although they concluded that an altered glycemia should be included in the definition, they did not reach an agreement about which measure and which cut-off value should be used. Figure 2 summarizes the consensus-based definition for MHO in children by Damanhoury et al. (27).

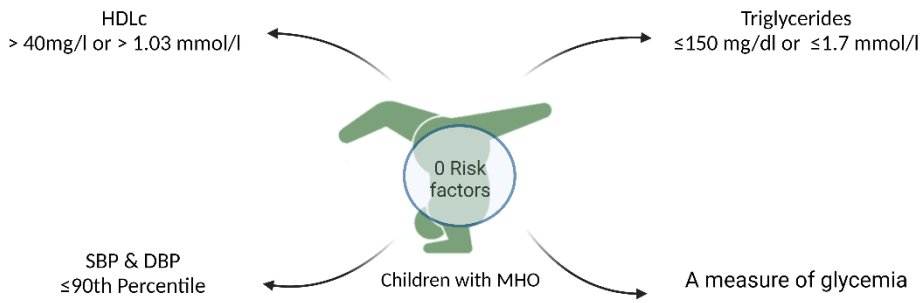

Figure 2. **Definition of MHO based on the consensus of Damanhoury et al.** Adapted from Damanhoury et al. (27) Created with BioRender.com

The prevalence of MHO is challenging to specify due to the different studies' controversy over the definition. Age, gender, ethnicity, and other sociodemographic factors could affect the prevalence in adulthood and childhood. A systematic review (28), based on different definitions, concluded that the prevalence could range from 6 to 75% in adulthood. The prevalence in the paediatric population could vary in different studies from 3 to 87%, depending on the criteria used to define the MHO (21).

Vukovik et al. (21) reviewed the predictors for presenting MHO. They exposed that the most robust factors among the previously published works were the presence of lower waist circumference measurements, lower BMI, younger age, and lower body fat measurements(21). However, several other factors have been proposed, such as early adiposity rebound (29,30), diet and lifestyle (31–34).

1.3 Mechanisms underlying the development of cardiovascular alterations

Although numerous predictor factors have been observed in adults and children, the underlying mechanisms for why some children with obesity present cardiometabolic alterations while others are free of them, are still poorly understood. It has been suggested that a preserved insulin sensitivity, a specific fat distribution, normal adipose tissue function, or the gut microbiota composition could be involved in preserving the MHO phenotype (15). Moreover, a connection between genetics and behavioural factors plays a crucial role in the development of the alterations (17). This section will investigate the suggested mechanisms underlying the maintenance of the MHO: Insulin resistance, adipose tissue functionality and structure, low-grade inflammation, and gut microbiota.

- ## Insulin resistance

Insulin is a hormone secreted by the pancreatic beta cells in response to increased blood glucose levels. Insulin works as an anabolic hormone, inhibiting lipolysis and hepatic gluconeogenesis and increasing glucose uptake by the liver, the muscle, and adipose tissue. Insulin resistance is the decreased tissue response to insulin-mediated actions (35). It is well known that insulin action is applied to several organs and pathways. Therefore, the development of insulin resistance has been proposed as one of the main underlying mechanisms for developing type 2 diabetes (T2D) and other metabolic alterations in childhood obesity (35,36).

It has been reported in previous studies that MUO children present higher HOMA-IR than children with MUO (37–39). As possible mechanisms, the authors suggested that the capacity to maintain the glycaemic control of the MHO could be due to a hereditary functionality of the β-pancreatic cells (40). However, the actual mechanism is still unelucidated.

- ## Adipose tissue

Beyond lipid storage, adipose tissue is an essential endocrine organ related to the development of cardiometabolic alterations. Its functionality plays a crucial role in cardiovascular disease and insulin resistance development (41). The adipose tissue releases different adipokines that participate in several inflammatory and metabolic interactions. The adipokines are diverse, and this group includes hormones such as leptin and adiponectin, peptides such as angiotensin or resistin, and inflammatory cytokines such as Interleukin-6 (IL-6), tumour necrosis factor α (TNFα) or chemerin (42,43).

Adipose tissue distribution is an important determinant of the metabolic phenotype in childhood obesity (41). There is evidence that increased adipose

abdominal and visceral fat is associated with an unhealthy phenotype and higher cardiometabolic risk (37,44). It is known that in a state of overnutrition, the expansion of the adipose tissue results in increasing the adipocyte size (hypertrophy) and/or enhancing the number of adipocytes (hyperplasia) (20). Long-time exposure to nutrient excess requires an expansion of the adipose tissue, either by hyperplasia and/or hypertrophy. Figure 3 shows how, in normal conditions, the lipids are stored in subcutaneous adipose tissue (SAT). If there is a dysfunction in fat accumulation, lipids tend to be stored in ectopic organs such as the visceral adipose tissue, the liver, the skeletal muscle, or the pancreas. This dysfunction leads to the release of proinflammatory, diabetogenic, and atherogenic signals that led to organ damage and the development of unhealthy obesity and its cardiovascular risk factors (20). However, a healthy expansion of the SAT leads to increasing the subcutaneous adipose tissue storage and secretion of beneficial adipokines such as adiponectin (20,45). The mechanisms underlying this capacity are still unelucidated, but it is suggested that age, sex, and genetic factors could modulate body fat distribution.

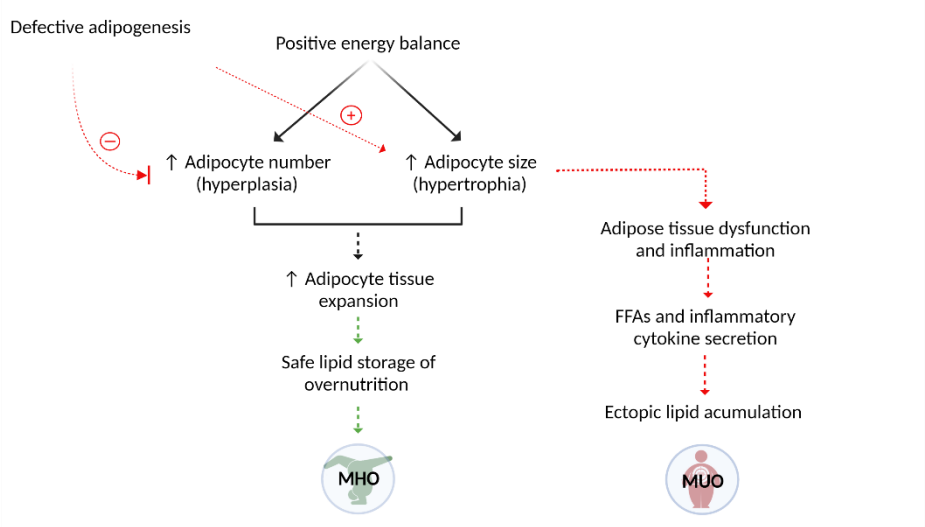

Figure 3. **Adipogenesis.** The figure summarizes impaired adipogenesis as a mechanism underlying the development of cardiometabolic alterations. Adapted from Iacobini et al, 2019 (20). Created with BioRender.com

- ## Chronic low-grade inflammation

Leptin is an anorexigenic hormone involved in appetite regulation. Moreover, it plays a role in glucose homeostasis and insulin sensitivity (46). Contrary to what would be expected, leptin levels are associated with higher BMI and body fat in adults and children (47–49). This association is caused by an increased leptin release by the adipose tissue besides leptin resistance during obesity. This inhibits its functionality and is linked to cardiovascular risk and inflammation (47,50). In contrast, adiponectin has been described as anti-inflammatory, anti-atherogenic and anti-diabetic adipokine (51,52). An increase in this adipokine has been reported after a weight loss treatment (53).

Children and adults with obesity are characterized by an increased release of inflammatory adipokines by the adipose tissue, such as IL-6 and TNFα, and a

decrease in adiponectin concentrations. These facts lead to a low-grade inflammatory status. This inflammation is one of the most important mechanisms underlying the development of comorbidities associated with obesity (54).

Several potential mechanisms underlying the development of inflammation in obesity have been suggested (55). The adipose tissue inflammation could be stimulated by signals released by the adipocyte's expansion, hypoxia or death, or mechanical stress due to increased TG levels (41,56).

Another mechanism triggering lower-grade inflammatory response is the recognition by the Toll-like receptors (TLRs) of some components from the gut (57). The most studied cell components are the lipopolysaccharides (LPS) present in the membrane of certain gram-negative species. The detection of LPS by TLRs stimulates the release of cytokines such as TNFα, IL-6, interleukin 1 beta (IL-1B) or monocyte chemoattractant protein-1 leading to an inflammatory state (58). In obesity, an impairment of the gut barrier leads to the translocation of LPS to the blood system from the gut. In agreement with this mechanism, it has been shown that children and adults with obesity present higher blood LPS indicating higher translocation of bacterial components and the mentioned gut barrier impairment (55,59,60).

- ## Gut microbiota

The relationship between gut microbiota and health was first reported by Hippocrates, who reported that "all diseases begin in the gut." However, during the last decades, the composition and functionality of the gut microbiota have emerged as essential players in the metabolic homeostasis of the human body (61).

The fermentation of undigested carbohydrates by the gut microbiota produces different metabolites such as short-chain fatty acids (SCFA) or bile acids that are a source of energy or could be integrated into different metabolic functions affecting the metabolism and immunity (62). These functions are related to body weight control, energy intake, glucose, lipid metabolism, and, as previously mentioned, low-grade inflammatory status. Some factors like diet (63), physical activity (64), or drug treatment (65) could affect the gut microbiota composition and functionality, causing dysbiosis, which could result in a "pre-disease" state linked to the development of obesity and cardiometabolic disorders (20). The last shreds of evidence have shown that the gut microbiota from adults (66,67) and children (68–70) with obesity present different characteristics than the microbiota of individuals with normal weight. In addition, some characteristics differ between subjects with healthy and unhealthy obesity (71,72). These facts suggest that the gut microbiota could play an important role in differentiating between MHO and MUO because of its metabolic implications.

The following sections will introduce the human microbiome and the gut microbiome and its different characteristics and implications in the human body.

2. The human microbiome

The human microbiome is the collection of bacteria, archaea, fungi, protists, and viruses that colonize the human body, including the skin, saliva, lungs, oral mucosa, conjunctiva, mammalian glands, seminal fluid, uterus, vagina and the gut.

It has been estimated that the 0.3% of all body weight is composed of bacteria. Moreover, a recent publication has demonstrated that the ratio of human to bacterial cells is closer to 1:1 (73).

Almost all of the human body is colonized by different bacterial communities from birth and the neonatal state throughout all life (74). The composition of the microbiota varies across the different parts of the body. As is shown in Figure 4, the different parts of the body present different predominant species depending on the characteristics of the environment. The characteristics of the skin's microbiome depend on the humidity and composition of the skin. The most representative skin genera are *Staphylococcus*, *Corynebacterium* and *Veillonela* (75). The oral microbiome is the second most populated site of the body. More than 700 bacteria species have been identified, and the major bacterial genera are *Streptococcus*, *Peptostreptococcus*, *Bifidobacterium*, *Corynebacterium* and *Desulfobacterium* (76). The respiratory system is also colonized by different bacteria, including *Streptococcus*, P*revotella*, *Veillonela* and *Neisseria* (77). In the vaginal environment, the predominant bacteria are the *Lactobacillus*, *Staphylococcus*, *Enterococcus* and *Bifidobacterium*, which are the most important representants of vaginal newborns (78). Despite the acid pH of the stomach, it is populated by genera such as *Prevotella*, *Streptococcus* or the well-known *Helicobacter pylori* (79). The microbiota composition changes along the gastrointestinal tract responding to the

different characteristics of the environment. The gut microbiota is the site with more quantity of bacteria (80). Specifically, the colon is the body's most complex ecosystem hosting most of the 10 to 100 trillion microorganisms of the human gastrointestinal tract (81). *Bacteroides*, *Bifidobacterium*, *Clostridium*, *Enterobacteriales* and *Akkermansia* are the most important genera of the gut.

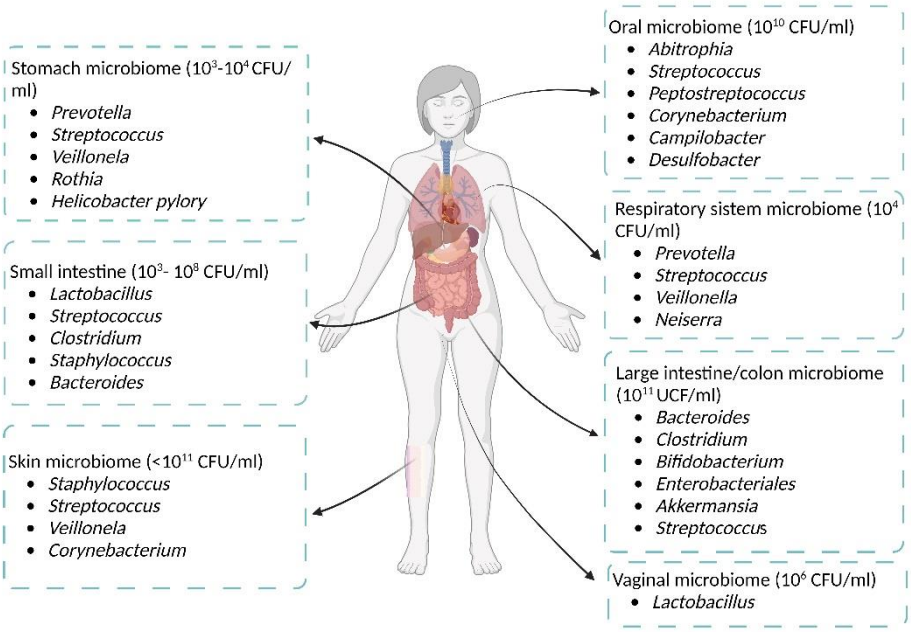

Figure 4. **Genera along the human body**. This figure shows the most representative genera around the body sites and their abundances (75–80). Adapted from Ruan et al., 2020 (82). Created with BioRender.com

The development of new technologies such as metagenomics or metabolomics or the new bioinformatics tools has helped to improve the knowledge about the bacteria inhabiting the human body. These new techniques have allowed to understand that there is a constant relationship between the bacteria that inhabit the human body and different physiological processes associated with health and disease. Extensive works such as The

Human Microbiome Project (81) or the Metagenomics of the Human Intestinal Tract project (83) have been funded, aiming to recollect all the gens and genomes of the microbiome. Other projects have been posteriorly initiated, adding data to these two initial works to complete the metagenome of all the body sites (84).

Considering that the most important human microbiome population is found in the gut and the compelling evidence associating it with health and disease, this thesis is focused on the gut microbiome. The gut microbiome characteristics and its development and host interactions are described below.

3. The gut microbiota

The gut microbiota is the collection of bacteria, archaea, fungi, protists, and viruses that colonize the intestinal tract. It is composed of more than 4000 different bacterial species. The 40% of the gut microbiome is shared among all the populations around the world (85).

As is shown in Figure 5, there is a scientific classification, the "taxonomic classification", for all the species based on the shared characteristics. They are grouped in taxa and assigned to a taxonomic rank. These ranks are shown in the above figure and are Domain, Phylum, Class, Order, Family, Genus, and Specie. The most specific taxonomic ranks are genus and species. The Genus includes a scientific name in Italic and capitalized "*Faeaclibacterium"*. Species are the most specific, and the name is preceded by the genus first letter, as shown in Figure 5, *"C.minuta*" (86).

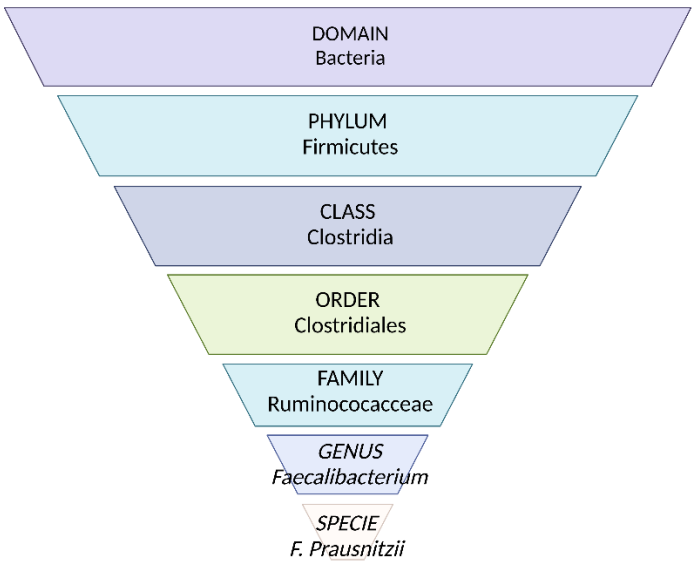

Figure 5. **Taxonomic classification of *Faecalibacterium prausnitzii***. Taxonomically, bacteria are classified according to similar characteristics into different ranks. Created with BioRender.com

The human gut microbiota consists of several different microbes, including archaea, bacteria, eukarya, viruses and parasites. A large study has estimated that there are more than 300000 species in the human gut microflora. A healthy gut environment favours the growth of bacteria from seven divisions (phyla): Firmicutes, Bacteroidetes, Actinobacteria, Fusobacteria, Proteobacteria, Verrucromicrobia and Cyanobacteria (87–89). Firmicutes and Bacteroidetes alone are the most abundant phyla, and they take over 90% of the number of bacteria in the large intestine (90). The most abundant species in the Bacteroidetes phylum belong to the genera *Bacteroides* and *Prevotella*. Moreover, the most abundant in the Firmicutes are the genera *Clostridium*, *Eubacterium* and Ruminococcus. While the phyla are widespread, within a population, genus and species tend to be more subject-specific (90).

3.1 Introduction to methods to analyse the gut microbiota

This section will describe the study of gut microbiota and the different concepts involved in its analysis.

First, it must be understood the difference between microbiota and the microbiome. While the microbiota is all the microscopic organisms that are populating an environment, the microbiome is the collection of genomes and genes of all the microorganisms found in the host's gut and its structural elements and metabolites (91,92). The investigations around these concepts have led to the creation of a new investigatory field, "metagenomics", which mainly examining the genetic material obtained from the environment or living samples. The metagenomics experiments enable the characterisation of the composition of the entire microbiome communities, including the gut microbiome (93).

It must be considered that metagenomics does not provide information on the biological functions or interactions between the host and the gut microbiota. Therefore, there has been a need to integrate other "omic" sciences, such as the metatranscriptome of the metaproteome, to understand the gut microbiota interactions with the host (93). Moreover, metabolomics focus on profiling the metabolites produced by the microbiota and how are interact with the host.

Nowadays, there are several techniques for the analysis of the microbiome. The most common methods are the marked gene analysis which includes the 16S ribosomal RNA gene sequencing or the Shotgun metagenomics (94).

Figure 6 shows the different methods for human microbiome analysis. The 16s rRNA gene obtains raw sequences that pass through quality filters to avoid sequencing artefacts. The resulting filtered sequences are clustered into operational taxonomic units (OUT) (95). The sequences are grouped into OTUS with 97% of similarity (106). OTU is the taxonomic lowest level. Then, a taxonomic identity is assigned for each OUT based on homology to known 16s rRNAs gene databases such as SILVA (96), Greengenes (97) or the Ribosomal Database Project (98) to assign the taxonomical classification of phylum, class, order, family, genus or species. Then, the resulting data is used to quantify the relative abundance of each OUT and the population diversity in each sample (94).

The shotgun analysis includes the analysis of metagenomic, metatranscriptomic and viromic. The metagenomic analysis helps to obtain DNA sequences that can be mapped to a reference genome library or could be used to describe new genomes. The metatranscriptomic analysis is applied to determine the active pathways or genes of the microbiome. It obtains RNA, which is compared to a reference library of pathways, genes and

microorganisms, showing which ones are active in a concrete moment in the human microbiome. Moreover, this technique could help to identify new metabolic pathways (94).

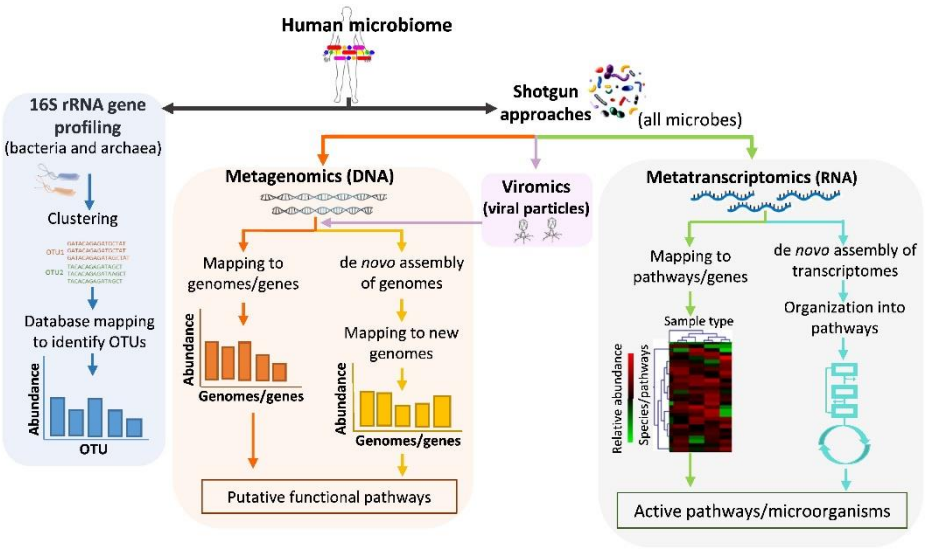

Figure 6. **Summary of the methods for the human microbiome analysis**. Summary of the nowadays most used technics for analysing and interpreting the microbiome. Extracted from Bikel et al., 2015 (94).

3.2 Microbiota related concepts

Sequencing the microbiota genome provides the relative abundances of different species included in one sample. However, other parameters are used to study the complexity of a sample. These other parameters included different samples' diversity, richness, and evenness.

The most analysed parameters in the first stages of the microbiota analysis are alpha and beta diversity.

- Alpha and Beta diversity

Alpha diversity of a sample could be defined as the number of different organisms present in a sample. Beta diversity can be understood as the similarity or dissimilarity between two or more communities. Besides this, the gamma diversity reflects the total variability in all the analysed faecal samples (Figure 7).

Alpha diversity summarizes an ecological community's structure concerning a sample's richness and evenness. The higher the index, the more diverse are the species in the samples (99).

- **Richness**: represents the number of different microorganisms that it can be found in a sample. It could be assessed by a different index such as the **CHAO1** index, and the relative observed number of species (**OBS**) or the **ACE** richness index.
- **Evenness**: representing how the microorganisms are distributed, for instance, if some groups are overrepresented. It could be assessed by **Shannon's index or Simpson's index**.

Beta diversity compares the composition of different samples and explains the differences in the microbial composition between them. Usually, the beta-diversity is completed with a graphical image helping interpret the differences. The most used parameters to calculate it are Jaccard's or Bray Curty's distance calculations and the Principal Coordinate analysis Plot for the visualization. The most used one is the principal coordinates analysis (PCoA) which classifies the samples by all the profiles, or the principal component analysis, which takes care of different parameters such as abundance (100).

A low microbiota diversity has been described as a common characteristic in some diseases such as Crohn's disease (101), inflammatory bowel disease (IBD) (102), colorectal cancer (103), T2D, and obesity (104).

- ## Dysbiosis

Healthy gut microbiota has not been defined yet, but it has been established that it could be that microbiota that comes from individuals with no signs of disease. The structure includes dominant bacteria Bacteroidetes and Firmicutes, followed by Proteobacteria and Actinobacteria. Dysbiosis could be defined as the changes in the composition of resident communities compared to those found in healthy individuals that may shift their function to disease (105,106).

- ## The resilience of the gut microbiota

Resilience is the propriety of an ecosystem to maintain its state and recover from perturbations. Diet, infections, or antibiotics can alter the gut microbiota. Kummar et al. (107) summarized that resilient microbiota would return to its original equilibrium after the perturbation, and a non-resilient microbiota will shift to an altered state of "dysbiosis" and contribute to the development of obesity and its comorbidities.

- ## Probiotic

The term probiotic was introduced in 2001 as " live microorganisms that, when administered in adequate amounts, confer a health benefit on the host". The beneficial effects of the probiotics create a more favourable gut environment linked to the maintenance of a healthy digestive tract and immune system (108).

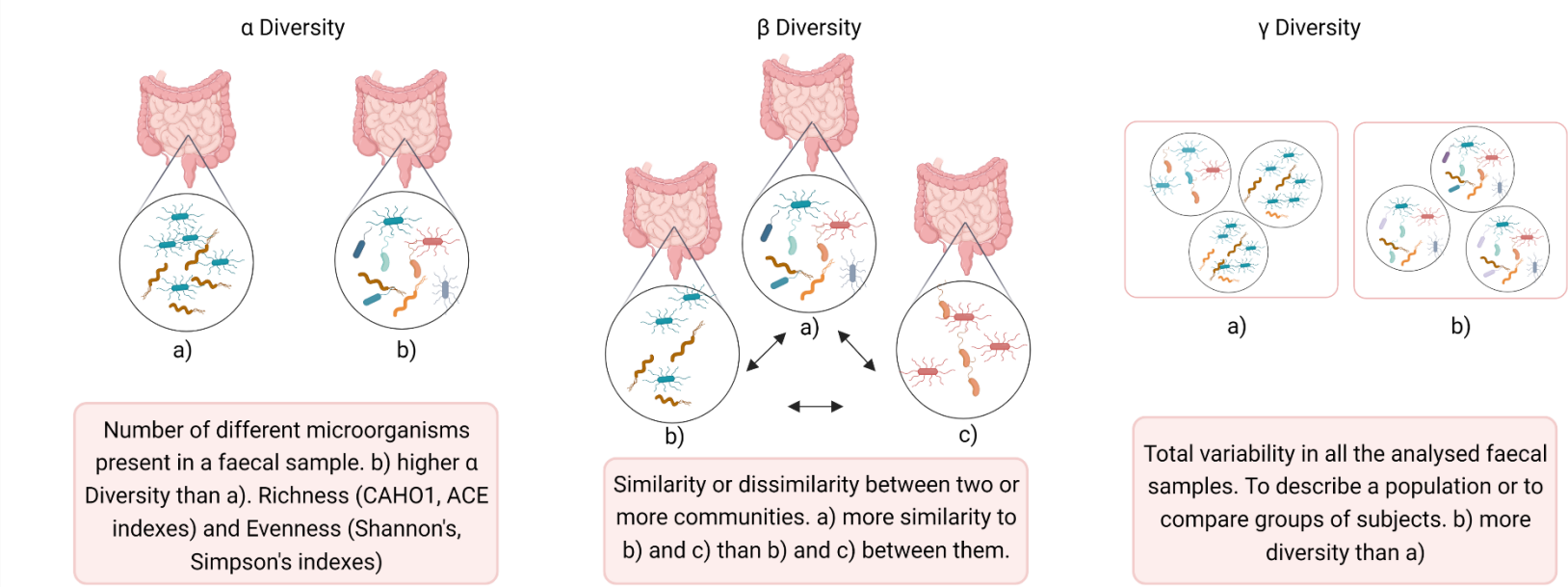

Figure 7. **Diversity of the microbiota samples**. This figure summarizes the three most used metrics for microbiota analysis. Created with BioRender.com

- Prebiotic

A panel of experts in 2016 introduced the definition of prebiotic. It was defined as "a substrate that is selectively utilized by host microorganisms conferring a health benefit". Some fermentable carbohydrates have been reported as presenting a prebiotic effect, but the most extensively studied are the non-digestible oligosaccharides fructans and galactans (109).

- Postbiotic

In 2019, postbiotic was defined as the "preparation of inanimate microorganisms and/or their components that confers a health benefit on the host". According to the authors of the consensus statement, "Inanimate" refers to the fact that live microorganism was present but have now been killed, without implying a loss of function. Postbiotics are involved in the modulation of resident microbiota, enhancement of epithelial barrier, modulation of immune responses, modulation of metabolic responses and systemic signalling via the nervous system (110).

- Enterotypes

It has been suggested that everyone is characterized by clusters of bacteria forming an enterotype. Nowadays, three dominant bacteria clusters in the human gut are recognized: Bacteroides (enterotype I), Prevotella (enterotype II) and Ruminococcus (enterotype III). Each enterotype is composed of different bacteria classified by their functions. There is controversy in separating the enterotypes because there is a considerable variation of the gut microbiota along with life. It has been suggested that Enterotype I drive energy by glycolysis while enterotypes II and III can also degrade mucin glycoproteins. The three enterotypes are associated with different dietary patterns described in the following sections (111).

3.3 Functions of the gut microbiota

The gut microbiota is involved in several functions of the human body through different mechanisms, such as maintaining the gut integrity or shaping the intestinal epithelium, harvesting energy, protecting against pathogens, regulating host immunity, and regulating lipid and glucose metabolisms (85). However, the gut microbiota changes throughout life due to different circumstances and could trigger a variety of different diseases, including Crohn's disease, inflammatory bowel disease (101,112), colon cancer (103,113), non-alcoholic fatty liver disease (114) and obesity (88) or cardiovascular disease (115,116). This section will summarize the most relevant proposed functions of the gut microbiota in the host metabolism, although many others have been reported.

- ### Nutrient metabolism

One of the main functions of the gut microbiota is to “digest” the undigested products from the diet that arrive in the colon (117). Colonic bacteria hold a saccharolytic activity that confers the ability to ferment non-digested carbohydrates obtaining beneficial products such as SCFA (118–120). However, bacteria could obtain energy from other sources resulting in other non-healthy metabolites such as indoles or amines (121).

Some bacteria have a proteolytic activity that can degrade the proteins from the diet, endogenous proteins, or mucins to small peptides, SCFA, branched-chain fatty acids (BCFA) and gas such as ammonium, CO_2 and H_2S (122). Usually, the degradation of proteins by the gut microbiota has negative connotations producing non-beneficial products.

There is a small proportion of fatty acids that arrives in the colon. It has been reported that gut microorganisms possess lipases that can degrade the diet TG and phospholipids into free lipids (121). Moreover, gut bacteria can influence lipid metabolism by metabolising bile acids, which facilitates digestion and absorption of dietary lipids, fatty lipids, cholesterol and fat-soluble vitamins (123).

- Vitamin synthesis

Some gut microbiota species have been associated with synthesising vitamin K and other vitamins included in the B group, such as biotin, cobalamin, folates, niacin acid, pantothenic acid, pyridoxine, riboflavin and thiamine (124).

- Immunomodulation

It has been described that, since early infancy, the gut microbiota plays an important role in the modulation of the immune system. Several mechanisms involve the interplay between gut microbiota and local and systemic immune systems. Making an overview, there is a cross-regulation between gut bacteria and the immune system that starts with the capacity of the immune system to tolerate beneficial commensals but prevent the growth of pathogens (117). Gut microbiota can also regulate the immune system via the synthesis of antimicrobial proteins and local immunoglobulins, controlling the overgrowth of bacteria and preventing a systemic immune response (125). Unregulated immune responses could trigger pathologies such as IBD or metabolic alterations. On the other hand, LPS are a major component of the membrane of gram-negative bacteria and a well-known pathogen. As previously mentioned, after the gram-negative bacteria release LPSs by outer membrane vesicles (126) or due to normal growth or antibiotic exposure (127), it can initiate an immune response and trigger an inflammatory response. (58).

Beyond this mechanism, gut microbiota can protect against pathogens without activating the immune system in different ways (128). For instance, certain bacteria can secrete molecules with bacteriostatic or bactericidal activity (129). Moreover, SCFA produced by the host microbiota could inhibit the growth of pathogens such as *Escherichia coli* (130).

3.4 Gut-microbiota derived metabolites

The gut microbiota-derived metabolites have gained importance during the last years because of their implications for the host metabolism and immune system (58,131,132). There are several metabolites and products associated with microbiota. Firstly, metabolites produced by microbiota foods fermentation, such as SCFAs, BCFAs or indoles. Secondly, metabolites such as secondary bile acids that are obtained from modifying previous molecules from the host. Moreover, components of the bacteria which interact with the host, such as LPS (133). Here the most representing metabolites are exposed, especially the SCFAs and the bile acids and their interactions with the host physiology. However, as represented in Figure 8, several other metabolites such as ethanol, gases and some organic acids could also interact with the host (134).

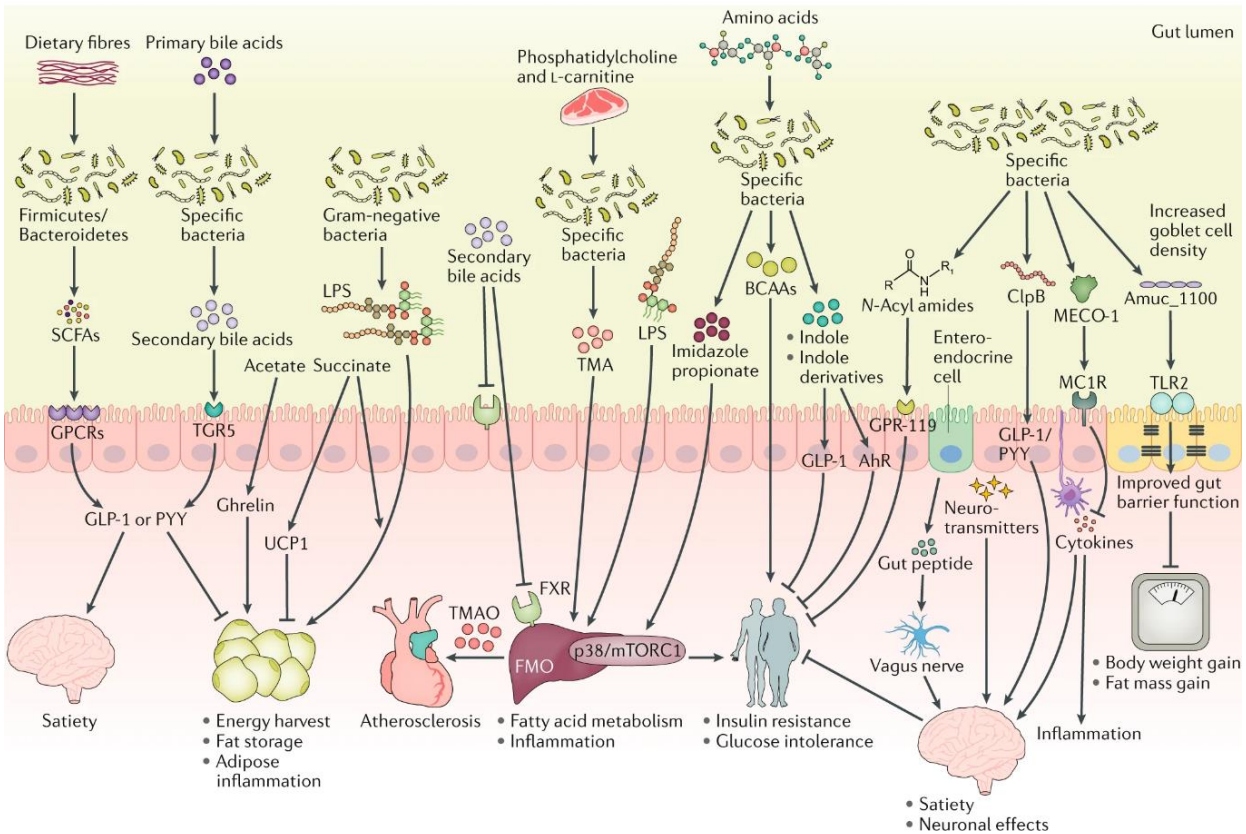

Figure 8. **Summary of gut microbiota derivates.** An overview of the most relevant metabolites derived from the gut microbiota, involved in the host homeostasis. Obtained from Yong Fan et al., 2020 (135).

- Short-chain fatty acids

The SCFAs are the primary end products of the fermentation of prebiotics, including plant-derived oligosaccharides, polysaccharides, resistant starch, and inulin by the SCFA-producing bacteria. Acetate, propionate, and butyrate are the most abundant SCFAs detected in faeces, representing 95% of all the SCFAs, and presenting a relatively constant proportion of 60:20:20, respectively (118,122,135). SCFAs provide 10% of the host's daily energy requirements and are involved in different gut health functions (136). For instance, SCFA promotes maintaining the integrity of the intestinal barrier, producing mucus, preventing inflammation, and reducing the risk of colorectal cancer. Furthermore, SCFA regulates the lipid and glucose metabolisms and the release of hormones such as leptin or insulin (137–140).

Butyrate is the SCFA currently identified as most important. It is the main energy source for the colonic cells; it exerts immuno-modulatory effects and stimulates the maintenance of tight conjunctions (141). Propionate could be used as a glucose precursor in the colon and enhances gut anorexigenic hormone release such as peptide YY (PYY) and glucagon-like peptide-1 (GLP-1) (142,143). However, it is mainly sent to the liver, where it contributes to gluconeogenesis (144). The most abundant SCFA is acetate, which is involved in cholesterol synthesis and liponeogenesis and, together with propionate, is involved in appetite regulation (143). SCFA-producing bacteria are principally those species belonging to the Clostridium clusters from Firmicutes phyla as *Clostridium leptum*, *Faecalibacterium prausnitzii*, or *Roseburia spp* (145). Although some bacteria, such as the *Bifidobacteria* genus, do not produce SCFA, they contribute to its production via metabolic cross-feeding, breaking down the fibre, and providing necessary substrates for the SCFA producing-bacteria, such as oligosaccharides or lactate (146).

SCFA in the host metabolism act mainly through coupling selective G-protein-coupled receptors (GPR), such as GPR4, GPR43, GPR119 and GPR109A, as summarized by Cunningham et al. (104). It has recently been described that GPR41 and GPR43 are the most important SCFA receptors and have been renamed free fatty acid receptor 3 (FFAR3) and free fatty acid receptor 2 (FFAR2) respectively. The affinity of the SCFA differs according to the different FFARs (104). Different effects have been described from the binding of the SCFAs with their specific receptors: promotion of the secretion of GLP-1 increasing insulin secretion (143); reduction of appetite due to the stimulation of the secretion of PYY, and the inhibition of neuropeptide Y (NPY) (142); improvement of the gut barrier homeostasis (137) and other functions, such as the regulation of the inflammatory response (147). The most important functions are deeper described below and summarized in Figure 9.

SCFA in the maintenance of the gut barrier

As represented in Figure 10, the gut barrier is composed of different layers with different functions. There is a thick external mucus composed mainly of glycosylated mucin proteins. The internal part of the mucus layer hosts the gut microbiota and some immune components such as immunoglobulins (148). Next to the mucus layer, there is a second layer mainly composed of glycosylated proteins that prevent the interaction of pathogenic microorganisms with the epithelium (148). A single layer of epithelial cells separates the lumen from the systemic circulation, known as the gut epithelium. Although mainly composed of enterocytes, the gut epithelium also contains other specialized cells such as Paneth or Golber cells. The cells of this epithelial layer are connected by transmembrane proteins such as tight junctions, adherent junctions or gap junctions. Beyond the epithelium is the lamina propria, which contains immune cells (149).

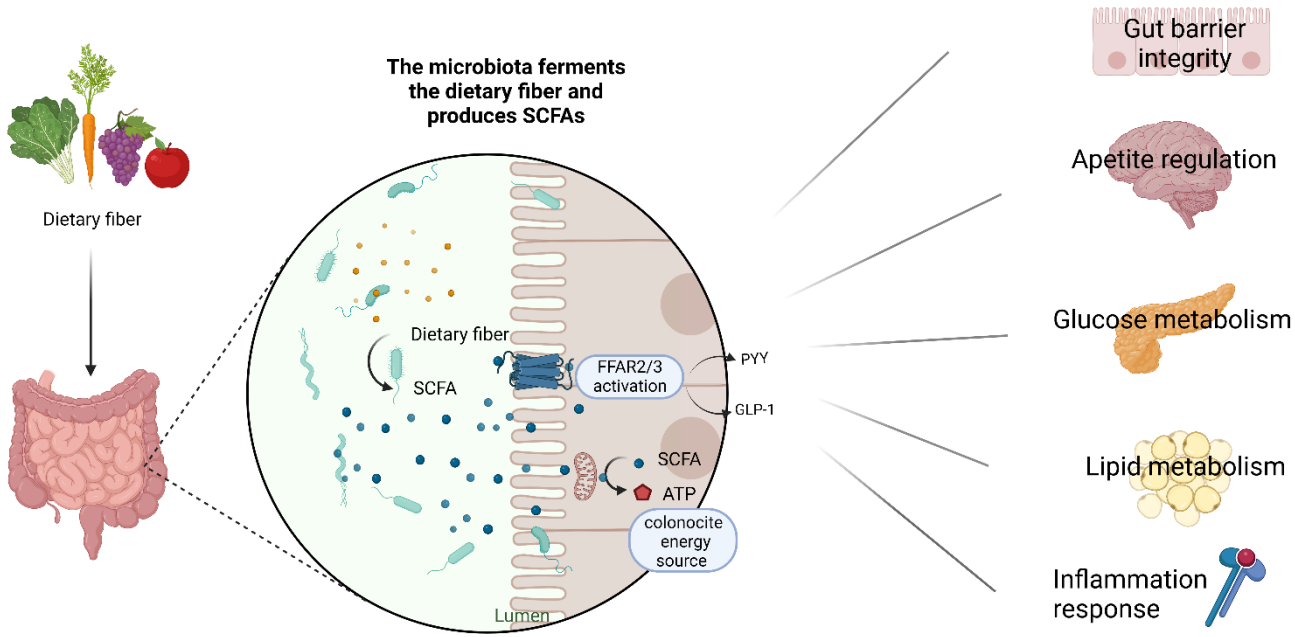

Figure 9. S**ummary of SCFAs functions in human health**. SCFAs can be used as an energy source by colonocytes. They can be involved in different functions: lipid metabolism stimulating leptin production; appetite regulation by the stimulation of PYY and GLP-1; lipid metabolism stimulating the secretion of insulin; maintenance of the gut barrier integrity through the stimulation of mucin secretion and the maintenance of junction proteins; release of anti-inflammatory cytokines involved in the immune response. SCFA: short chain fatty acids; PYY: peptide Y; GLP-1: glucagon-like peptide 1; ATP: adenosine triphosphate; FFAR2/3: free fatty acid receptor 2 and 3. Created with BioRender.com

A disruption in the intestinal barrier and its junctions increases intestinal permeability, leading to the translocation of harmful components such as LPS to the systemic circulation, known as metabolic endotoxemia. Once in the blood, LPS triggers an immune response stimulating the production of proinflammatory cytokines such as IL-6 or TNFα and the activation of macrophages (150). It has been shown that a continuous infusion of LPS leads to a pro-inflammatory state in specific tissues. As has been mentioned before, cardiometabolic alterations such as type 2 diabetes, atherosclerosis or fatty liver disease have been associated with this proinflammatory state in specific tissues (151).

Between the functions of the SCFAs in the human body, there is the maintaining of the gut barrier integrity. One proposed mechanism is its ability to regulate tight junctions stimulating fatty acid beta-oxidation at the colonocytes. The fatty acid beta-oxidation triggers a hypoxic environment that induces the hypoxia-inducible factor 1, which upregulated the expression and function of junction proteins. However, suppose there are limited SCFAs, due to poor dietary fibre intake. In that case, the colonocytes need to switch to non-consuming oxygen glycolysis to obtain energy, create a non-hypoxic environment, and lose the expression of junction proteins triggering the immune response (139).

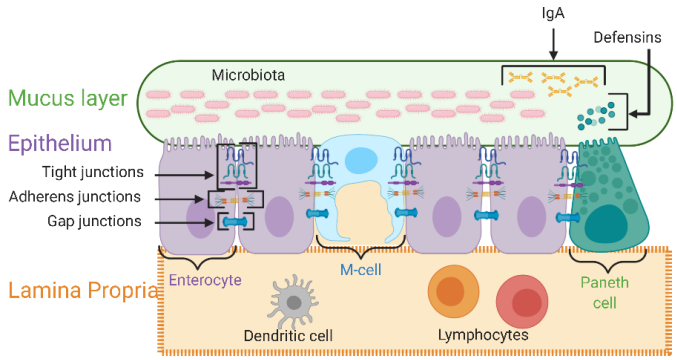

Figure 10. **Key layers of the intestinal barrier**. Extracted from Sharma et al. 2020 (149).

SCFA in appetite regulation

SCFAs can modulate the food intake by activating the FFAR2 and FFAR3 in the gut. The activation of FFAR2 and FFAR3 triggers the release of GLP-1 (143) and PYY (147). GLP-1 regulates blood glucose levels indirectly by the stimulation of insulin and inhibition of glucagon secretion by the β pancreatic cells. PYY acts through the nervous system as an anorexigenic hormone and could stimulate glucose absorption leading to the satiety state. On the other hand, SCFAs binding with FFAR3 in the adipocytes stimulates leptin production, which acts directly in the hypothalamus reducing the food intake by inhibiting the NPY (147). Supporting this mechanism, it has been reported that supplementation with butyrate is related to the prevention of diet-induced obesity in mice and may be mediated by appetite reduction (152,153). In a complete work, Chambers et al. (154) demonstrated that propionate significantly stimulates PYY and GLP-1 secretion from colonic cells *in vitro.* In the same work (154), they observed that the acute supplementation with inulin-propionate ester significantly reduced food caloric intake by more than 10% in a group of healthy adult participants.

SCFA in glucose and lipid metabolism

Blood glucose is obtained by gluconeogenesis and the consumption of blood glucose. The regulation of blood glucose is a very complex mechanism mainly performed by glucagon and insulin, both hormones secreted by the pancreas.

SCFAs are involved in glucose homeostasis by coupling to FFAR2 and FFAR3. The activation of FFAR2 and FFAR3 activates GLP-1 release from the enteroendocrine L-cells (142). GLP-1 stimulates insulin release and suppresses the glucagon secretion of the pancreatic cells. It has been reported that T2D patients showed significantly lower levels of GLP-1 than healthy individuals (155). This fact has been confirmed in impaired glucose tolerance

in adults (156) and adolescents with obesity (157). Beyond the stimulation of GLP-1 secretion, Vadder et al. (158) added that the binding of butyrate to the GPR41 led to intestinal gluconeogenesis. Moreover, propionate activated intestinal gluconeogenesis via a gut-brain process after binding with FFAR3 (158). SCFAs can increase glucose uptake through the stimulation of the expression of the glucose transporter type 4 (GLUT4) in skeletal muscle tissue (159) via the activation of AMP-activated protein kinase (AMPK). Another proposed mechanism is that SCFAs can act in the hepatocytes, decreasing glycolysis and gluconeogenesis and stimulating glycogen synthesis (144).

Regarding lipid metabolism, it has been exposed that acetate can inhibit lipolysis by activating FFAR2 in the adipose tissue in animal models. Ge et al. (160) reported in a mouse model that activating FFAR2 by acetate may reduce plasma free fatty acids. Moreover, in a model of human incubate adipocytes, acetate was established as the responsible for the antilipolytic effects of SCFA (161).

SCFA role in inflammation

Obesity is characterized by intestinal and systemic inflammation. As previously summarized, the epithelial barrier's impairment could lead to an inflammatory state (150). The SCFAs can modulate the inflammatory response by several mechanisms. As previously described, one of them is the stimulation of barrier integrity (139). The binding of SCFAs, especially butyrate, with FFAR2 or FFAR3 also inhibits histone deacetylases (HDACs), downregulating the expression of pro-inflammatory cytokines, including IL-6, TNFα and upregulates anti-inflammatory cytokines, such as IL-10 (140,162).

- ## Secondary Bile acids

Bile acids are synthesized in the liver from cholesterol. In hepatic tissue, bile acids are conjugated to glycine and taurine or sulphate before being secreted. These conjugated bile acids are cholic acid and chenodeoxycholic acid. After its synthesis, the primary bile acids are secreted and stored in the gallbladder. With food intake, the primary bile acids are released into the duodenum to facilitate the digestion and absorption of lipids, fatty acids, cholesterol, liposoluble vitamins and other hydrophobic components (163). The main part of the bile acids is reabsorbed in the distal ileum and transported back to the liver via enterohepatic circulation, where they are involved in cholesterol and bile acid synthesis. Around 1-5% of the primary bile acids that arrive in the colon can be transformed into secondary bile acids by the colonic bacteria (123,124). The secondary bile acids present antimicrobial activity and cytotoxicity and have been related to increased risk of colorectal cancer and cholesterol gallstone formation. The microbial transformation of bile acids could be done by the action of the bile salt hydrolase present in certain bacteria genera such as *Bifidobacterium*, *Bacteroides* or *Clostridium and Lactobacillus and Listeria* (164). The deconjugate bile acids, which can be re-absorbed and returned to the liver to start the circle again. The variation of the bile acid composition by the gut microbiota affects the nutrient absorption and the metabolic status of the host (124).

- ## Tryptophan derivates

Tryptophan is an essential amino acid obtained only from the diet. In the gastrointestinal tract, some bacteria such as *Lactobacillus spp, Clostridium or Ruminococcus* can degrade tryptophan into different indoles, including indole propionic acid (IPA) indoleacetic acid, indole ethanol, indole aldehydes and indole acrylic acid, and also to tryptamine (165,166). These end products can

act as signalling molecules that modulate enteroendocrine enterocyte function, maintain epithelial integrity, stimulate the immune system, and regulate glucose, lipid metabolism, and food intake (166–168). In this line, *in vitro* studies have shown that indoles can modulate the GLP-1 release from the enteroendocrine cells, increasing its secretion in a short exposure (168). A study by Toumainen et al. (169) observed that higher IPA was inversely associated with the incidence of T2D during a mean of 7 years of follow-up in a cohort of participants with impaired glucose tolerance. Moreover, they showed a positive correlation between IPA and dietary fibre intake and lower low-grade inflammation.

- Trimethylamine- N-oxide (TMAO)

Lyases in some gut species can degrade choline, carnitine and L-carnitine in red meat, eggs, dairy and seafood into trimethylamine (TMA). The portal circulation transports TMA to the liver, which is oxidized into trimethylamine-N-oxide (TMAO). Several bacteria included in Firmicutes, Proteobacteria, and Clostridiales have been defined as TMA-producers. It has been reported that the production of TMAO by metabolising TMA in the liver depends on the composition and diversity of the gut microbiota (170). A recent systematic review showed that higher circulating TMAO might be an independent factor for the prediction of cardiovascular events and mortality (171).

There are different mechanisms by which TMAO could affect cardiovascular health. However, those mechanisms have not been completely elucidated yet. TMAO may increase the production of pro-inflammatory cytokines such as TNF-α and IL-1 and may decrease anti-inflammatory cytokines such as IL-10 (138). The potential consequences of these alterations are atherosclerosis development, which has been widely studied in previous works (138,171), T2D, and an alteration of the bile acid metabolism (172). As the renal pathway

must excrete TMAO, it has also been associated with chronic kidney disease (173).

- ### Branched-chain amino acids

The BCAAs, leucine, isoleucine, and valine are essential amino acids that can be synthesised by gut bacteria such as *Clostridium,* Bacteroides or *Ruminococcus* (174,175). Human studies have observed that BCAA concentrations are associated with higher BMI, T2D and cardiometabolic diseases (131,176). Liu et al. (175) observed that serum BCAA was increased in adults with obesity and the gut microbiota of these individuals showed a higher capacity to produce these aminoacids compared to the participants with normal weight.

BCAAs are used as substrates for protein and BCFA synthesis and can also act as a signalling molecule affecting glucose metabolism, among others. It has been hypothesized that BCAAs are associated with insulin resistance mediating the activation of the “mammalian target of rapamycin complex” (mTORC1). This activation leads to a decrease in glucose uptake; therefore, it contributes to insulin resistance (177).

3.5 Modulators of the gut microbiota

Gut microbiota develops during the host's lifelong. This section will sail through different factors that could be involved in the development and modulation of gut microbiota (Figure 11).

In early life, the gut microbiota is more unstable than in adulthood and evolves until 3-5 years of age, when it becomes similar to the microbiome of an adult (178). It was hypothesized that the womb, placenta, amniotic fluid, and meconium were sterile for a long time. In the last years, several reports appeared suggesting that there are bacterial communities colonizing these sites during pregnancy (179,180). However, some authors concluded that there was no evidence to support the existence of a placental microbiome (181). Some authors suggested that the most robust evidence against microbiomes in the fetal environment is the possibility of obtaining germ-free animals via cesarean sections (182).

The possible prenatal colonization is still debatable, but in any case, the biggest colonization begins just after birth. The composition and development of the infant's gut microbiota can be influenced by several prenatal factors, such as maternal health status, diet, weight, smoking, and antibiotics during pregnancy or the delivery method (183). Different afterbirth factors have been linked to the development of the infant microbiome, including the gestational age at birth, the antibiotic usage, the geographical location or the presence of pets or siblings. However, the early infancy diet has been described as the main afterbirth modulator (184).

- Perinatal factors

Several perinatal factors are involved in the future development of the gut microbiome. It has been reported in both animal (185) and human studies (185) that maternal diet can modulate the gut microbiome of children. Furthermore, the maternal diet a modify the composition and diversity of the human milk microbiota, which is an essential modulator of the infant's gut microbiota (186,187). Beyond the diet, it has been reported that excessive weight gain during pregnancy could be related to the concentrations of C*lostridium* and low concentrations of Bacteroides during early life (188).

The gestational age has also been pointed out as a modifier of bacterial diversity and composition after birth (87). Preterm babies showed low diversity and increased abundance of pathogenic bacteria, such as Proteobacteria, independently of the delivery mode or the formula feeding (189,190). However, gut microbiota modulates over time, and it has been observed that the gut profile of preterm children did not differ from those of term newborns after a 4-month follow-up (190).

The mode of delivery is an important modulator of the gut microbiome in the neonatal period (191). In a vaginal birth, the neonate interacts with the microbiota present in the birth canal, such as *Lactobacillus and Bifidobacterium* (87,192). However, in a cesarian section, the newborn stays in contact with the mother's skin microbiome, characterized by *Staphylococcus, Corynebacterium, or Veillonela* (191,193,194).

Another significant modulator of the gut microbiota is diet since birth. Breastfeeding is the "gold standard" of nutrition for the newborn. It is associated with several benefits in children, such as preventing gastrointestinal alterations, atopic dermatitis or obesity (195–197). Most of the benefits associated with breastfeeding are linked to its composition.

Significantly, human milk oligosaccharides, the third most abundant solid components in breast milk, are the primary substrate for beneficial bacteria such as *Bifidobacterium.* The result of its fermentation is the production of SCFAs and their beneficial effects on the child's metabolism (198).

- Age

Gut microbiota is changing along with life and increases diversity and richness. Toddlers are enriched in Actinobacteria, and as previously mentioned, they present a less stable composition. During adulthood, there is an increase in the abundance of Firmicutes, and during the elderly, a decrease in the diversity and a decrease of health-related bacteria such as *Bifidobacterium* (199). (Figure 12).

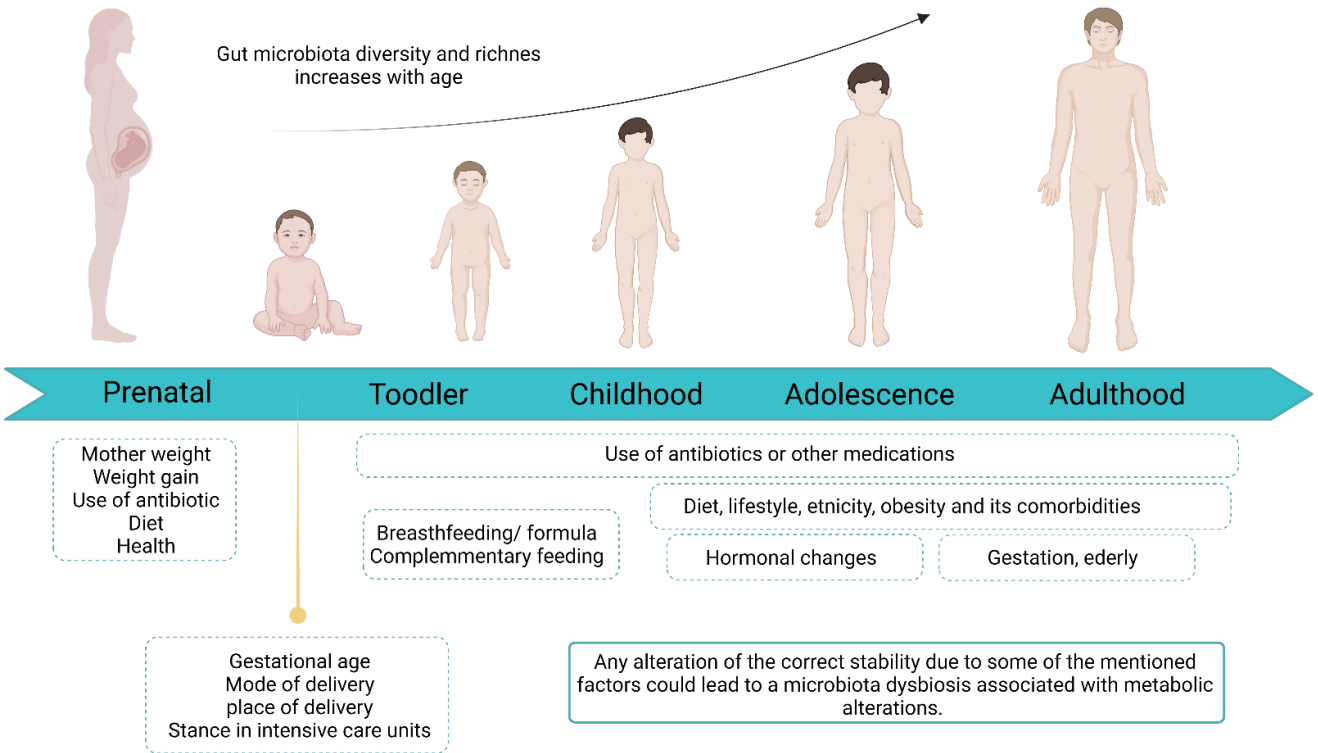

Figure 11. **Factors influencing the gut microbiota along life.** Created with BioRender.com

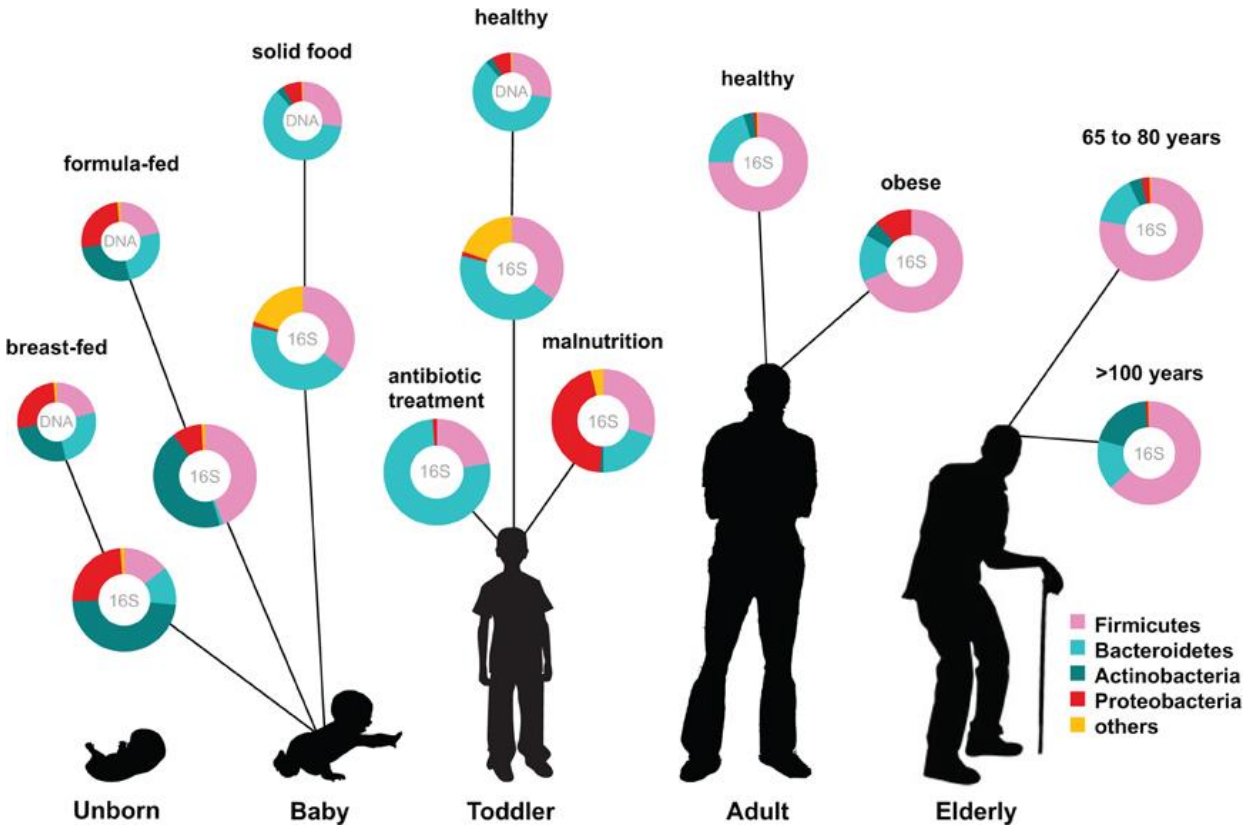

Figure 12**. Gut microbiota changes along life stages.** This figure is an overview of the most abundant phylum's relative abundance of the gut microbiome composition in different stages of human life. Figure extracted from Ottman, et al. 2012 (284).

- ## Geography

A recent review (200) characterized the composition of the pediatric gut microbiome using data worldwide. They observed that as in adulthood, in children, the most abundant representants were shared among all the populations: Firmicutes, Bacteroides, Actinobacteria and Proteobacteria, followed by Verrucomicrobiota, Tenericutes and Fusobacteria. However, they observed that specific characteristics were associated with specific areas. For instance, Firmicutes could vary from 31.6% in African children to 67.7% in European children or 69.0% in North American children. Moreover, was observed a decrease in the diversity of the microbiome from the rural and farming areas to the industrialized urban zones (200,201).

In specific populations, Yatsunenko et al. (202) observed that the gut microbiota of Amazonian and Malawian adult populations presented higher diversity than the United States adult population, a fact that is considered a marker of health status. They suggest that this alteration seems to be present since childhood. In children populations, Filippo et al. (203) compared the faecal microbiota of European and African children observing lower proportions of Firmicutes and a higher abundance of Bacteroidetes in African children. These regional differences could be partly explained by differences in diet and lifestyle factors which will be discussed below.

- ## Physical activity

Sports practice is associated with changes in the gut microbiota composition and the bacterial products that tend to have a healthy profile with beneficial effects for the host in adulthood (204,205) and childhood (206). Not only does the practice of sport by itself modifies the gut microbiota, but also the intensity. Liang et al. (207) observed in a group of professional athletes that those who obtained higher qualifications presented higher richness (number

of bacteria) and diversity (number of different bacteria) than those who presented lower qualifications. Following this trend, in the adult population, it has been reported that active participants resented higher *Akermansia muciniphyla* or Christensenellaceae family than those participants with sedentary behaviours, both reported as health-promoting bacterias (206,208). However, the mechanisms by which gut microbiota could be modulated by physical activity are still to be elucidated

- Dietary patterns

Previously, in this thesis, in the section dedicated to the gut microbiota functions, it has been summarized how the gut microbiota can affect human health due to the different end products from the nutrient metabolism. Dietary macro and micronutrients are the energy source of gut microbes. Specific species have higher proteolytic activity, such as *Bacteroides* (122). On the other hand, dietary carbohydrates increase the SCFA-producing species such as *Clostridium leptum*, *Faecalibacterium prausnitzii*, or *Roseburia spp* (145). Moreover, dietary fats may indirectly impact the gut microbiota, promoting increased bile-tolerant bacteria such as *Clostridium* or *Bacteroides* (164). Although the macronutrients are the main modulators of the gut microbiota composition, micronutrients such as polyphenols or different vitamins also have to be considered for their beneficial effects on the gut microbiota composition (209). This evidence indicates that the diet's composition can shape the gut microbiota.

Previous studies in the adult population have reported that after an increase in fibre intake, *Bifidobacterium* genus abundance is increased due to its high adaptation to fibre-rich diets (210–213). Yanq Qi et al. (209) reviewed the different effects of the fibre. They observed that besides Bifidobacterium, the galactooligosaccharides, a fermentable fibre, could increment some of the

health-beneficial bacteria such as *Akkermansia muciniphyla*, *Fecalibacterium spp*. *Prevotella, Roseburia* or *Lactobacillus spp*. Interventional studies on children are scarce. However, Nicolucci et al. (214) found that a 16 weeks-intervention with a prebiotic was associated with a significant increase in *Bifidobacterium spp* and a decrease in *Bacteroides vulgatus* abundances.

One factor that should be considered in the analysis of the influence of diet on the microbiota is that our diet is very complex and does not only include a single food or macronutrient. It is a combination of foods together with customs and cooking methods of these same foods. Zuo et al. (215) observed that changes in dietary patterns are responsible for more than 50% of the microbiota variability. In addition, it must also be considered that gut microbiota itself can modify how the human body absorbs and metabolizes the nutrients that reach the intestine (216–218).

Different works have observed that rural diets are associated with Prevotella enterotype while Bacteroides is predominant in Western diets in adults (202,219) and children (203,220). Rural diets are characterized by low animal fat and protein intake and are rich in starch fibre and plant protein. In contrast, a Western diet is characterized by high animal protein, fats and sugars consumption and is associated with a worse metabolic profile and obesity (221).

Vegan and vegetarian diets are, as rural patterns, rich in fibre and low in animal protein and fat. Several studies have analyzed the different compositions of gut microbiota of vegans and vegetarians compared to omnivores. Trefflich et al. (222) in 2019 summarized that Bacteroidetes and Actinobacteria phyla are associated with higher fibre and low fat intake, while taxa belonging to Firmicutes and Proteobacteria were inversely associated. The genus *Prevotella* was increased in vegetarian and vegan diets compared to omnivore diets (223).

The Mediterranean diet is characterized by high amounts of fruits and vegetables rich in fermentable carbohydrates (224). Several studies reported that adherence to the Mediterranean diet resulted in modulation of the gut microbiota, with an increase of health-related bacteria such as *Bifidobacterium*, *Prevoella*, *Roseburia*, *Bacteroides* or *Faecalibacterium* and a decrease of non-health-related bacteria such as *Ruminococcus* (225–227).

Beyond specific associations, several studies have been performed on adults and children (63,210,212,214,225), showing how changes in dietary patterns could impact the gut microbiota, which may contribute to alleviating metabolic deterioration (228).

- ## Antibiotic and drug therapy

The gut microbiome can metabolize drugs by the production of enzymes and could influence the host's capacity for drug metabolism affecting, for instance, hepatic function (65,229).

It is well known that antibiotic therapy could modify the composition and functionality of the gut microbiota producing long-term effects. In neonates, antibiotic therapy could alter bacterial development inducing a higher risk of allergy, inflammatory bowel disease or obesity (230–232). Recently, it has been also added that commonly used drugs such as non-steroidal anti-inflammatory drugs could affect the microbiota composition. (229) For instance, it has been observed that the treatment with ibuprofen causes an increase in the *Propionibactearciae* or *Pseudomonaceae* and *Rinkenellaceae* species compared with the nonusers or the users of another drug such as naproxen (229).

In summary, the gut microbiota is modulated by non-modifiable and modifiable factors. The following sections will review the interaction between gut microbes and the host and how it could affect cardiometabolic health.

3.6 Dysbiosis, obesity and metabolic alterations

As previously mentioned, several exogenous and endogenous factors may affect the gut's microbial composition. The correct function of the gut microbiota relies on a stable composition that in humans consists of bacteria from the phyla Bacteroides, Firmicutes, and Actinobacteria (85). As previously mentioned in microbiota-related concepts, an imbalance of this microbial composition and a shift to disease status can be described as dysbiosis (106). Several metabolic activities may be affected by dysbiosis, resulting in obesity and metabolic disorders such as dyslipidaemia, diabetes, non-alcoholic fatty liver disease or hypertension in both adults (233,234) and children (235,236). In an extensive review, Ballini et al. (88) suggested that mechanisms by which gut microbiota could be associated with obesity and related disorders are: alterations of the gut barrier permeability, the immune system functionality or the production of signalling molecules involved in the host's metabolism. Energy harvesting from food refers to the gut microbiota's capacity to extract energy from aliments that the human digestive process has not. Therefore, higher bacterial energy extraction may indirectly lead to a higher energy supply from the diet to the subject (237,238).

The first observations about the differences in gut microbiota in obesity were in mice. Ley et al. (239) observed that the ob/ob mice exhibited different microbiota characteristics than their lean homologous. In that work, the obese animal model presented a significant reduction in Bacteroidetes phyla and an increase in Firmicutes. The authors suggested that de ob/ob mice were more efficient in harvesting energy from food than the lean mice. However, the most relevant work to prove causality between gut microbiota and obesity was performed by Turnbaugh et al. (238). In this study, they observed that the transference of gut microbiota from obese mice to germ-free mice increased

the total fat mass and body weight of the germ-free mice. They observed an increased dietary calorie use and extraction and a higher concentration of faecal acetate and butyrate after the faecal transplantation.

The gut microbiota is involved in several pathways related to metabolic health. Weng et al. (240) demonstrated that a group of microbiota-depleted mice presented lower weight loss after a caloric restriction intervention. Moreover, they observed that mice with altered gut microbiota presented a worse metabolic profile, including elevated fasting blood glucose and total cholesterol, higher body fat and lower metabolic rate than the control group.

Studies in humans, mainly in adult subjects, try to identify a "healthy" microbiota profile associated with leanness and good metabolic status. Most authors agree that lower diversity and richness are characteristic of the gut microbiota of people with obesity (241–244). Le Chatelier et al. (67) conducted a study with 169 obese and 126 non-obese individuals. The authors observed that those with lower bacterial richness presented more overall adiposity, insulin resistance, dyslipidaemia, and more inflammation than those with higher bacteria richness.

Several works of different populations and ages have added that specific taxa, such as Firmicutes, are more abundant in subjects with obesity. In contrast, others, such as Bacteroidetes, tend to be higher in normal-weight populations. The alteration of the Firmicutes: Bacteroidetes ratio has been suggested as a characteristic of obesity in several studies, although there are controversial results (66,68,70). Recently, data from the review of Magne et al. (245) concluded that no differences exist in the Firmicutes: Bacteroidetes ratio between obese and normal-weight adults (245). Certain bacteria such as *Fecalibacterium* and *Lachnospiraceae* are in higher abundance in subjects with obesity and are associated with high capacity of energy extraction and body

fat distribution, respectively (69,70,241,246). A higher abundance of Verrucomicrobiota phylum and its specie *Akkermansia muciniphyla* are common characteristics among lean adults' and children's populations (247,248). *Akkermansia* has also been associated with the beneficial distribution of the adipose tissue and healthier metabolic status, particularly in fasting plasma glucose and triglycerides in overweight and obese adults (247).

In this line, little recent evidence revealed that children and adults with MUO have different gut microbiota characteristics than their MHO peers. There is an interesting study which included 317 MHO and 430 MUO adult subjects (71). They observed significant differences in gut microbial diversity and composition between groups. To our knowledge, there are only two recent studies conducted on children, and they observed the same trend as in adults (72,249).

3.7 Changes in gut microbiota and effect on metabolic health

Diet is the main basis for treating many non-communicable diseases nowadays. Evidence supports dietary interventions' efficacy in improving metabolic health (250,251). However, it is known that exists a vast inter variability between subjects' responses to treatment.

Previous works have shown how gut microbiota modifications through dietary interventions exert beneficial metabolic effects (217,225). However, some authors (218,252,253) suggested that the already existing gut microbiota may modulate the effect of the diet on the metabolism.

In adult subjects with overweight and obesity, it has been shown that richness is a potential predictor of the efficacy of the weight-loss dietary intervention (254). Moreover, specific taxa such as *Akkermanisa muciniphyla* have been associated with improved metabolic outcomes after a 6-week caloric restriction intervention (247). To date, evidence in children is scarce. Nadal et al. (255) reported that Lactobacillus abundance was higher in children with more significant weight loss.

All in all, it seems that having some gut microbiota characteristics and species may favour or impair the efficacy of dietary interventions, leading to the idea that subjects may benefit from different interventions according to their gut microbiota. Personalized nutrition is emerging as a novel approach to treating obesity and cardiovascular disease, intending to tailor dietary advice by incorporating the individual's exogenous and endogenous factors (256).

The research on the interactions between gut microbiota and metabolic health has gained interest during the last decades. Furthermore, it is a new field of interest in preventing cardiovascular disease in children with obesity.

Hypothesis & Objectives

Hypothesis

Based on the role that gut microbiota plays in human health and the scarce evidence in children's populations, the hypothesis of this thesis are:

1. In children and adolescents with obesity, the composition of the gut microbiota is associated with cardiometabolic alterations
2. The composition of the gut microbiota may determine the effectiveness of nutritional intervention on obesity

Objectives

This thesis aimed to characterize gut microbiota profiles of children and adolescents with obesity associated with cardiometabolic risk and with a worse response to a nutritional intervention

For this purpose, we addressed the following specific objectives:

1. To characterize the gut microbiota of children with obesity from the clinical trial Obemat 2.0 according to its metabolic health status.
2. To analyse associations between the gut microbiota and metabolic health.
3. To analyse the role of the gut microbiota as a predictor of the success of a multicomponent intervention to improve obesity and its comorbidities.

Methods

UNIVERSITAT ROVIRA I VIRGILI
GUT MICROBIOTA: A CONNECTION BETWEEN OBESITY AND CARDIOVASCULAR HEALTH IN CHILDREN
Mireia Alcázar López

Methods overview

This thesis is based on the MICROBEKIDS study. This study aimed to evaluate whether the motivational intervention of the OBEMAT2.0 clinical trial (PI15/00970) was more effective than the conventional intervention in increasing the gut microbiota diversity and, therefore, improving BMI and MetS components. The secondary objectives were to analyse the role of the gut microbiota as a predictor of cardiometabolic health and treatment success.

- Obemat 2.0 clinical trial

The Obemat study (257) aimed to evaluate the efficacy of a multicomponent motivational intervention for treating childhood obesity compared to the usual intervention for childhood obesity. It was a randomized, non-blinded clustered clinical trial for treating children with obesity for 12 (+3) months. The study holds coordinated primary care centres and hospital specialized services, integrating individual motivational interviews, educational groups, and eHealth wearable tools.

The structure of this study was based on two arms: a control group following the usual recommendations in primary care; and an intervention group receiving a structured motivation-based interview supported by educational materials, combined with group therapy and eHealth. The educational materials were published elsewhere (258,259)

The recruited study sample was 303 children with obesity (n= 137 for the control group and 166 for the intervention group). They had a baseline comprehensive clinical assessment before the intervention (ages 8 to 14) and a final assessment after 12 months of therapy (ages 9 to 15) performed by trained nutritionists. Moreover, they were invited to participate in a collection of biological samples (faecal and blood) to investigate childhood obesity

(COLOBEPED, reference C.0004585). Those who accepted were included in the Microbekids study, the work that includes this thesis.

We performed a cross-sectional observational study to achieve this thesis's first and second specific objectives. We included anthropometrical parameters, systolic and diastolic blood pressure, blood samples and faecal samples collection from the baseline assessment. Further methods are reported in Manuscript 1.

To achieve the last objective of this thesis, we prepared a longitudinal, observational prospective study secondary to the MICROBEKIDS study. For this work, we included anthropometrical parameters, systolic and diastolic blood pressure, biochemistry and faecal samples from those children that attended both the baseline and final assessments and followed the study as *Per Protocol*, attending to at least 9 of the 11 intervention visits. Further methods are reported in Manuscript 2.

Ethics

This thesis followed the rules of the Declaration of Helsinki (260) and was approved by the ethics committees responsible for the activity of all the involved study centres. All parents or legal guardians signed the informed consent before study enrolment, and children aged 12 years or above signed informed assent to participate in the study.

Results

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GUT MICROBIOTA: A CONNECTION BETWEEN OBESITY AND CARDIOVASCULAR HEALTH IN CHILDREN
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Manuscript 1

Gut microbiota is associated with metabolic health in children with obesity

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Original article

Gut microbiota is associated with metabolic health in children with obesity

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SUMMARY

Background and aims: We aimed to describe and characterize the gut microbiota composition and diversity in children with obesity according to their metabolic health status.

Methods: Anthropometry, Triglycerides, HDL cholesterol, HOMA-IR, and systolic and diastolic blood pressure (SBP, DBP) were evaluated (and z-score calculated) and faecal samples were collected from 191 children with obesity aged from 8 to 14. All children were classified depending on their cardiometabolic status in either a "metabolically healthy" (MHO; n = 106) or "metabolically unhealthy" (MUO; n = 85) group. Differences in gut microbiota taxonomies and diversity between groups (MUO vs MHO) were analysed. Alpha diversity index was calculated as Chao1 and Simpson's index, and β-diversity was calculated as Adonis Bray–Curtis index. Spearman's correlations and logistic regressions were performed to study the association between cardiometabolic health and the microbiota.

Results: Children in the MUO presented significantly lower alpha diversity and richness than those in the MHO group (Chao1 index p = 0.021, Simpson's index p = 0.045, respectively), whereas microbiota β-diversity did not differ by the cardiometabolic health status (Adonis Bray–Curtis, R^2 = 0.006; p = 0.155). The MUO group was characterized by lower relative abundances of the genera *Christensenellaceae R7 group* (MHO:1.42% [0.21–2.94]; MUO:0.47% [0.02–1.60], p < 0.004), and *Akkermansia* (MHO:0.26% [0.01–2.19]; MUO:0.01% [0.00–0.36], p < 0.001) and higher relative abundances of *Bacteroides* (MHO:10.6% [4.64–18.5]; MUO:17.0% [7.18–27.4], p = 0.012) genus. After the adjustment by sex, age, and BMI, higher *Akkermansia* (OR: 0.86, CI: 0.75–0.97; p = 0.033), Christensenellaceae R7 group (OR: 0.86, 95% CI: 075–0.98; p = 0.031) and Chao1 index (OR: 0.86, CI: 0.96–1.00; p = 0.023) represented a lower risk of the presence of one or more altered cardiovascular risk factors.

Conclusion: Lower proportions of *Christensenellaceae* and *Akkermansia* and lower diversity and richness seem to be indicators of a metabolic unhealthy status in children with obesity.

1. Background

Obesity has become a major public health problem during the last decades affecting both adults and children. The importance of the obesity pandemic relies on the fact that its relation with cardiovascular disorders is one of the main mortality risk factors worldwide [1]. Childhood obesity tracks to adolescence and adulthood [2] thus, most adolescents with obesity may have a higher risk of metabolic disorders such as insulin resistance, dyslipidaemia, and hypertension in adult life [3,4].

It has been suggested that a combination of genetic and environmental factors and an unhealthy dietary pattern with insufficient physical activity could be the main reasons for the development of obesity and its metabolic comorbidities [5,6]. Recently, other factors such as sleep duration [7], stress [8], poor calcium intake [9], or the gut microbiota composition [10] are

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suggested as new players in the development of obesity and its comorbidities.

There is an emerging interest in the role of gut microbiota in the regulation of metabolic health. Specific bacterial taxa produce short-chain fatty acids (SCFA) by the fermentation of undigested fibre in the gut [11]. SCFA represent an important source of energy for the host [12] and are involved in different metabolic pathways linked to anti-inflammatory effects, appetite control, or regulation of glucose and lipid metabolisms [13].

The presence of an alteration of the gut microbiota composition, known as dysbiosis, has been described both in adults [14,15] and children [16–19] with obesity. Obesity dysbiosis is characterized by the increased levels of *Firmicutes* vs *Bacteroidetes*, a lower bacterial diversity [14,16,19], and lower absorption of SCFA [20]. The consequences of obesity dysbiosis have been linked with the development of metabolic alterations such as dyslipidaemia and insulin resistance or hypertension [21–24].

However, it has been observed that not all the subjects with obesity present co-morbidities; in fact, it has been estimated that only the 47% of the patients with obesity will develop metabolic syndrome, and 10% of the adults with obesity will not develop any of the disorders involved in it [25]. This absence is more often observed in young and physically active patients, and it has been associated with the new concept called "metabolically healthy obesity" (MHO) [26,27]. Recently it has been described that children categorized as MHO present better metabolic profiles than those classified as "metabolically unhealthy obesity" (MUO), including lower systolic and diastolic blood pressure and better lipid profile [28]. MHO has been frequently defined as obesity without metabolic comorbidities and cardiovascular disease, like type 2 diabetes, dyslipidaemia, hypertension, and atherosclerosis [29]. As there were non-standardized criteria to identify children with MHO, in 2018, Damanhoury et all published a consensus-based definition. The consensus concluded that MHO in children should reflect the absence of any cardiometabolic alteration including dyslipidaemia, hypertension, or glucose intolerance [30] like. We hypothesized that the gut microbiota could be one of the triggering factors of metabolic alterations in children with obesity.

The reports investigating the composition of the gut microbiota according to the metabolic health status of the population with obesity are scarce in adults [31,32] and to our knowledge, there are only two recent works in small samples of children with obesity (overall 20 of 65 children with obesity) investigating this association [33,34]. Even the two studies associated a lower diversity to the metabolic unhealthy obesity condition, there is no specific taxonomic that could be associated with the onset of metabolic alteration.

Therefore, our study aimed to describe the gut microbiota composition and diversity in children with obesity and to characterize the differences in the gut microbiota according to their metabolic health status.

2. Methods

2.1. Study design and participants

This was a cross-sectional observational study, secondary to a randomized clustered clinical trial on a motivational intervention to treat children with obesity. To perform the present study, we used the baseline anthropometry and biochemistry data along with the microbiota composition of the participants enrolled in the OBEMAT2.0 clinical trial [35].

Data from 315 children with obesity (170 males; 145 females) aged 8 to 14 were obtained from the clinical trial OBEMAT2.0 at baseline. Children were recruited from June 2016 to March 2018 from primary health care centres belonging to the "Camp de Tarragona" healthcare area. Obesity was considered according to BMI values equal to or higher than 97th percentile from Hernandez et al. according to the National Clinical Practice Guidelines [36]. The visit was conducted at hospital Universitari de Tarragona Joan XXIII and Hospital Universitari Sant Joan de Reus between June 2016 and March 2018, where participants of the Obemat2.0 trial were invited to take part in a voluntary faecal collection; 191 of the participants accepted and brought a sample to the centre.

2.2. Anthropometrical and biochemical measurements

Bodyweight was evaluated using a digital scale (SECA 769) with a precision of 50 g in underwear. Height was measured by a wall-mounted stadiometer (SECA 216) with 0.1 cm of precision. The waist was measured as the mid-point between the iliac crest and lower rib with a Holtain waist circumference non-extensible tape with a precision of 1 mm [37]. Body mass index (BMI) was calculated as weight over squared height (kg/m^2). BMI was converted into z scores using the World Health Organization 2007 reference data [38].

A trained nurse extracted a blood sample from participants in fasting conditions. High-density lipoproteins cholesterol (HDL) (mg/dL), triglycerides (mg/dL), and glucose (mg/dL) were measured in the respective laboratories of local study centres using routine clinical diagnostic enzymatic methods, and insulin (μIU/ml) was quantified by immunoradiometric assays. The Homeostasis Model Assessment of Insulin resistance (HOMA-IR) was calculated as HOMA-IR = (Inulin mIU x Glucose (mmol/L)/22.5 as previously reported [39].

Trained study personnel measured systolic (SBP) and diastolic blood pressure (DBP) (mmHg) at least 20 min after arriving at the study centre. Blood pressure was assessed in duplicate (with a time slot of 5 min between measures) using a Dinamap Pro 100 device on the left arm, while the child remained to sit down with the arm laying comfortably. The mean value of the two duplicate measures was then calculated.

Biochemical parameters, SBP, and DBP were standardized as z-scores using the Stavnsbo references [40].

2.3. Faecal DNA collection and extraction

The study participants were instructed to self-collect a faecal sample and then store it at -20 °C until the visit. Participants were provided with tubes, isolation bags, and icy patches to transport the samples to the clinic, where the faecal samples were stored at -80 °C until their analysis.

DNA was extracted from approximately 200 mg of stool samples using the MagAttract PowerSoil DNA kit (Qiagen, Venlo, Netherlands) for KingFisher Duo Primer Purification System (Thermo Fischer Scientific Inc, Waltham, MA, USA) following the manufacturer's protocol at the ICTS infrastructure with the equipment of the Centre for Omic Sciences (COS), Joint Unit of the Universitat Rovira i Virgili and Eurecat.

2.4. Sequencing and bioinformatics analysis

DNA libraries were obtained following the 16srDNA GENE Metagenomic Sequencing Library Preparation Illumina protocol (Cod. 15044233 Rev. A). The gene-specific sequences were targeting the variable V3 and V4 regions. The primers were selected according to Klindworth et al., 2019 [41]. Microbial genomic DNA (5 ng/μl in 10 mM Tris pH 8.5) was used to initiate the protocol. The

multiplexing step was performed using Nextera XT Index Kit (FC-131-1096) (Illumina, San Diego, CA, USA). One μl of the PCR product was run on a Bioanalyzer DNA 1000 chip to verify the size (the expected size on a Bioanalyzer trace was ~550 bp). The libraries were sequenced using a 2 × 300pb paired-end run (MiSeq Reagent kit v3 (MS-102-3001)) on a MiSeq- Illumina Sequencer (FISABIO sequencing service, Valencia, Spain) according to the manufacturer's instructions. The quality assessment was performed using prinseq-lite program [42] and sequences were selected with a minimum length of 50. Sequence data were analysed using quiime 2 pipelines by Boylen et al. [43]. The metataxonomic analyses were performed using some quiime2 plugins. Denoising, paired-ends joining and chimera depletion was performed starting from paired ends data using the DADA2 pipeline [44]. Taxonomic assignment was conducted using the Silva v138 database with the addition of the specie level classification by the same database [45]. Taxa representing less than 0.01% of the reads across all the samples were filtered.

2.5. Cardiometabolic health

To assess the children's cardiometabolic risk, we created a continuous cardiometabolic risk score (Cmet Risk) based on Eisenmann et al. [46]. This score was the sum of the standardized SBP, DBP triglycerides, HOMA-IR, and HDL cholesterol z-scores, this last one multiplied by -1 (as HDL cholesterol is inversely related to cardiometabolic risk). A higher score was indicative of a less favourable cardiometabolic profile.

Al the participants included in the study were classified by their metabolically status in children with metabolically healthy obesity (MHO) and children with metabolically unhealthy obesity (MUO). The parameters included in this classification were Triglycerides, HDL, SBP, DBP, and HOMA-IR. We considered a child as belonging to the MUO if at least one of the parameters previously described was over ≥1.5SD of the z-score (or ≤ 1.5SD for HDL).

2.6. Statistical analysis

Descriptive data were reported as the median and interquartile range for continuous variables and as a percentage for categorical variables. For testing differences in health outcome parameters, the relative abundance of bacteria taxa, and alpha diversity between MUO and MHO groups, Mann–Whitney U-test was performed. Only bacteria with a P-value < 0.01 were considered significantly different.

Spearman's rank correlations were used to find associations between the alpha diversity index and the cardiovascular risk factors and the metabolic risk score as a continuous variable.

Beta diversity analysis was conducted by permutational multivariate analysis to assess the effect of the metabolic status on the gut microbial composition at amplicon sequence variant (ASV) level (Adonis test) using Bray–Curtis distance in the MicrobiomeAnalyst online platform [47]. The clustering of the samples according to the metabolic status was performed by principal component analysis at the ASV level.

Associations between the cardiometabolic risk factors (as continuous variables) and the bacteria with at least 0.01% of abundance were assessed using Spearman's rank correlation. The bacteria that were significantly associated with metabolic risk score or the cardiometabolic risk factors and the alpha diversity indexes were included in logistic regression models performed in R version 4.1 [48]. These models were adjusted for age, sex, and BMI (z-score). Results were expressed by odds ratio (OR) and 95% confidential interval (95% IC).

2.7. Ethics

The study followed the rules of the Declaration of Helsinki [49] and was approved by the ethics committees responsible for the activity of all the involved study centres. All parents or legal guardians signed the informed consent before study enrolment and children aged 12 years or above signed informed assent to participate in the study.

3. Results

3.1. Description of the population

One hundred ninety-one participants of the Obemat 2.0 trial were classified by their metabolically status. Characteristics of the study participants are described in Table 1. The median age of all the participants was 10 [9.0–12.0] years and did not significantly differ between groups. Almost half (45%) of all the participants presented MUO. Significant differences for all the cardiometabolic risk factors were found between the MHO and the MUO group, including the z-BMI ($p = 0.003$) and waist ($p < 0.001$). Table S1 shows the number of participants that presented each altered cardiovascular risk factor.

3.2. Differences in microbiota composition and diversity depending on cardiometabolic risk

In terms of alpha diversity, the MUO group presented lower richness than the MHO group measured as Chao1 ($p = 0.021$), ACE ($p = 0.020$), and Simpson ($p = 0.045$) indices (Fig. 1A).

Assuming an alpha risk of 0.05 and beta risk of 0.33, a difference between groups (MHO and MUO) of 6.6 has a statistical power of 67%.

Studying each metabolic risk factor independently, those children with altered triglycerides levels presented lower alpha diversity than those with normal levels ($p = 0.027$ in Chao 1 and $p = 0.047$ in Shannon index) (Table S2). A similar trend was observed in children with altered SBP compared to children with not altered SBP ($p = 0.035$, Chao1 index). No differences in microbial diversity were found between children with other metabolic alterations.

Despite the overall microbiota β-diversity was not significantly affected by cardiometabolic status (Adonis Bray–Curtis, $R^2 = 0.006$; $p = 0.155$) (Fig. 1B), differences in specific taxa were found between groups. Relative abundance of the most abundant phylum according to the metabolic condition of the participants is presented in Fig. 2A. Firmicutes were the most abundant taxa in both groups followed by Bacteroidota, both represented 90% of all bacteria. Higher relative abundances of Firmicutes ($p = 0.002$) and Verrucomicrobia ($p = 0.001$) phyla were found in the MHO group compared to MUO, which showed enrichment in Bacteroidetes phylum ($p = 0.002$). Descriptive data are shown in Table S3.

At lower taxonomical levels (Fig. 2B-E), participants with metabolic alterations (MUO) presented a higher relative abundance of the order of Bacteroidales ($p = 0.010$) and the genus of Bacteroides ($p = 0.012$). The relative abundance of the Christensenellaceae family and its lower taxonomic level Christensenellaceae R-7 group ($p = 0.004$), was more than twice higher in the MHO than in the MUO group (MHO:1.42 [0.21–2.94]; MUO:0.47 [0.02–1.60]; $p = 0.004$). The *Akkermansia* genus and the higher taxonomic levels were also under-expressed in the MUO group (MHO: 0.26% [0.01–2.19]; MUO: 0.01% [0.00–0.36]; $p = 0.001$) (Table S3).

Indeed, the relative abundance of the Christensenellaceae family and the genus *Christensenellaceae_R-7_group* significantly

Results

Table 1
Characteristics of the study participants separated by their cardio-metabolic status.

	Whole Sample (n = 191)	MHO (n = 106)	MUO (n = 85)	P-Value
	median [IQR]	median [IQR]	median [IQR]	
Gender (male/female)	110/81	60/46	57/35	0.872
Age (y)	10.0 [9.00; 12.0]	10.0 [9.00; 11.0]	11.0 [9.00; 12.0]	0.102
BMI z-score	2.54 [2.29; 2.83]	2.45 [2.22; 2.75]	2.64 [2.36; 3.00]	0.003
Waist z-sore	2.05 [1.63; 2.41]	1.86 [1.60; 2.21]	2.24 [1.87; 2.65]	<0.001
Triglycerides z-score	0.31 [−0.23; 1.10]	−0.03 [−0.45; 0.41]	0.79 [0.26; 1.89]	<0.001
HDL z-score	−0.62 [−0.97; −0.05]	−0.35 [−0.82; 0.27]	−0.84 [−1.16; −0.48]	<0.001
SBP z-score	0.47 [−0.10; 1.10]	0.29 [−0.15; 0.67]	0.82 [−0.05; 1.86]	<0.001
DBP z-score	−0.13 [−0.61; 0.44]	−0.38 [−0.71; 0.24]	0.26 [−0.31; 0.83]	<0.001
HOMA-IR z-score	0.50 [0.05; 1.25]	0.25 [−0.05; 0.62]	1.24 [0.45; 2.25]	<0.001
Cardiometabolic Risk z-score	2.24 [0.27; 3.95]	0.63 [−0.74; 2.02]	4.13 [2.84; 6.07]	<0.001

MHO: metabolically healthy obesity group; MUO metabolically unhealthy obesity group. IQR: interquartile range. BMI: body mass index. HDL: High Density Lipoproteins cholesterol. SBP: systolic blood pressure. DBP: diastolic blood pressure. HOMA-IR: Homeostatic Model Assessment of Insulin Resistance. P-value calculated by Mann Withney's U-test.

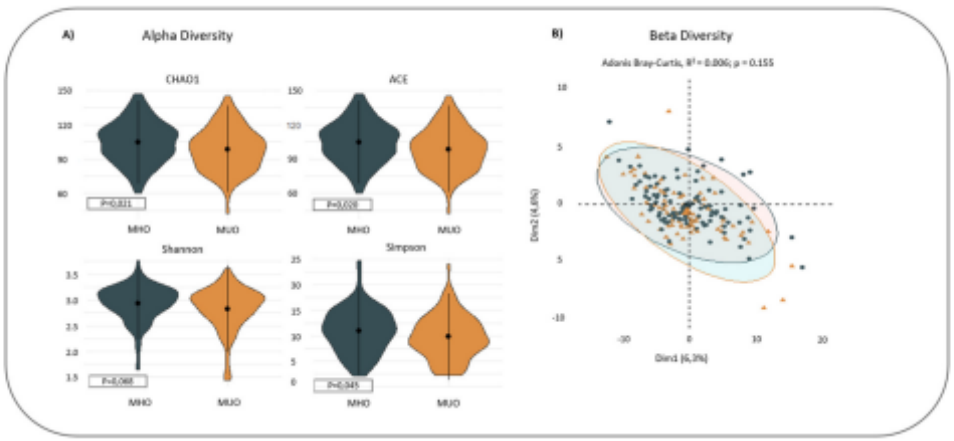

Fig. 1. Characterization of beta-diversity and alpha diversity of gut microbiome of participants in the Metabolically Healthy Obesity and Metabolically Unhealthy Obesity (MHO and MUO) groups. A) Alpha diversity index. Violin plots represent the differences at the genus level among children with MHO and MUO using the Mann–Whitney U test B). The clustering of the samples according to the metabolic status was performed by principal component analysis at the amplicon sequence variable, ASV, level.

differed between children with normal and high serum triglycerides concentrations (1.46 [0.41–2.89] vs. 0.64 [0.14–1.71]; p = 0.029). Children with altered triglycerides presented as well higher abundances of *Coprococcus* (0.72 [0.36–1.14]; 0.99 [0.54–1.74]; p = 0.045) and less *Bacteroides dorei* (0.37 [0.00–1.76]; 0.13 (0.00–0.39); p = 0.029). Children with altered HOMA-IR presented higher levels of *Bacteroides* (5.12 [1.86–10.5]; 7.6 [3.19–13.1; p 0.036), and *Alistipes* (3.75 [1.26–6.65]; 5.30 [2.90–9.34]; p = 0.042) genera in comparison with those participants without this alteration. The same trend was observed in those participants with altered SBP, who showed higher abundances of *Bacteroides* genus, and lower abundances of Christensenellacea R-7 group (Table S4).

3.3. *Associations of some microbial taxa with the cardiometabolic health*

Figure 3 represents the associations between some bacterial taxa, alpha diversity, and cardiometabolic risk factors. An inverse correlation of the Chao1 index with the metabolic risk score (Rho = −0.15; p = 0.03) and triglycerides (Rho = −0.15; p = 0.03) and a direct correlation with HDL (Rho 0.15; p = 0.04) was observed. The association with the metabolic risk score and the alpha diversity index remained significant after the adjustment by sex, age, and BMI (Table S5).

The correlation analyses revealed that there were several bacteria associated with the cardiometabolic risk score. The metabolic risk score was positively associated with Bacteroidota (Rho = 0.15; p = 0.03) and Proteobacteria (Rho = 0.14; p = 0.042) phyla and inversely associated with Firmicutes (Rho = 0.14; p = 0.047). These trends represent the associations that we found into the corresponding lower taxonomic levels Bacteroidales order (Rho = 0.15; p = 0.03), Clostridia (Rho = −0.15; p = 0.033), and Gammaproteobacteria (Rho = 0.16; p = 0.026) classes. Moreover, the Verrucomicrobiae class was also negatively associated with the metabolic risk score (Rho = −0.15; p = 0.041).

In lower taxonomic levels, we observed how *Christensenellaceae R7-group* (Rho = −0.18; p = 0.01) and *Akkermansia* genus (Rho = −0.15; p = 0.045) were negatively associated with the metabolic risk score. *Christensenellaceae R-7 group* also was associated inversely with the triglyceride levels (Rho = −0.15; p = 0.04) (Fig. 3).

Although we have not observed a significant association between the BMI and the diversity, the abundance of the *Christensenellaceae R7-group* was inversely associated with the BMI of our participants (Rho = −0.15; p = 0.04).

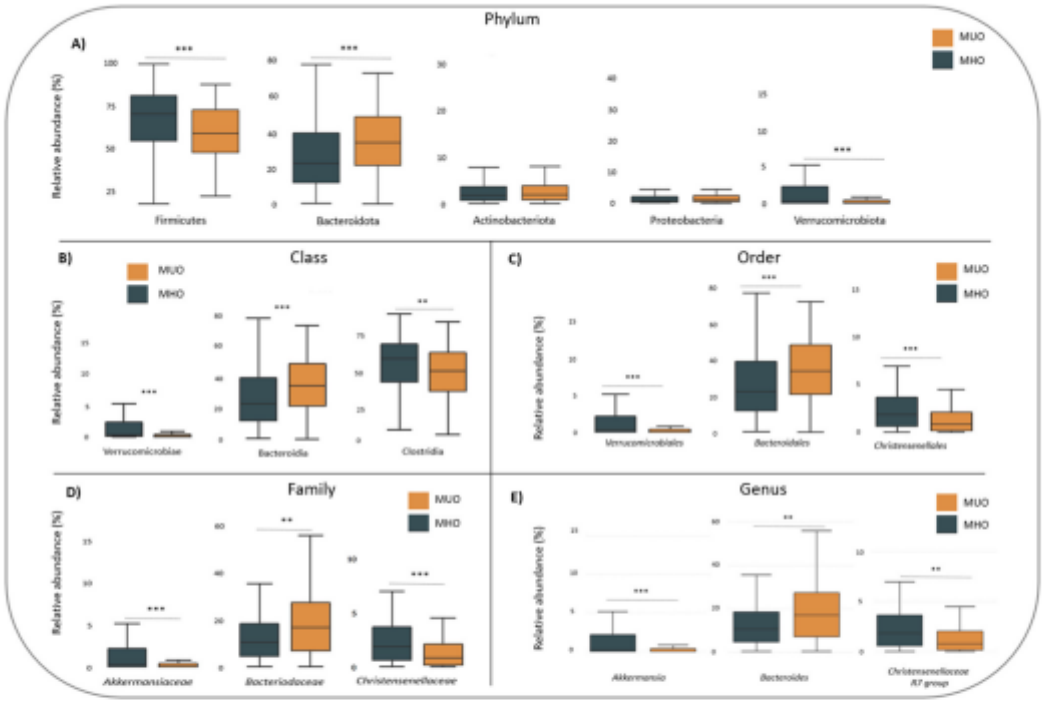

Fig. 2. Box plot representation of the differences at Phylum, Class, Order, Family, and genus levels between the MUO and the MUO group. Mann Whitney U test was used to calculate the significance. **p < 0.05; ***p < 0.01.

Logistic regression models of different bacteria on MHO vs. MUO adjusted by sex, BMI, and age were performed (Table 2). BMI was the variable that most affected the cardiovascular risk. However, we observed that the association of Bacteroidales, *Christensenellaceae R-7*, and *Akkermansia* were consistent after the adjustment. The greater presence of *Akkermansia* and *Christensenellaceae R7* was associated with having a lower risk of presenting any alteration in one or more of the different cardiometabolic risk factors. The greater presence of Bacteroidales was associated with a higher risk of being classified in the MUO group.

4. Discussion

Our study reveals that children with metabolic unhealthy obesity were characterized by lower diversity and lower relative abundance of Christensenellales (*Christensenellaceae R-7*) and Verrucomicrobiales *(Akkermansia)* than those without any cardiometabolic alteration. Furthermore, children with metabolic unhealthy obesity showed, at the same time, an enrichment of *Bacteroides*. To the best of our knowledge, this is the work with the higher number of participants with obesity that have studied the association of the gut microbiota with metabolic health in children.

We observed that the most abundant phyla in the overall sample were Firmicutes and Bacteroidetes, consistently with what was previously described [18,19,50]. Our participants presented lower proportions of Bacteroidetes in comparison with Firmicutes, which might be explained by the obesity condition. Moreover, we have detected specific characteristics of the microbiota profile depending on the metabolic health group. Supporting the literature [33,34] we have observed that the MUO group presented lower diversity than the MHO group. Rampelli et al. observed how a worse lifestyle was associated with low diversity and a worse inflammatory profile which could trigger the development of obesity and its comorbidities. Moreover, low microbial diversity is considered one of the main markers of intestinal dysbiosis and it has been associated with several alterations such as higher insulin resistance, adiposity, dyslipidaemia, and worse inflammatory profile [33,51].

When we looked into detail the microbiota profile of children with MUO and MHO. We observed that children with MUO presented a higher proportion of Bacteroides and children with MHO higher proportion of Christensenellaceae and Akkermansia. Consistently, the diversity was inversely associated with the abundance of Bacteroides and directly with Akkermansia and Christensenellace.

Consistently with previous works, the group with MUO presented a higher abundance of the *Bacteroides* genus and upper taxonomic levels in comparison with the MHO [34]. It has been proposed *Bacteroides* genus as a biomarker of a western diet, rich in fat and protein [52]. Actually, Some *Bacteroides* spp. would be especially efficient readapting their metabolism to different gut environments [53].

Christensenella genera have been reported as health-related bacteria in adulthood [54,55]. Christenesenellaceae family was consistently found to be increased in healthy populations compared to disease groups in several cohort studies [33,56,57]. Some authors have previously observed that Christensenellaceae were depleted in participants with metabolic syndrome [58,59] and inversely correlated with metabolic risk in adult populations [60].

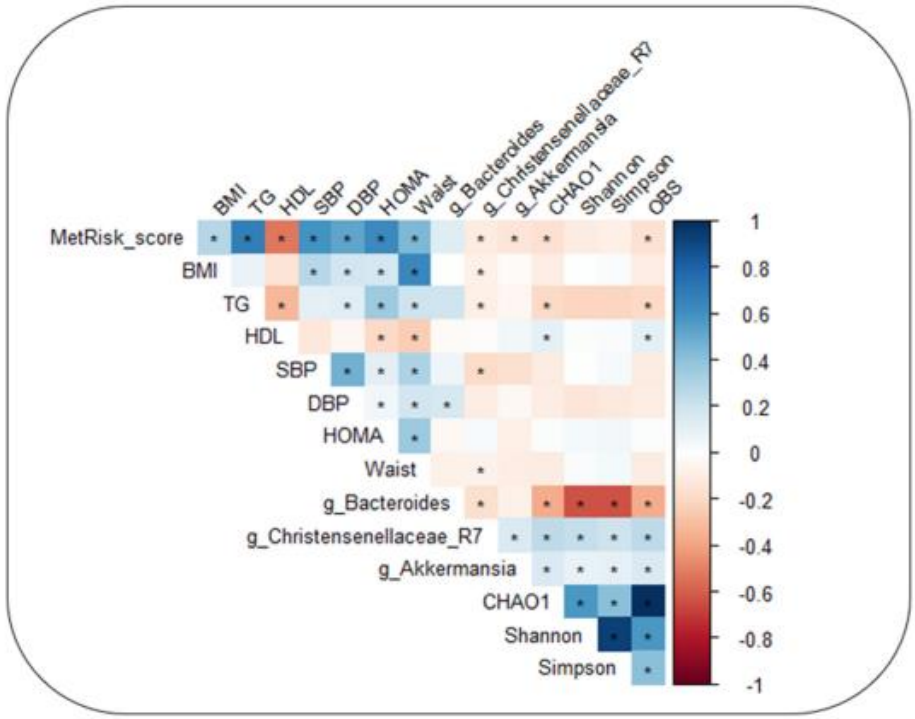

Fig. 3. Paired correlations between bacteria, alpha diversity, and cardiometabolic risk factors. The heatmap represents Spearman's rank correlation coefficients between the different parameters. Positive or negative correlations are represented in blue and red respectively. BMI: Body mass index, TG: triglycerides HDL: high-density lipoproteins, SBP: systolic blood pressure, DBP diastolic blood pressure. HOMA insulin resistance index, MetRisk_score: cardiometabolic risk score. Cardiovascular factors are expressed as z-score. * Indicates Spearman's correlation p value < 0.05.

Table 2
Logistic regression models of bacterial abundances with effect on the presence of one or more altered metabolic risk factors (being classified as Metabolically Healthy Obesity).

Predictors	Bacteroides model			Christensenellaceae model			Akkermansia model			Mixed model		
	OR	95% CI	P	OR	95% CI	P	OR	95% CI	P	OR	95% CI	P
Intercept	0.00	0.00–0.01	**<0.001**	0.00	0.00–0.05	**<0.001**	0.00	0.00–0.07	**0.001**	0.00	0.00–0.03	**<0.001**
Age (years)	1.38	1.11–1.72	**0.004**	1.34	1.08–1.66	**0.008**	1.29	1.04–1.61	**0.022**	1.30	1.05–1.64	0.019
Sex [girl]	1.30	0.68–2.50	0.431	1.40	0.74–2.68	0.307	1.31	0.69–2.50	0.406	1.43	0.74–2.80	0.297
BMI	4.50	2.05–10.45	**<0.001**	3.86	1.79–8.79	**0.001**	3.76	1.75–8.51	**0.001**	4.36	1.96–10.25	**<0.001**
Bacteroides	1.03	1.01–1.05	**0.001**							1.03	1.00–1.05	**0.023**
Christensenellaceae_R-7				0.86	0.75–0.98	**0.031**				0.99	0.83–1.18	0.878
Akkermansia							0.86	0.74–0.97	**0.033**	1.00	0.84–1.19	0.965
Interaction (Akkermansia*Christensenellacea_R-7)										0.90	0.78–0.99	0.076
Observations	191			191			191			191		
R^2 Tjur	0.131			0.102			0.108			0.160		

In fact, Fun J et al., observed that high levels of this family were associated with low triglyceride levels and elevated high-density lipoproteins [61]. Regarding the works performed in children, it has been observed that Christensenellaceae is associated with healthy metabolic status [56]. Yuan X et al. concluded that MHO and normal-weight children showed higher levels in comparison with those MUO children [33]. Our data add evidence in children to support the hypothesis that higher proportions of Christensenellaceae could prevent the presence of metabolic alterations. It has been suggested that the relative abundance of this family is one of most associated with the host genetic variations [62] and it is commonly cited among the heritable components of the gut microbiota [63]. This, among others, considered heritable families, has been inversely associated with visceral fat and other metabolic alterations [64] suggesting a potential role of host genes to modify the relationship between their microbiome and metabolic health. Although the mechanisms are still not clearly understood, our data support the hypothesis suggesting an important role of the

Tarragona, Institut Català de la Salut); González S, Ruiz-Morcillo E, Ainsa ME, Vilalta P, Aranda B (CAP Sant Salvador, Tarragona, Institut Català de la Salut); Boada A, Balcells E (ABS Alt Camp Est, Vilarodona, Institut Català de la Salut).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clnu.2022.06.007.

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Supplementary material for

Gut microbiota is associated with metabolic health in children with obesity

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[1] Full detail given in Acknowledgement section.

Supplementary Table 1. Distribution of the participants by the cardiometabolic risk factors.

Cardiometabolic risk factors	Health outcome parameter < 1.5SD	Health outcome parameter ≥1.5SD**
	N (%)	N (%)
Triglycerides	159 (83%)	32 (17%)
HDL cholesterol**	177 (93%)	14 (7%)
HOMA-IR	155(81%)	36 (19%)
SBP	159 (83%)	32 (17%)
DBP	181 (95%)	10 (5%)
Waist	33 (17%)	158 (83%)

SD: standard deviation. HOMA-IR: Homeostatic model assessment for insulin resistance; SBP: systolic blood pressure; DBP: diastolic blood pressure **HDL altered is considered a z-score ≤ -1.5SD of the mean.

Supplementary Table 2. Description of the alpha diversity index by cardiovascular

			CHAO1	Shannon	Simpson
Gender	Boy	N= 110	97.0 [85.2;113]	2.9 [2.75;3.15	10.0 [7.50;13.0]
	Girl	N = 81	106.0 [96.0;116]	3.0[2.74;3.15]	10.8 [7.34; 13.5]
	p.value		**0.027**	0.525	0.728
Triglycerides z-score	Altered	N=32	95.5 [78.0;108]	2.9 [2.39;3.13]	8.5 [4.25;12.0]
	Normal	N=159	104 [91.0;116]	3.00 [2.75;3.17]	10.8 [7.67;13.5]
	p.value		**0.027**	**0.047**	**0.039**
HDL z-score	Altered	N=14	92.0 [86.8;118]	3.0 [2.79;3.16]	10.9 [8.82;14.1]
	Normal	N=177	103 [90.0;115]	3.0 [2.72;3.15]	10.4 [7.39;13.4]
	p.value		0.417	0.393	0.409
HOMA-IR z-score	Altered	N=155	101 [88.0;116]	3.0 [2.75;3.16]	10.4 [7.43;13.4]
	Normal	N=36	103 [94.0;111]	3.0 [2.70;3.13]	11.3 [7.60;13.1]
	p.value		0.96	0.886	0.784
SBP z-score	Altered	N=159	104 [91.5;116]	3.00 [2.75;3.17]	11.0 [7.52;13.6]
	Normal	N=32	94.0 [80.8;109]	2.9 [2.67;3.05]	8.8 [7.16;10.8]
	p.value		**0.035**	**0.11**	**0.049**
DBP z-score	Altered	N=181	101 [89.0;115]	3.0 [2.75;3.17]	10.7 [7.43;13.5]
	Normal	N=10	104 [86.2;111]	2.8 [2.55;2.98]	8.4 [7.12;10.8]
	p.value		0.955	0.067	0.149
Waist circumference z-score	Altered	N=158	99.5 [88.0;111]	2.97 [2.75;3.14]	10.4 [7.67;13.3]
	Normal	N=33	113 [97.0;121]	3.0 [2.51;3.18]	10.5 [5.66;14.3]
	p.value		0.049	0.964	0.685

Biochemical parameters, waist, SBP, and DBP were expressed as z scores following the Stavnsbo references (261).

Supplementary Table 3. Main taxonomic differences between children with metabolically healthy obesity and children with metabolically unhealthy obesity.

	MHO	MUO	p-value
	median [IQR]	median [IQR]	
p__bacteroidota	22.7 [12.0;39.4]	34.1 [21.5;48.5]	0.002
p__bacteroidota; c__bacteroidia	22.7 [12.0;39.4]	34.1 [21.5;48.5]	0.002
p__bacteroidota; c__bacteroidia; o__bacteroidales	22.7 [12.0;39.4]	34.1 [21.3;48.5]	0.002
p__bacteroidota; c__bacteroidia; o__bacteroidales;f__bacteroidaceae	10.6 [4.6;18.5]	17.0 [7.18;27.4]	0.012
p__bacteroidota; c__bacteroidia; o__bacteroidales;f__bacteroidaceae;g__bacteroides;__	4.2 [1.5;8.5]	6.2 [3.1;12.4]	0.01
p__firmicutes	69.4 [53.9;80.0]	58.1 [47.1;71.8]	0.002
p__firmicutes; c__bacilli; o__erysipelotrichales;f__erysipelatoclostridiaceae;g__erysipelatoclostridium;s__[clostridium]_	0.01 [0.00;0.03]	0.00 [0.00;0.02]	0.046
p__firmicutes; c__bacilli; o__rf39	0.01 [0.00;0.13]	0.00 [0.00;0.05]	0.043
p__firmicutes; c__bacilli; o__rf39;f__rf39	0.01 [0.00;0.13]	0.00 [0.00;0.05]	0.043

MHO: children with metabolically healthy obesity; MUO: children with metabolically unhealthy obesity. Values represent the percentage of the abundance. IQR: interquartile range
P-value for differences between groups calculated by Mann-Whitney U test.

Supplementary Table 3 continuation. Main taxonomic differences between children with metabolically healthy obesity and children with metabolically unhealthy obesity.

	MHO	MUO	p-value
p__firmicutes; c__clostridia	58.8 [44.0;68.6]	51.1 [38.0;62.8]	0.018
p__firmicutes; c__clostridia; o__christensenellales	1.85 [0.59;3.67]	0.82 [0.17;2.09]	0.001
p__firmicutes; c__clostridia; o__christensenellales;f__christensenellaceae	1.85 [0.59;3.67]	0.82 [0.17;2.09]	0.001
p__firmicutes; c__clostridia; o__christensenellales;f__christensenellaceae;g__christensenellaceae_r7	1.42 [0.21;2.94]	0.47 [0.02;1.60]	0.004
p__firmicutes; c__clostridia; o__oscillospirales;f__butyricicoccaceae;g__butyricicoccus;s__butyricicoccus_sp.	0.13 [0.03;0.37]	0.07 [0.00;0.25]	0.032
p__firmicutes; c__clostridia; o__oscillospirales;f__ucg-010	0.06 [0.01;0.39]	0.02 [0.00;0.14]	0.017
p__firmicutes; c__clostridia; o__oscillospirales;f__ucg-010;g__ucg-010;s__unidentified	0.01 [0.00;0.07]	0.00 [0.00;0.02]	0.046
p__firmicutes; c__clostridia; o__peptostreptococcales-tissierellales;f__anaerovoracaceae	0.16 [0.10;0.28]	0.12 [0.06;0.23]	0.048
p__firmicutes; c__clostridia; o__peptostreptococcales-tissierellales;f__anaerovoracaceae;g__family_xiii_ad3011_group;s__uncultured_bacterium	0.06 [0.02;0.11]	0.05 [0.01;0.10]	0.046

MHO: children with metabolically healthy obesity; MUO: children with metabolically unhealthy obesity. Values represent the percentage of the abundance. IQR: interquartile range
P-value for differences between groups calculated by Mann-Whitney U test.

Supplementary Table 3 continuation. Main taxonomic differences between children with metabolically healthy obesity and children with metabolically unhealthy obesity.

	MHO	MUO	p-value
p__firmicutes; c__clostridia;o__peptostreptococcales-tissierellales;f__anaerovoracaceae;g__family_xiii_ucg-001;s__uncultured_bacterium	0.02 [0.00;0.06]	0.01 [0.00;0.03]	0.018
p__verrucomicrobiota	0.27 [0.01;2.25]	0.02 [0.00;0.36]	0.001
p__verrucomicrobiota; c__verrucomicrobiae	0.26 [0.01;2.25]	0.01 [0.00;0.36]	0.001
p__verrucomicrobiota; c__verrucomicrobiae;o__verrucomicrobiales	0.26 [0.01;2.19]	0.01 [0.00;0.36]	0.001
p__verrucomicrobiota; c__verrucomicrobiae; o__verrucomicrobiales;f__akkermansiaceae	0.26 [0.01;2.19]	0.01 [0.00;0.36]	0.001
p__verrucomicrobiota; c__verrucomicrobiae; o__verrucomicrobiales;f__akkermansiaceae;g__akkermansia	0.26 [0.01;2.19]	0.01 [0.00;0.36]	0.001
p__firmicutes; c__clostridia;o__peptostreptococcales-tissierellales;f__anaerovoracaceae;g__family_xiii_ucg-001;s__uncultured_bacterium	0.02 [0.00;0.06]	0.01 [0.00;0.03]	0.018
p__verrucomicrobiota	0.27 [0.01;2.25]	0.02 [0.00;0.36]	0.001
p__verrucomicrobiota; c__verrucomicrobiae	0.26 [0.01;2.25]	0.01 [0.00;0.36]	0.001
p__verrucomicrobiota; c__verrucomicrobiae; o__verrucomicrobiales;f__akkermansiaceae	0.26 [0.01;2.19]	0.01 [0.00;0.36]	0.001
p__verrucomicrobiota; c__verrucomicrobiae; o__verrucomicrobiales;f__akkermansiaceae;g__akkermansia	0.26 [0.01;2.19]	0.01 [0.00;0.36]	0.001

MHO: children with metabolically healthy obesity; MUO: children with metabolically unhealthy obesity. Values represent the percentage of the abundance. IQR: interquartile range
P-value for differences between groups calculated by Mann-Whitney U test.

Supplementary Table 4. Main taxonomic differences between children with altered levels of triglycerides, HOMA-IR, or SBP and those with normal levels

	Normal	Altered	P-value
	Median [IQR]	**Median [IQR]**	
Triglycerides z-score	**N= 159**	**N=32**	
d__Bacteria;p__Firmicutes;c__Clostridia;o__Christensenellales	1.46 [0.41;2.89]	0.64 [0.14;1.71]	0.029
d__Bacteria;p__Firmicutes;c__Clostridia;o__Christensenellales;f__Christensenellaceae	1.46 [0.41;2.89]	0.64 [0.14;1.71]	0.029
d__Bacteria;p__Firmicutes;c__Clostridia;o__Christensenellales;f__Christensenellaceae;g__Christensenellaceae_R-7_group	1.43 [0.40;2.87]	0.64 [0.14;1.71]	0.03
d__Bacteria;p__Firmicutes;c__Clostridia;o__Christensenellales;f__Christensenellaceae;g__Christensenellaceae_R-7_group;__	0.97 [0.14;2.42]	0.28 [0.01;1.26]	0.025
d__Bacteria;p__Firmicutes;c__Clostridia;o__Lachnospirales;f__Lachnospiraceae;g__Coprococcus	0.72 [0.36;1.14]	0.99 [0.54;1.74]	0.042
d__Bacteria;p__Firmicutes;c__Clostridia;o__Lachnospirales;f__Lachnospiraceae;g__Coprococcus;__	0.72 [0.36;1.14]	0.99 [0.54;1.74]	0.042
d__Bacteria;p__Firmicutes;c__Clostridia;o__Oscillospirales;f__Ruminococcaceae;__	0.40 [0.09;1.44]	0.26 [0.03;0.68]	0.038

Altered: health outcome ≥1.5SD of the mean of z score. Normal: health outcome < 1.5SD of the mean of the z score. HDL altered is considered a z-score ≤ -1.5SD of the mean. Z scores were calculated following Stavnsbo references (261).Values represent the percentage of the abundance. IQR: interquartile range. P-value for differences between groups calculated by Mann-Whitney U test.

Supplementary Table 4 continuation. Main taxonomic differences between children with altered levels of triglycerides, HOMA-IR, or SBP and those with normal levels

	Normal	**Altered**	**P-value**
	Median [IQR]	**Median [IQR]**	
d__Bacteria;p__Firmicutes;c__Clostridia;o__Peptococcales;f__Peptococcaceae	0.01 [0.00;0.06]	0.00 [0.00;0.01]	0.011
d__Bacteria;p__Firmicutes;c__Clostridia;o__Oscillospirales;f__UCG-010;g__UCG-010;__	0.01 [0.00;0.06]	0.00 [0.00;0.01]	0.043
HOMA-IR z-score	Altered (n=155)	Normal (n=36)	
d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Bacteroidales;f__Bacteroidaceae;g__Bacteroides;__	5.12 [1.86;10.5]	7.62 [3.19;13.1]	0.036
d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Bacteroidales;f__Rikenellaceae	3.88 [1.57;7.14]	5.30 [3.05;9.34]	0.028
d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Bacteroidales;f__Rikenellaceae;g__Alistipes	3.75 [1.26;6.65]	5.30 [2.90;9.34]	0.042
d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Bacteroidales;f__Bacteroidaceae;g__Bacteroides;s__Bacteroides_vulgatus	0.72 [0.05;1.95]	1.72 [0.36;3.68]	0.008
d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Bacteroidales;f__Rikenellaceae;g__Alistipes;s__Alistipes_sp.	0.13 [0.00;0.49]	0.33 [0.10;0.79]	0.029
d__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Streptococcaceae;g__Streptococcus;__	0.12 [0.04;0.33]	0.08 [0.02;0.13]	0.031

Altered: health outcome ≥1.5SD of the mean of z score. Normal: health outcome < 1.5SD of the mean of the z score. HDL altered is considered a z-score ≤ -1.5SD of the mean. Z scores were calculated following Stavnsbo references (261).Values represent the percentage of the abundance. IQR: interquartile range. P-value for differences between groups calculated by Mann-Whitney U test.

Supplementary Table 4 continuation. Main taxonomic differences between children with altered levels of triglycerides, HOMA-IR, or SBP and those with normal levels

	Normal	Altered	P-value
	Median [IQR]	**Median [IQR]**	
d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Bacteroidales;f__Rikenellaceae;g__Alistipes;s__Alistipes_obesi	0.10 [0.02;0.28]	0.17 [0.06;0.41]	0.041
d__Bacteria;p__Firmicutes;c__Clostridia;o__Lachnospirales;f__Lachnospiraceae;g__Lachnoclostridium	0.03 [0.01;0.09]	0.08 [0.02;0.14]	0.007
d__Bacteria;p__Firmicutes;c__Clostridia;o__Lachnospirales;f__Lachnospiraceae;g__Lachnoclostridium;__	0.02 [0.00;0.06]	0.06 [0.02;0.11]	0.018
Systolic Blood Pressure z-score	**Altered (n=159)**	**Normal (n=32)**	
d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Bacteroidales;f__Bacteroidaceae	11.4 [5.49;22.2]	21.3 [7.62;28.2]	0.033
d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Bacteroidales;f__Bacteroidaceae;g__Bacteroides	11.4 [5.49;22.2]	21.3 [7.62;28.2]	0.033
d__Bacteria;p__Firmicutes;c__Clostridia;o__Christensenellales	1.56 [0.47;3.44]	0.63 [0.14;1.39]	0.001
d__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Streptococcaceae;g__Streptococcus;__	0.12 [0.04;0.33]	0.08 [0.02;0.13]	0.031

Altered: health outcome ≥1.5SD of the mean of z score. Normal: health outcome < 1.5SD of the mean of the z score. HDL altered is considered a z-score ≤ -1.5SD of the mean. Z scores were calculated following Stavnsbo references (261).Values represent the percentage of the abundance. IQR: interquartile range. P-value for differences between groups calculated by Mann-Whitney U test.

Supplementary Table 4 continuation. Main taxonomic differences between children with altered levels of triglycerides, HOMA-IR, or SBP and those with normal levels

	Normal	Altered	P-value
	Median [IQR]	**Median [IQR]**	
d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Bacteroidales;f__Rikenellaceae;g__Alistipes;s__Alistipes_obesi	0.10 [0.02;0.28]	0.17 [0.06;0.41]	0.041
d__Bacteria;p__Firmicutes;c__Clostridia;o__Lachnospirales;f__Lachnospiraceae;g__Lachnoclostridium	0.03 [0.01;0.09]	0.08 [0.02;0.14]	0.007
d__Bacteria;p__Firmicutes;c__Clostridia;o__Lachnospirales;f__Lachnospiraceae;g__Lachnoclostridium;__	0.02 [0.00;0.06]	0.06 [0.02;0.11]	0.018
Systolic Blood Pressure z-score	**Altered (n=159)**	**Normal (n=32)**	
d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Bacteroidales;f__Bacteroidaceae	11.4 [5.49;22.2]	21.3 [7.62;28.2]	0.033
d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Bacteroidales;f__Bacteroidaceae;g__Bacteroides	11.4 [5.49;22.2]	21.3 [7.62;28.2]	0.033
d__Bacteria;p__Firmicutes;c__Clostridia;o__Christensenellales	1.56 [0.47;3.44]	0.63 [0.14;1.39]	0.001
d__Bacteria;p__Firmicutes;c__Clostridia;o__Christensenellales;f__Christensenellaceae	1.56 [0.47;3.44]	0.63 [0.14;1.39]	0.001

Altered: health outcome ≥1.5SD of the mean of z score. Normal: health outcome < 1.5SD of the mean of the z score. HDL altered is considered a z-score ≤ -1.5SD of the mean. Z scores were calculated following Stavnsbo references (261).Values represent the percentage of the abundance. IQR: interquartile range. P-value for differences between groups calculated by Mann-Whitney U test.

Supplementary Table 4 continuation. Main taxonomic differences between children with altered levels of triglycerides, HOMA-IR, or SBP and those with normal levels

	Normal	Altered	P-value
	Median [IQR]	**Median [IQR]**	
d__Bacteria;p__Firmicutes;c__Clostridia;o__Christensenellales;f__Christensenellaceae;g__Christensenellaceae_R-7_group	1.55 [0.45;3.37]	0.59 [0.12;1.25]	0.001
d__Bacteria;p__Firmicutes;c__Clostridia;o__Christensenellales;f__Christensenellaceae;g__Christensenellaceae_R-7_group;__	1.10 [0.12;2.82]	0.18 [0.01;0.94]	0.002
d__Bacteria;p__Firmicutes;c__Clostridia;o__Oscillospirales;f__Oscillospiraceae;g__UCG-002;__	0.59 [0.16;2.32]	0.23 [0.07;0.58]	0.012
d__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridia_UCG-014	0.38 [0.01;3.20]	0.02 [0.00;1.16]	0.031
d__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridia_UCG-014;f__Clostridia_UCG-014	0.38 [0.01;3.20]	0.02 [0.00;1.16]	0.031
d__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridia_UCG-014;f__Clostridia_UCG-014;g__Clostridia_UCG-014	0.38 [0.01;3.20]	0.02 [0.00;1.16]	0.031
d__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridia_UCG-014;f__Clostridia_UCG-014;g__Clostridia_UCG-014;__	0.38 [0.01;3.14]	0.02 [0.00;1.16]	0.022
d__Bacteria;p__Firmicutes;c__Clostridia;o__Oscillospirales;f__Oscillospiraceae;g__UCG-002;s__uncultured_rumen	0.33 [0.02;1.13]	0.13 [0.00;0.41]	0.029
d__Bacteria;p__Firmicutes;c__Clostridia;o__Oscillospirales;f__Oscillospiraceae;g__UCG-005;__	0.16 [0.05;0.37]	0.07 [0.02;0.17]	0.009

Altered: health outcome ≥1.5SD of the mean of z score. Normal: health outcome < 1.5SD of the mean of the z score. HDL altered is considered a z-score ≤ -1.5SD of the mean. Z scores were calculated following Stavnsbo references (261).Values represent the percentage of the abundance. IQR: interquartile range. P-value for differences between groups calculated by Mann-Whitney U test.

Supplementary Table 4 continuation. Main taxonomic differences between children with altered levels of triglycerides, HOMA-IR, or SBP and those with normal levels

	Normal	Altered	P-value
	Median [IQR]	**Median [IQR]**	
d__Bacteria;p__Firmicutes;c__Clostridia;o__Oscillospirales;f__Oscillospiraceae;g__UCG-005;__	0.16 [0.05;0.37]	0.07 [0.02;0.17]	0.009
d__Bacteria;p__Firmicutes;c__Clostridia;o__Oscillospirales;f__UCG-010	0.06 [0.00;0.31]	0.01 [0.00;0.07]	0.008
d__Bacteria;p__Firmicutes;c__Clostridia;o__Oscillospirales;f__UCG-010;g__UCG-010	0.06 [0.00;0.31]	0.01 [0.00;0.07]	0.008
d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae;g__Prevotella	0.04 [0.01;4.19]	0.01 [0.00;1.38]	0.043
d__Bacteria;p__Firmicutes;c__Clostridia;o__Oscillospirales;f__Ruminococcaceae;g__[Eubacterium]_siraeum_group	0.02 [0.00;0.15]	0.00 [0.00;0.03]	0.019
d__Bacteria;p__Firmicutes;c__Clostridia;o__Oscillospirales;f__Ruminococcaceae;g__uncultured;__	0.02 [0.00;0.07]	0.00 [0.00;0.03]	0.042
d__Bacteria;p__Firmicutes;c__Clostridia;o__Peptostreptococcales-Tissierellales;f__Anaerovoracaceae;g__Family_XIII_UCG-001	0.02 [0.00;0.04]	0.00 [0.00;0.02]	0.007
d__Bacteria;p__Firmicutes;c__Clostridia;o__Peptostreptococcales-Tissierellales;f__Anaerovoracaceae;g__Family_XIII_UCG-001;s__uncultured_bacterium	0.02 [0.00;0.04]	0.00 [0.00;0.02]	0.007
d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae;g__Prevotella;__	0.01 [0.00;3.24]	0.00 [0.00;0.05]	0.021
d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Bacteroidales;f__Rikenellaceae;g__Alistipes;s__Alistipes_indistinctus	0.01 [0.00;0.07]	0.00 [0.00;0.02]	0.039

Altered: health outcome ≥1.5SD of the mean of z score. Normal: health outcome < 1.5SD of the mean of the z score. HDL altered is considered a z-score ≤ -1.5SD of the mean. Z scores were calculated following Stavnsbo references (261).Values represent the percentage of the abundance. IQR: interquartile range. P-value for differences between groups calculated by Mann-Whitney U test.

Supplementary Table 5. Logistic regression models of alpha diversity indexes with effect on the presence of one or more altered metabolic risk factors (being classified as Metabolically Unhealthy Obesity).

Dependent variable	Presence of MUO								
Predictors	OR	95% CI	p	OR	95% CI	p	OR	95% CI	p
(Intercept)	0.01	0.00 – 0.28	**0.010**	0.01	0.00 – 0.55	**0.027**	0.02	0.00 – 2.72	0.123
age y	1.36	1.10 – 1.70	**0.005**	1.35	1.10 – 1.69	**0.006**	1.35	1.09 – 1.68	**0.006**
sex [girl]	1.43	0.75 – 2.77	0.278	1.34	0.71 – 2.55	0.375	1.33	0.70 – 2.53	0.384
BMI	3.93	1.81 – 8.98	**0.001**	4.14	1.91 – 9.50	**<0.001**	4.14	1.91 – 9.47	**<0.001**
CHAO1	0.98	0.96 – 1.00	**0.023**						
Shannon				0.42	0.18 – 0.92	**0.033**			
Simpson							0.02	0.00 – 0.87	**0.050**
Observations	191			191			191		
R^2 Tjur	0.104			0.102			0.099		

Reply letter to the editor- Manuscript 1

Reply – Letter to the editor "Comment on Gut microbiota is associated with metabolic health in children with obesity"

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Letter to the editor: "Comment on Gut microbiota is associated with metabolic health in children with obesity

José Maurício Lucas da Silvaa, João Henrique da Costa Silva , Mayara Luclécia da Silvaa, Viviane de Oliveira Nogueira Souza , Waleska Maria Almeida Barrosa.

Dear Editor, With great satisfaction, we analyzed the study by Alcazar et al. (1) which aimed to describe the composition and diversity of the intestinal microbiota in children with obesity and characterize the differences in the intestinal microbiota according to their metabolic health status. Thus, 191 participants of both sexes were included, aged between 8 and 14 years.

Initially our attention was focused on the division of two groups in this research, where one of these was characterized by metabolically healthy individuals (MHO) and the other metabolically unhealthy (MUO). It has also been described that lower proportions of Christensenellaceae and Akkermansia associated with a greater number of Bacteroides, appear to be indicative of an unhealthy metabolic state in obese children. It is known that the intestinal microbiota can be modified according to lifestyle, thus it was described in the study by GuevaraCruz et al. (2) that changes in life habits are able to limit the occurrence of dysbiosis of the intestinal microbiota by reducing bacteroides and increase in the abundance of Akkermansia. Based on this, when evaluating individuals from metabolic and microbiota aspects, it is necessary to take into account, influence of eating habits and physical activity, since these, may cause interaction between metabolic changes and colonization of bacteria in the intestine (3). Thus, it would be possible to determine in a reliable way whether the individuals who are allocated in the MHO and MUO groups are undergoing food intervention or physical activity, according to intestinal microbiota. Another interesting, factor would be the

application of a semi-structured questionnaire containing clinical information, that could be answered either by the child's guardian or by the child himself, related to the use of vitamin supplementation, in view, vitamin D may cause changes the composition and diversity of the intestinal microbiota (4). In addition, it was observed that in the present study it was not described the use of a statistical test to to evaluate the distribution of normality of the sample and to define the use of parametric and nonparametric tests. Therefore, we highlight the importance of analyzing the distribution of normality, and its use is undeniable, because when there is no occurrence of this, the interpretations and inferences of the data may not be reliable or valid (5). Finally, we reiterate the relevance of the study by Alcazar et al. (1) and its scientific contribution to describe the composition and diversity of the intestinal microbiota in children obesity, since this theme is quite relevant in the current context. Funding Statement This work was supported by Fundação de Amparo à Ciência e Tecnologias do Estado de Pernambuco (FACEPE). Conflict of Interes

The authors have no conflicts of interest to report. Author Contributions JMLS idealized the letter, writing of the manuscript. JHCS, MLS, VONS and WMAB writing and revision of the manuscript. All authors read and approved the final version the manuscript.

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Reply – Letter to the editor "Comment on Gut microbiota is associated with metabolic health in children with obesity"

Authors: Alcazar M, Escribano J, Ferré N, Closa-Monasterolo R, Selma-Royo M,Luque V.

We are grateful to Mr Lucas Da Silva and colleagues for their interest in our publication entitled "Gut microbiota is associated with metabolic health in children with Obesity".

We are aware and fully agree, that there are crucial factors such as eating habits and physical activity influencing the gut microbiota variability. Furthermore, we add, that at the same time, those lifestyle factors may affect cardiovascular health (1), as well (Figure 1), establishing a complex interrelationship between exposures and effects. The authors suggested including vitamin D deficiency among other factors influencing the gut microbiota. We appreciate this suggestion, which stresses again the complexity of analysing all possible interrelated factors influencing the association between microbiota and Health. For example, Vitamin D deficiency interacts as well with diet, physical activity, microbiota and gut barrier functions (2).

Figure 1. Factors potentially associated with gut microbiota and cardiovascular health in children with obesity (2–4). Image created with Biorender.com

The published study, to which the letter referred, aimed to evaluate whether the gut microbiota was different between children with MHO and with MUO before starting a weight-loss intervention (5). Further analyses of the project will try to associate the interactions between lifestyle factors with microbiota composition and cardiometabolic health.

Another aspect that concerned the authors of the Letter, is that we did not report on the use of a statistical test to evaluate the distribution of normality of the sample. It is well known that a huge inter-individual variance exists in gut microbiota diversity and relative abundances of their components, and although we did not report on the normality assessment performed, variables were reported as median and interquartile ranges, and non-parametric tests were used for testing differences and paired correlations.

Uncertainties in the association between exposures and health effects are the driving force keeping these investigations active.

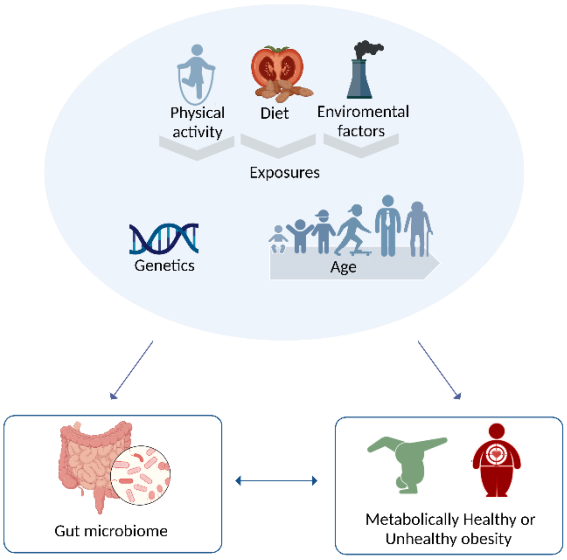

Figure 1. **Factors potentially associated with gut microbiota and cardiovascular health in children with obesity (2–4).** Image created with Biorender.com

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Manuscript 2

Children's gut microbiota predicts the efficacy of the obesity treatment

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Study importance questions (requested by the journal)

What is already known about this subject?

- Dietary interventions may modify the gut microbiota to a more favourable profile linked to the improvement of metabolic alterations
- It is not known whether gut microbiota plays a role as a cause, as a mediator and/or as a consequence in the relation between lifestyle and health outcomes
- In adults, a specific profile of gut microbiota has been associated with the response to a weight loss intervention, suggesting a potential cause-mediation link between diet and cardiometabolic risk

What are the new findings in your manuscript?

- In children with obesity, higher diversity before a nutritional intervention was associated with higher improvement in the metabolic health
- A specific profile characterized by high *Faecalibacterium* may predict a successful intervention

How might your results change the direction of research or the focus of clinical practice?

- Our results add evidence to the importance of the gut microbiota in tailored treatments in children with obesity.
- A possible tailored treatment would be to perform interventions (i.e., as supplements) to specifically improve unfavourable gut microbiota profiles before promoting lifestyle changes, to increase its success rate.

Abstract

Objective: Our study aimed to evaluate whether gut microbiota could help to predict the success of an intervention for weight loss in a cohort of children with obesity.

Methods: Data from 44 children with obesity (8-14y) were obtained from the Microbekids study. The gut microbiota was determined by 16s rRNA gene sequencing. Body Mass Index (BMI), waist and biochemistry were assessed at baseline and final (1 year later) visits. A MetScore was calculated as the sum of systolic and diastolic blood pressure, waist, Triglycerides, HOMA-IR, and -HDL-cholesterol z-scores. BMI or MetSCore responders (BMI-R, MetScore-R) were those with an improvement more significant than the median from the overall study sample.

Results: BMI-R presented higher abundances of *Veillonella* and lower abundances of *Sutterella* (p=0.041, p=0.019). *Bilophila* and *Sutterella* concentrations were negatively associated with BMI loss (r=0.031, p=0.043; r=0.33, p=0.035). MetScore-R presented higher abundances of *Faecalibacterium* (p=0.038) and *Eubacterium coprostanoligenes* (p=0.017), both compared to non-responders. Alpha diversity (r=0.37, p=0.01), *Faecalibacterium* (r=-0.33, p=0.029) and *Eubacterium coprostanoligenes* (r=-0.47, p=0.001) were associated with greater MetScore improvement, while Bacteroides (r=0.34, p=0.024) were associated with less MetScore improvement.

Conclusion: Gut microbiota composition may predict the success of a nutritional intervention for weight loss in children with obesity.

Introduction

Dietary habits, together with physical activity, represent some of the most important modifiable factors associated with the development of obesity (1,2). Thus, the efforts to treat and prevent obesity have been centred on lifestyle programmes, including dietary recommendations aiming to reduce body weight and improve metabolic health in both adults and children (3). However, it has been observed that each person may show different responses to the same treatment (4). To tailor obesity interventions to a specific individual or group's needs, personalized nutrition integrates several host-specific variables that could affect the response to intervention (5). Holistically, personalized nutrition would consider exogenic and endogenic factors such as metabolomics, genetics, lifestyle, and gut microbiota (5).

Gut microbiota is involved in several functions of the human body, such as harvesting energy, regulating host immunity, and regulating metabolism (6). Therefore, it has been proposed as an important player in the development of obesity and its associated comorbidities (6,7). Specific gut microbiota profiles could result in a pre-disease state linked to the development of obesity and cardiometabolic disorders (8).

Several studies have been performed on adults and children (9,10) showing how changes in dietary patterns could have an impact on the gut microbiota that may contribute to the alleviation of metabolic deterioration. However, recent studies have observed that the response to an intervention could be influenced by the already existing microbiota (11,12). A work combining the role of anthropometrics, dietary habits, physical activity, blood parameters, and gut microbiota in a cohort of 800 adults revealed that postprandial glycaemic response could be accurately predicted by incorporating gut microbiome features in the machine-learning algorithms (13). In this line, microbes richness has shown the potential to predict the efficacy of a weight-loss dietary intervention in adults with obesity, which exerted improvements in the inflammatory profile (14). Stanislavski et al. concluded that gut microbiota before a behavioural weight-loss intervention predicted the improvement in waist circumference. Specific taxa such as *Akkermansia muciniphila* have been associated with improved metabolic outcomes after a 6-week calorie restriction (15).

In childhood obesity, we recently showed that microbiota diversity, and certain taxa, were associated with better cardiometabolic profiles (16). Nadal et al. (17) reported that Lactobacillus abundances were higher in those children with more significant weight loss. However, to our knowledge, the association between the gut microbiota composition before a weight loss intervention and the success of such intervention has hardly been analysed in

children. Therefore, our study aimed to evaluate if gut microbiota could help to predict the success of an intervention for weight loss in a cohort of children with obesity.

Methods

Study design and participants

This work was a longitudinal observational study secondary to a randomized clinical trial on a weight loss intervention for children with obesity. The present work used the baseline and final anthropometry and biochemistry data, along with the microbiota composition of the participants enrolled in the Obemat2.0 clinical trial (18) (clinicaltrials.gov NCT03749200) and Microbekids trial (clinicaltrials.gov NCT03749291).

Data from 305 children with obesity (163 males; 140 females) aged 8 to 14 were obtained from the clinical trial OBEMAT2.0 at baseline. Children were recruited from June 2016 to March 2018 from primary healthcare centres belonging to the "Camp de Tarragona" healthcare area. Obesity diagnosis criteria were BMI ≥ 97th percentile, according to Hernandez et al. (19), as indicated by the National Clinical Practice Guidelines. Baseline assessments were conducted at hospital Universitari de Tarragona Joan XXIII and Hospital Universitari Sant Joan de Reus. During the baseline assessment, the trial participants were invited to take part in a voluntary faecal and blood sample extraction; the same procedure was done at the final assessment one year

later. Participants who followed the intervention Per Protocol (attending to at least nine of the eleven intervention visits, as well as the baseline and the final assessments), participated in the baseline faecal sample collection, participated in both baseline and final blood extraction, and had no missing data for any of the anthropometric data, were included in the analyses.

Anthropometry, blood pressure and biochemical parameters

Body weight was measured using a digital scale (SECA 769, precision 50 g) in underwear. Height was measured by a wall-mounted stadiometer (SECA 216, precision 0.1 cm). The waist was measured as the mid-point between the iliac crest and lower rib with a Holtain non-extensible tape (precision 0.1cm). Body mass index (BMI) was calculated as weight over squared height (kg/m2). BMI z-score was calculated according to the World Health Organization references (20).

A trained nurse extracted a blood sample from participants in fasting conditions. High-density lipoproteins cholesterol (HDL) (mg/dL), triglycerides (mg/dL), glucose (mg/dL) and insulin (µIU/ml) were measured in the laboratories of local study sites using routine clinical diagnostic methods. Insulin was quantified by immunoradiometric assays, and the others were measured using routine clinical diagnostic enzymatic methods. The Homeostasis Model Assessment of Insulin resistance (HOMA-IR) was calculated as HOMA-IR = (Insulin mIU x Glucose (mmol/L)) / 22.5 (21).

Study personnel measured systolic blood pressure (SBP) and diastolic blood pressure (DBP) (mmHg) at least 20 min after arriving at the study centre. Blood pressure was assessed in duplicate (with a time slot of 5 min between measures) using a Dinamap Pro 100 device on the left arm while the child was sitting down with the arm lying comfortably. The mean value of the two measures was calculated.

Biochemical parameters, SBP, and DBP, were standardized as z-scores using the Stavnsbo et al. references (22).

Faecal DNA collection and extraction

All participants who agreed to participate in the faecal sample collection received a stool sampler, ice bag, and ice box to collect their samples one day before the scheduled meeting and save it in a -20oC freezer. On the day of the date, the samples were stored in -80oC freezers of the respective Biobanks.

DNA libraries were obtained following the 16srDNA GENE Metagenomic Sequencing Library Preparation Illumina protocol (Cod. 15044233 Rev. A). The gene-specific sequences targeted the variable V3 and V4 regions. The primers were selected according to Klindworth et al., 2019 (23). Microbial genomic DNA (5 ng/µl in 10 mM Tris pH 8.5) was used to initiate the protocol. The multiplexing step was performed using Nextera XT Index Kit (FC-131-1096) (Illumina, San Diego, CA, USA). One µl of the PCR product was run on a Bioanalyzer DNA 1000 chip to verify the size (the expected size on a

Bioanalyzer trace was ~550 bp). The libraries were sequenced using a 2x300pb paired-end run (MiSeq Reagent kit v3 (MS-102-3001)) on a MiSeq- Illumina Sequencer (FISABIO sequencing service, Valencia, Spain) according to the manufacturer's instructions. The quality assessment was performed using prinseq-lite program (24) and sequences were selected with a minimum length of 50. Sequence data were analysed using the quiime 2 pipelines by Boylen et al. (25). The metataxonomic analyses were performed using some quiime2 plugins. Denoising, paired ends joining, and chimaera depletion was performed starting from paired ends data using the DADA2 pipeline (26). Taxonomic assignment was conducted using the Silva v138 database with the addition of the specie level classification by the same database (27). Taxa representing less than 0.01 % of the reads across all the samples were filtered.

Cardiometabolic 126res t

To assess the children's cardiometabolic risk, we created a continuous variable, the cardiometabolic risk score (MetScore), based on Eisenmann et al. (28). This score was calculated as the sum of the standardized SBP, DBP, triglycerides, HOMA-IR, and HDL cholesterol z-scores, this last one multiplied by -1 (as HDL cholesterol is inversely related to cardiometabolic risk). A higher score was indicative of a less favourable cardiometabolic profile.

Response to the intervention

Participants included in the study were classified as responders and non-responders according to their improvement in BMI z-score and Metabolic risk score. BMI-responder (BMI-R) or MetSCore-responder (MetScore-R) were defined as subjects having a reduction of the parameter (BMI z-score or Metabolic risk score, respectively) greater than the median reduction for each parameter in the overall study sample. The median difference for BMI was -0.36. The median difference for the MetScore was -1.14. Those children that did not meet these criteria were classified as non-responders (BMI-NR, MetScore-NR).

Statistical analysis

Kolmogorov-Smirnov test was performed to assess the normality of the variables. Descriptive data were reported as the median and interquartile range (25^{th}-75^{th} percentiles) for continuous variables and as frequency and percentage (n, %) for categorical variables.

Wilcoxon signed-rank tests were performed for testing differences in anthropometrical and biochemical parameters, the relative abundance of bacterial taxa and alpha diversity between responders and non-responders' groups, and baseline and final assessments.

Bacterial taxa abundances were expressed as the relative abundance (%) in overall composition Alpha diversity indices were represented as CHAO1,

Shannon's and Simpson's indices (expressed as inverse Simpson's index). Beta diversity analysis between responders and non-responders was conducted by permutational multivariate analysis at the amplicon sequence variant (ASV) level using Bray-Curtis's distance in the MicrobiomeAnalyst online platform (29). The clustering of the samples according to the response to the intervention was performed by principal component analysis at the ASV level. Spearman's rank correlations were used to find simple associations between the different alpha diversity indexes (CHAO1, Shannon, Simpson), the relative abundance of all the bacteria and the improvement of BMI and metabolic health parameters.

Bacteria significantly associated with BMI or MetScore reduction and with a relative abundance higher than 0.1% were included in different linear and logistic regression models. The models for the variability of the BMI z-score were adjusted for baseline age, sex, and baseline BMI (z-score). Models for the variability of the MetScore were adjusted by the difference in BMI (z-score) and BMI, sex age and MetScore at the baseline assessment. The selection of the variables for the best model was made by stepwise regression by bidirectional elimination.

Logistic regression models were performed to analyse the odds of a successful response to the intervention (e.g., classified either as BMI-R or MetScore-R) depending on the gut microbiota composition. Models to quantify the odds of being BMI-R were adjusted by baseline BMI, age, and gender, while models to

quantify the odds of being MetScore-R were adjusted by sex, BMI (z-score) reduction, and BMI, age and MetScore at the baseline assessment. The selection of variables for the best model was made by stepwise regression by bidirectional elimination.

Data management and statistical analyses were conducted using Rstudio v1.4.1717 (30).

Ethics

The study followed the rules of the Declaration of Helsinki (31) and was approved by the ethics committees responsible for the activity of all the involved study centres. All parents or legal guardians signed the informed consent before study enrolment as well as for participating in the registered samples collection (COLOBEPED, reference C.0004585), and children aged 12 years or above signed informed assent to participate.

Results

Characteristics of the study participants

Seventy-two participants accepted, brought a faecal sample at baseline, and participated in the blood extraction at both baseline and final assessments. Finally, forty-two participants (57% boys and 43 % girls) of the Microbekids study followed at least 9 of the 11 Obemat2.0 intervention visits and were included in this work (Figure S1). Table 1 shows the baseline and final assessment characteristics of all the participants. Median age was 10.0

[9.00;12.0] years, and BMI z-score was 2.57 [2.12;2.73] at study entry. There was a significant decrease in BMI z-score over 0.4SD ($p<0.001$), waist (0.34 SD; $p=0.010$), HOMA-IR (0.42 SD; $p=0.001$), a slight increase in diastolic blood pressure ($p=0.026$), and a significant improvement in MetScore of around 1 SD ($p=0.030$) associated to the lifestyle intervention. The median difference between the final and baseline MetScore and BMI was -1.14 [-2.70;0.82] and -0.36 [-0.56;-0.09], respectively.

All participants were classified according to their response to the intervention as BMI-R (n=21) or BMI-NR (n=21) and as MetScore-R (n=21) or MetScore-NR (n=21). Characteristics of the study participants according to the response group are described in Table S2. There were no statistically significant differences between the different response groups in anthropometrical or biochemical parameters at baseline assessment. At the final assessment, BMI-R subjects showed lower triglycerides ($p=0.008$), SBP and DBP ($P=0.011$; $p=0.004$, respectively) and lower MetScore ($p=0.001$) than the BMI-NR. MetScore-R showed significantly lower BMI ($p=0.001$), waist ($p=0.004$) and MetScore z-scores ($p=0.012$) at the final assessment compared to MetScore-NR subjects.

Gut microbiota characteristics by the response to the intervention

We did not observe significant differences in alpha diversity between responders and no responders at baseline. However, there was a trend to

higher values of CHAO1, Shannon and Simpson among the two responder groups compared to the two non-responders (**Figures 1A,1B).** No significant differences were found in the overall baseline microbiota β-diversity between responders and non-responders **(Figures 1C,1D).**

Figures 2A and 2B present the most abundant phyla, which were: Firmicutes, Bacteroidota, Actinobacteriota, Proteobacteriota, Desulfobacteria and Verrucomicrobiota. No significant differences between response groups were found at the phylum level. At the genus level, we found that BMI-R were characterized by lower abundances of *Sutterella* (p=0.019) and higher values of the *Veilonella* (p=0.041). MetScore-R subjects were characterized by higher relative abundances of *Eubacterium coprostanoligenes* (p=0.038) and *Faecalibacterium* (p=0.017) (**Figures 2C, 2D**).

Table 1. Characteristics of the study cohort at baseline and final assessments

	Baseline Assessment Median [IQR]	**Final assessment** Median [IQR]	p-value
	n= 42		
Gender (males/females)	24/18	24/18	
Age (y)	10.0 [9.00;12.0]	11.0 [10.0;13.0]	< 0.001
BMI z-score	2.57 [2.12;2.77]	2.13 [1.71;2.66]	<0.001
Waist z-score	1.87 [1.50;2.34]	1.53 [1.03;2.20]	0.010
Triglycerides z-score	0.17 [-0.31;0.68]	-0.04 [-0.53;0.44]	0.556
HDLc z-score	-0.41 [-0.93;0.21]	-0.46 [-0.81;0.14]	0.320
HOMA-IR z-score	0.60 [0.36;1.27]	0.18 [-0.38;0.63]	0.001
Systolic blood pressure z-score	0.51 [-0.35;1.31]	0.13 [-0.27;1.07]	0.099
Diastolic blood pressure z-score	-0.09 [-0.49;0.44]	0.13 [-0.27;1.07]	0.026
Metabolic Risk Score	4.18 [1.97;5.72]	3.18 [1.13;5.11]	0.030
Difference between Final BMI and Baseline BMI[a]	-0.36 [-0.56;-0.09]		
Difference between Final MetScore and Baseline MetScore[b]	-1.14 [-2.70;0.82]		

BMI: Body mass index; HDLc: High-density lipoprotein cholesterol, MetScore: Metabolic risk score. P-value calculated by paired samples Wilcoxon test. [a] Difference in BMI = Final BMI z-score – Baseline BMI z-score. [b]Difference of MetScore = Final MetScore – Baseline MetScore. A lower difference indicates a greater decrease in the BMI and the MetScore.

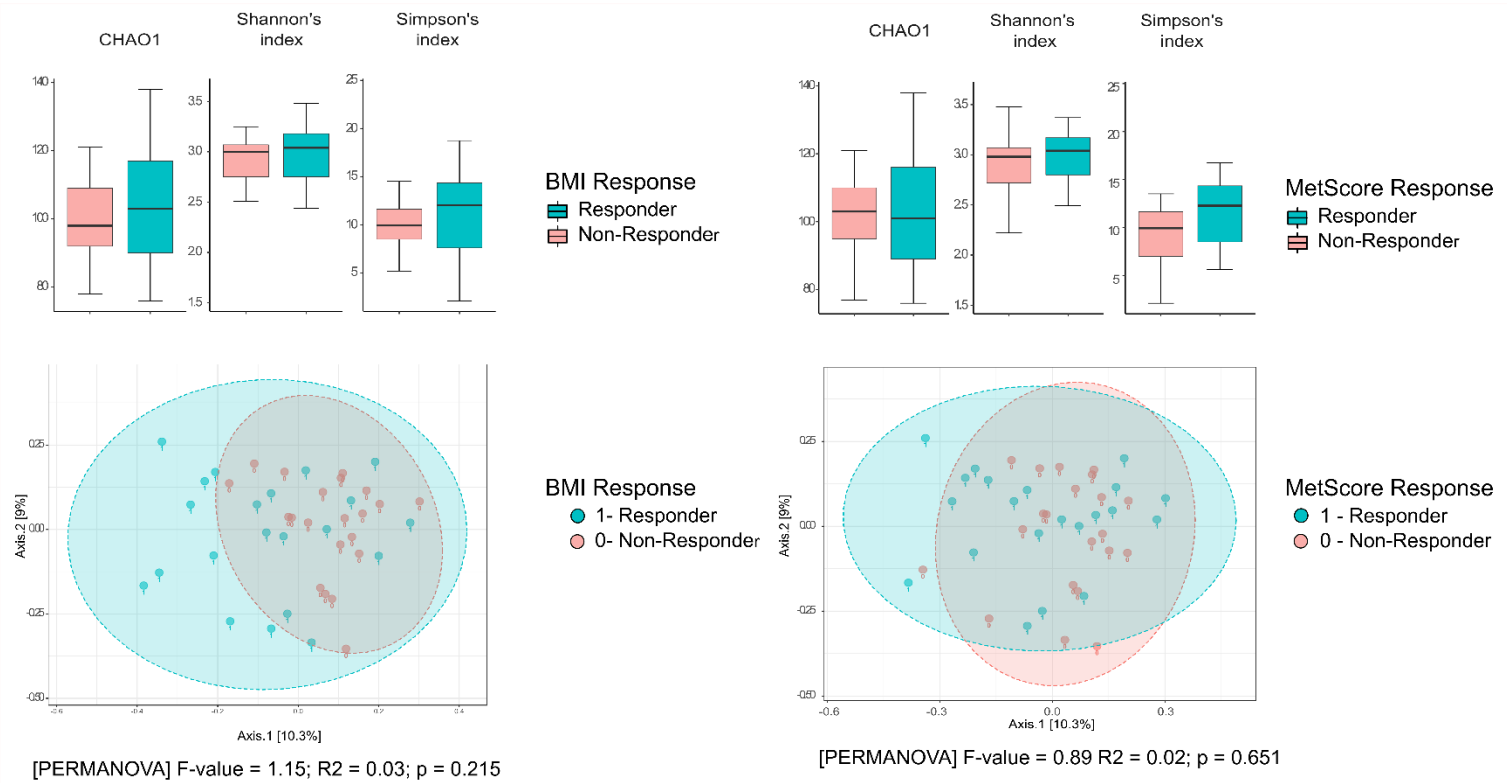

Figure 1. Alpha and Beta diversity of the baseline gut microbiota. The baseline alpha diversity indices for the microbiota samples are labelled by A) BMI response to the intervention and B) MetScore response to the intervention. Permutational MANOVA PCoA is represented labelled by C) BMI response to the intervention and D) MetScore response to the intervention.

Baseline gut microbiota as a predictor of the response to the intervention

Even though there were no significant differences in gut microbiota diversity between responders and non-responders, we have found statically significant associations between the baseline Shannon and Simpson's indices and improvements in MetScore (Rho=-0.37, p=0.01 and Rho=-0.42; p=0.005, respectively), SBP (Rho= -0.48, p=<0.001 and Rho=-0.45, p=0.002, respectively), and HDL (Rho = 0.40, p = 0.007 and Rho = 0.46, p=0.001, respectively) **(Figure 3).** Results from linear regression models showed that Shannon and Simpson's indices, adjusted by different clinical characteristics, could explain around 25% of the MetScore improvement **(Figure 4).** Results of the linear regression models are shown in **S2**. Binary logistic regression models showed that there was no higher risk of not responding to the intervention according to Shannon's and Simpson's indices (data not shown).

Figure 3 shows paired correlations between specific taxa (with relative abundance higher than 0.10), alpha diversity indices and the response to the intervention (BMI and MetScore reductions) and the changes in the cardiovascular risk factors. We have found that phylum Desulfobacteriota was significantly negatively associated with less BMI loss (rho=0.36; p-value= 0.018). At the genus level, *Bilophila* (rho=0.31, p = 0.043) and *Sutterella* genus (Rho 0.33; p=0.035) were following the same trend. The MetScore improvement was significantly associated with Bacilli's class (Rho= -0.33;

p=0.031). At genus level, *Ruminoccocus*, *Faecalibacterium* and *Eubacterium coprostanoligenes group* showed also significant associations with the metabolic improvement (Rho=-0.31, p=0.047; Rho= -0.33, p=0.029; Rho= -0.47, p=0.001, respectively). Higher abundances in *Bacteroides* and *Escherichia Shigella* genus before the intervention were associated with less MetScore improvement (Rho=0.34, p=0.024; Rho=0.42, p=0.005).

Linear regression models explaining BMI reduction by the genus (previously showing significant simple paired correlations), adjusted by the baseline BMI, sex and age, are presented in **Table 2**. Biophilia abundances were significantly associated with no BMI improvement by the intervention (p=0.005). The different genera were not associated with an increased risk of not responding to the intervention in logistic regression models (data not shown).

Table 3 shows linear regression models on the MetScore improvement explained by the genus (previously showing significant simple paired correlations), adjusted by baseline age, sex, MetScore and BMI and the difference in BMI. These models revealed that baseline abundances of *Faecalibacterium* and *Eubacterium coprostanoligenes* were significantly associated with the MetScore improvement (p=0.001 and p=0.005, respectively). On the other hand, *Escherichia shigella* and *Bacteroides* were associated with a lower response to the intervention. The combination of low levels of *Faecalibacterium* and *Eubacterium coprostanoligenes* with increased

levels of *Bacteroides* presented the highest prediction for MetScore improvement (R^2 adjusted= 50%). Logistic regression analyses of faecal microbiota on MetScore improvement are shown in Table 4. Higher abundances of the *Faecalibacterium* genus at baseline increased the odds of responding positively to the intervention (Odds ratio, 95%IC: 1.47[1.14-2.07], p=0.008). As in linear regressions, the combination of higher *Faecalibacterium* and *Eubacterium* with lower *Bacteroides* abundances may predict the success of the intervention.

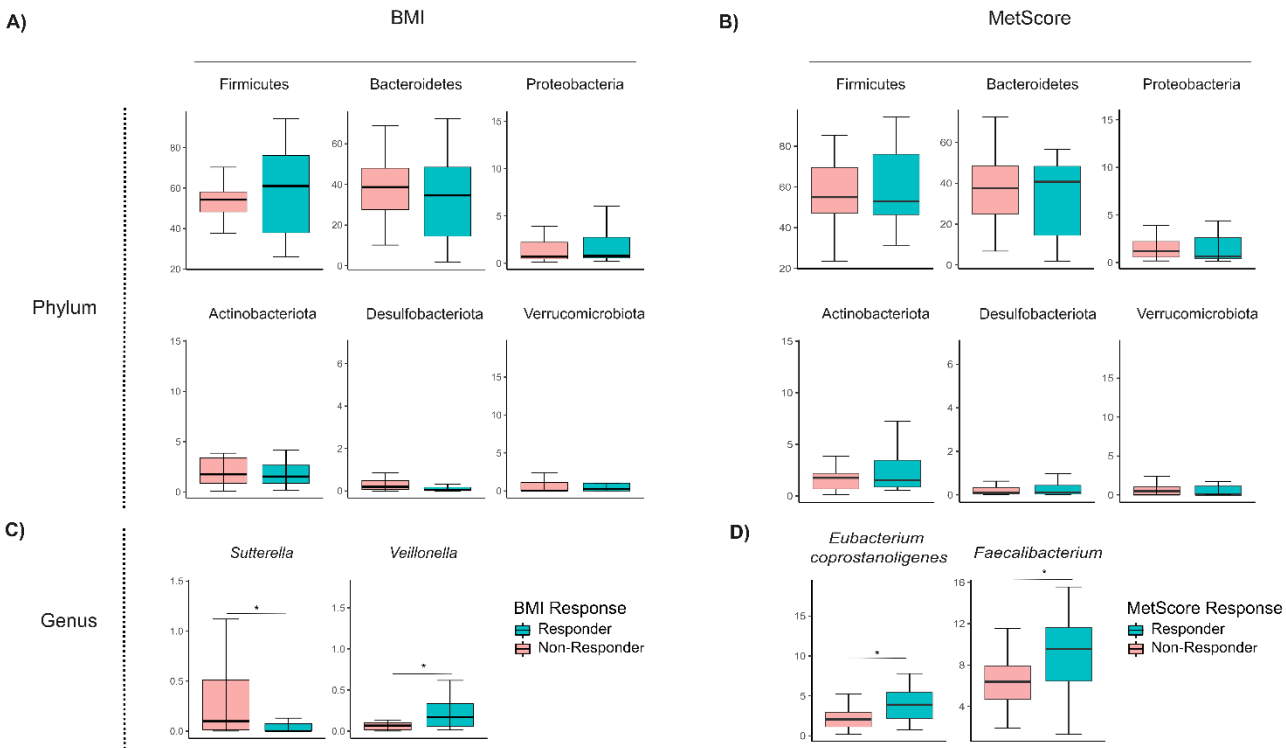

Figure 2. Characteristics of the baseline gut microbiota based on the response to the intervention. A) Average relative abundance of the most abundant phyla and average relative abundance of the significant different genera among the MetScore-R (n=21) and the MetScore-NR (n=21). A) Average relative abundance of the most abundant phyla and average relative abundance of the significant different genera among the BMI-R (n=21) and the BMI-NR (n=21). * p<0.05

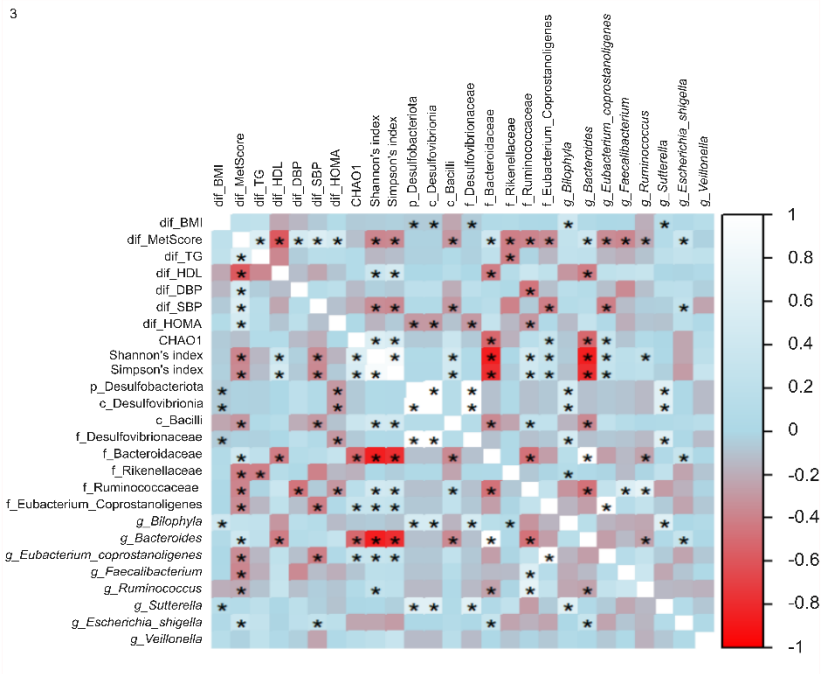

Figure 3. Paired correlations between alpha diversity, abundant taxa and the improvement of health outcomes. The heatmap represents Spearman's rank correlation coefficients between the different parameters. Positive or negative correlations are represented in red and blue, respectively. BMI: Body mass index, TG: triglycerides HDL: high-density lipoproteins, SBP: systolic blood pressure, DBP diastolic blood pressure. HOMA insulin resistance index, MetScore: cardiometabolic risk score. Cardiovascular factors are expressed as z-scores.
* Indicates Spearman's correlation p-value < 0.05

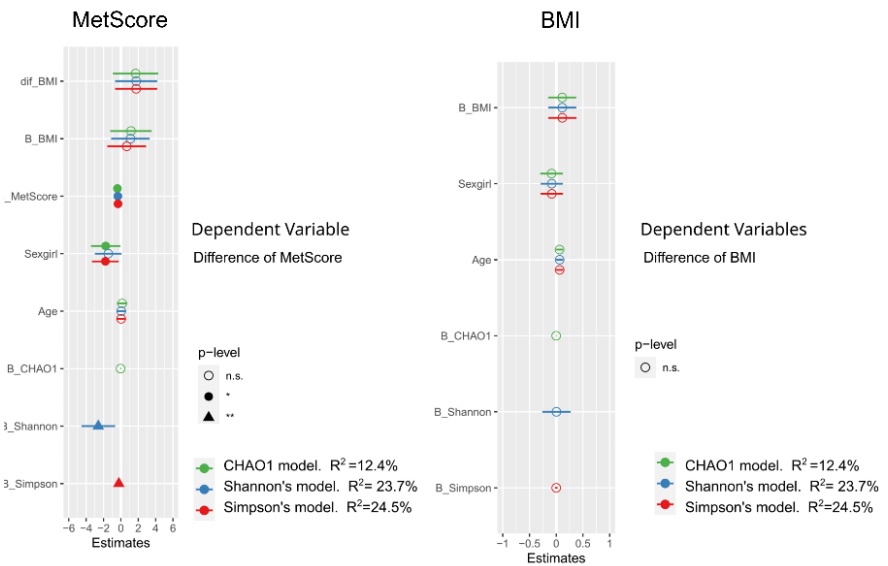

Figure 4. Associations between gut microbiota and the change of BMI and Metabolic risk score. a) Forest plots of the linear regression models (adjusted by baseline BMI, the difference in BMI, age and sex) was used to represent the variability of the MetScore. B) Forest plots of the linear regression models (adjusted by the Baseline BMI, sex, and age) were used to represent the variability of the BMI. Greater negative indices correlate with a greater decrease in the BMI or the Metabolic Risk score and vice versa.

Table 2. Multiple linear regression models for the improvement of the BMI

Dependent variable	**The difference in the BMI**					
	Bilophila		***Sutterella***		***Veillonella***	
Predictors	Estimates	p	Estimates	p	Estimates	p
(Intercept)	-1.36 (-2.34 – -0.37)	**0.008**	-1.30 (-2.37 – -0.22)	**0.019**	-1.37 (-2.47 – -0.27)	**0.016**
B_BMI	0.11 (-0.13 – 0.34)	0.355	0.09 (-0.16 – 0.35)	0.467	0.12 (-0.14 – 0.38)	0.352
Sex [girl]	-0.11 (-0.30 – 0.07)	0.228	-0.12 (-0.34 – 0.09)	0.247	-0.08 (-0.28 – 0.13)	0.458
Age	0.06 (0.00 – 0.12)	**0.038**	0.07 (0.00 – 0.13)	**0.042**	0.07 (0.00 – 0.13)	**0.045**
g Bilophila	**0.84 (0.27 – 1.41)**	**0.005**				
g Sutterella			0.20 (-0.10 – 0.50)	0.193		
g Veillonella					0.15 (-0.15 – 0.45)	0.321
Observations	42		42		42	
R^2 / R^2 adjusted	0.280 / 0.202		0.149 / 0.057		0.132 / 0.038	

BMI: Body mass index; B_BMI: Baseline BMI; B_MetScore: Baseline metabolic risk score; B_CHAO1: Baseline CHAO1 index; B_Shannon: Baseline Shannon's index; B_Simpson = Baseline Simpson's index; Difference of BMI = Final BMI z-score – Baseline BMI z-score

Table 3. Multiple linear regression models of the improvement of the MetScore

Dependent variable	**Improvement of the MetScore (no/yes)**											
	Faecalibacterium		***E.coprostanoligenes***		***E.shighella***		***Bacteroides***		***Ruminococcus***		***Best model***	
Predictors	Estimates	p	Estimates	p	Estimates	p	Estimates	p	Estimates	p	Estimates	p
(Intercept)	-3.68 (-12.50 – 5.14)	0.402	-2.99 (-12.21 – 6.23)	0.514	-1.55 (-11.34 – 8.25)	0.751	-3.01 (-12.38 – 6.37)	0.519	-2.38 (-12.55 – 7.79)	0.637	-3.85 (-11.45 – 3.75)	0.310
dif BMI	1.64 (-0.64 – 3.92)	0.154	1.66 (-0.73 – 4.06)	0.166	1.76 (-0.77 – 4.28)	0.166	1.67 (-0.77 – 4.10)	0.173	1.43 (-1.26 – 4.12)	0.287	1.54 (-0.43 – 3.51)	0.122
B BMI	1.90 (-0.23 – 4.04)	0.079	1.43 (-0.77 – 3.62)	0.195	0.71 (-1.62 – 3.04)	0.538	0.90 (-1.33 – 3.12)	0.419	1.06 (-1.34 – 3.46)	0.377	1.93 (0.07 – 3.79)	0.042
B MetScore	-0.47 (-0.78 – -0.16)	0.004	-0.42 (-0.74 – -0.10)	0.012	-0.33 (-0.67 – 0.00)	0.053	-0.36 (-0.68 – -0.04)	0.029	-0.35 (-0.70 – 0.00)	0.052	-0.49 (-0.75 – -0.22)	0.001
Sex [girl]	-1.72 (-3.17 – -0.27)	0.021	-1.75 (-3.27 – -0.23)	0.026	-1.29 (-2.90 – 0.32)	0.113	-1.36 (-2.91 – 0.18)	0.081	-1.62 (-3.30 – 0.05)	0.057	-1.77 (-3.03 – -0.50)	0.008
Age	0.39 (-0.14 – 0.92)	0.146	0.26 (-0.29 – 0.80)	0.343	0.10 (-0.48 – 0.67)	0.737	0.12 (-0.44 – 0.67)	0.671	0.18 (-0.42 – 0.77)	0.552	0.38 (-0.08 – 0.84)	0.105
g Faecalibacterium	-0.37 (-0.58 – -0.16)	0.001									-0.32 (-0.50 – -0.14)	0.001
g Eubacterium coprostanoligenes			-0.32 (-0.54 – -0.10)	0.005							-0.24 (-0.43 – -0.06)	0.013
g Escherichia shigella					0.54 (0.03 – 1.06)	0.040						
g Bacteroides							0.06 (0.02 – 0.11)	0.009			0.04 (-0.00 – 0.08)	0.068
g Ruminococcus									-0.25 (-0.69 – 0.19)	0.260		
Observations	42		42		42		42		42		42	
R2 / R2 adjusted	0.427 / 0.329		0.372 / 0.264		0.301 / 0.182		0.351 / 0.239		0.239 / 0.109		0.601 / 0.504	

BMI: Body mass index; B_BMI: Baseline BMI; B_MetScore: Baseline metabolic risk score; dif_BMI: Final BMI z-score – Baseline BMI z-score. Negative estimates indicate an inverse correlation with the improvement of the MetScore after the intervention.

Table 3 shows linear regression models on the MetScore improvement explained by the genus (previously showing significant simple paired correlations), adjusted by baseline age, sex, MetScore and BMI and the difference in BMI. These models revealed that baseline abundances of *Faecalibacterium* and Eubacterium coprostanoligenes were significantly associated with the MetScore improvement (p=0.001 and p=0.005, respectively). On the other hand, Escherichia shigella and Bacteroides were associated with a lower response to the intervention. The combination of low levels of *Faecalibacterium* and Eubacterium coprostanoligenes with increased levels of Bacteroides presented the highest prediction for MetScore improvement (R2 adjusted= 50%). Logistic regression analyses of faecal microbiota on MetScore improvement are shown in Table 4. Higher abundances of the *Faecalibacterium* genus at baseline increased the odds of responding positively to the intervention (Odds ratio, 95%IC: 1.47[1.14-2.07], p=0.008). As in linear regressions, the combination of higher *Faecalibacterium* and Eubacterium with lower Bacteroides abundances may predict the success of the intervention.

Table 4. Logistic regression models of the response in MetScore to the intervention

Dependent variable	**Improvement of the MetScore (no/yes)**											
	Faecalibacterium		***E.coprostanoligenes***		***Escherichia shigella***		***Bacteroides***		***Ruminococcus***		***Best model***	
Predictors	Odds Ratios	p	Odds Ratios	p	Odds Ratios	p	Odds Ratios	p	Odds Ratios	p	Odds Ratios	p
(Intercept)	0.02 (0.00 – 330.87)	0.425	0.02 (0.00 – 450.06)	0.471	0.02 (0.00 – 211.38)	0.420	0.05 (0.00 – 625.66)	0.529	0.02 (0.00 – 203.68)	0.426	0.00 (0.00 – 0.09)	**0.005**
dif BMI	0.10 (0.00 – 1.41)	0.109	0.11 (0.01 – 1.27)	0.096	0.14 (0.01 – 1.32)	0.103	0.13 (0.01 – 1.36)	0.107	0.17 (0.01 – 1.78)	0.155	0.07 (0.00 – 0.85)	0.058
B BMI	0.72 (0.06 – 8.38)	0.792	1.23 (0.11 – 14.45)	0.863	1.70 (0.18 – 16.97)	0.640	1.71 (0.18 – 17.29)	0.637	1.59 (0.16 – 15.97)	0.686		
B MetScore	1.33 (0.95 – 2.04)	0.131	1.24 (0.89 – 1.89)	0.239	1.14 (0.84 – 1.64)	0.427	1.17 (0.85 – 1.70)	0.373	1.15 (0.84 – 1.65)	0.405	1.34 (0.98 – 2.00)	0.097
Sex [girl]	5.89 (1.24 – 36.14)	**0.035**	5.10 (1.17 – 27.07)	**0.038**	3.59 (0.89 – 16.54)	0.082	4.32 (1.00 – 22.63)	0.061	4.24 (1.06 – 19.76)	**0.050**	8.74 (1.57 – 71.62)	**0.023**
Age	0.93 (0.49 – 1.73)	0.826	0.99 (0.54 – 1.78)	0.967	1.10 (0.64 – 1.93)	0.728	1.07 (0.60 – 1.92)	0.812	1.05 (0.61 – 1.81)	0.848		
g Faecalibacterium	1.47 (1.14 – 2.07)	**0.008**									1.50 (1.14 – 2.18)	**0.011**
g Eubacterium coprostanoligenes			1.30 (1.03 – 1.83)	0.068							1.23 (0.97 – 1.75)	0.150
g Escherichia shigella					0.57 (0.14 – 1.18)	0.348						
g Bacteroides							0.95 (0.89 – 1.00)	0.074			0.95 (0.87 – 1.02)	0.197
g Ruminococcus									1.17 (0.81 – 1.73)	0.398		
Observations	42		42		42		42		42		42	
R^2 Tjur	0.373		0.298		0.208		0.253		0.190		0.485	

BMI: Body mass index; B_BMI: Baseline BMI; B_MetScore: Baseline metabolic risk score; dif_BMI: Final BMI z-score – Baseline BMI z-score. Improvement of the MetScore = MetScore difference ≤ median of the difference.

Discussion

Our work shows that the gut microbiota of children with obesity before starting an obesity treatment may be helpful in predicting the responsiveness to the intervention. Children with higher BMI reduction presented a higher relative abundance of *Veillonella*, and children with greater metabolic health improvement presented higher abundances of the *Faecalibacterium* and Eubacterium coprostanoligenes group before the intervention. Furthermore, we observed that a specific baseline profile characterized by a high abundance of *Faecalibacterium*, and higher microbiota diversity was associated with a better response to the intervention in terms of cardiometabolic health. On the other hand, higher abundances of *Bilophila* were associated with worse responses in terms of reduction of BMI.

In children, it has been previously described that early gut microbiota composition may be a predictor of growth and overweight (32), and shreds of evidence suggest that gut microbiota could predict the effects of dietary intervention in pathologies such as inflammatory bowel syndrome (33). Chumpitazi et al. (33) showed that children with IBS that positively responded to a low FODMAP diet had greater gut microbiota richness. To our knowledge, there was only one previous study in children showing that weight loss was associated with gut microbiota (17). Nadal et al. (17) detected changes in the gut microbiota of children with obesity associated with the intervention and

the response to the intervention; furthermore, they observed higher Lactobacillus abundances before the intervention in subjects with greater body weight loss. To the best of our knowledge, the present work is the first study analysing the capacity of the gut microbiota to predict the response to an intervention.

Previous works have pointed out specific bacteria that could modulate the response to intervention in adults; however, the specific role of different gut microbiota compositions is still poorly understood.

With our work, we observed that *Bilophila* abundance was associated with less BMI improvement. *Bilophila* is a genus included in the Desulfobacterota phylum, an extremely bile-tolerant bacteria that have been associated with metabolic alterations and animal-based diets (34). Jian et al. (35) proposed that higher *Bilophila* abundances may predispose subjects to increase liver fat in response to overfeeding. Interestingly, although we have not observed associations of *Bilophila* with any cardiovascular risk, previous works in mice showed that *Bilophila wadsworthia*, the most important specie within the *Bilophila* genus, aggravated the effects of a high-fat diet in metabolic alterations via promoting intestinal barrier dysfunction, inflammation and bile acid dysmetabolism (36).

A special interest of the present work relies on the fact that, independently of the body weight loss, and the obesity severity before the intervention, the gut

microbiota composition and diversity were associated with the improvement of the metabolic risk following the intervention. Poorer gut microbiota diversity had been previously associated with obesity and cardiometabolic alterations in adults (37,38) and children (16). Le Chatelier et al. (38) observed that obesity is associated with a lower bacterial richness, and those individuals with lower bacterial richness gain more weight over time. A posterior work in line with our findings observed that higher gene richness at baseline was associated with a more marked improvement of adipose tissue and systemic inflammation (14). However, even though Korpela et al. (39) found that specific species could predict the decrease in cholesterol levels, they did not find significant associations between the baseline diversity and the response to the intervention. In our work, we observed that the genus Bacteroides was inversely correlated with both richness and diversity and the response to the intervention, indicating that not only diversity plays a significant role in the responsiveness to an intervention but also specific bacteria taxa.

Dao et al. (15) reported that a higher baseline abundance of *Akkermansia muciniphila* was associated with greater insulin sensitivity and body fat distribution after a six-week energy-restricted diet in adults. In this line, we would like to remark on the potential role of *Faecalibacterium* in the effectiveness of the intervention of this study. Those children who showed greater improvement in metabolic health showed higher baseline abundances of *Faecalibacterium*, the most abundant genus in our sample. Moreover, the

combination of higher *Faecalibacterium* and *Eubacterium coprostanoligenes* group together with lower levels of Bacteroides predicted higher metabolic improvement. The genus *Faecalibacterium* is a well-known butyrate producer, and its anti-inflammatory properties have been previously reported (40). In concrete, its most abundant specie, *Faecalibacterium prausnitzii,* has been described as the most important butyrate-producing bacteria in the human colon (41). Moreover, *Faecalibacterium prausnitzii* has been proposed as a protector against bacterial translocation (42). Its beneficial effects may be associated with its capacity to produce butyrate, which is the most important short-chain fatty acid produced by the gut microbiota and is the main source of energy for the colonic cell. Butyrate is associated with anti-inflammatory effects and the maintenance of gut barrier stability (42). Furthermore, we have observed that *Eubacterium coprostanoligenes* group was associated with improvement of metabolic health; it was previously reported that *Eubacterium coprostanoligenes group* was associated with the degradation of cholesterol to coprostanol (43). Coprostanol is poorly absorbed in the intestine, and some works have found an inverse correlation between plasma cholesterol and faecal coprostanol. Some authors are suggesting cholesterol conversion to coprostanol by gut microbiota as a new strategy for cholesterol control in humans (44).

We have found specific taxa associated with better response to the intervention. However, the variability of the response to the intervention

between different cohorts is diverse. The identification of specific signatures that contributes to individual response and their combination with other physiological parameters is nowadays a challenging field. Hughes et al. (45) highlighted the existing difficulty in comparing different works due to the variability of the methodology, the outcomes, and the population (45).

Possible mechanisms by which the baseline host's gut microbiota could influence the intervention's success could be multifactorial. It is known that specific bacteria such as F.prausnitzii (42) are health-promoter bacteria and may promote satiety, while others such as *B.wadsworthia* (36) are associated with inflammatory responses

One of the limitations of our work was the relatively small sample size of each group which could limit the generalisability of our findings. However, it is worth highlighting that this is the greater sample size of children with microbiota and health associated with changes in an intervention. The fact that the results observed in this study are consistent with previous literature may provide robustness to our study. Another limitation might be that adherence to dietary and physical activity recommendations was not monitored. Diet and physical activity might be responsible for at least part of the health improvements. Further analysis should give light on the complex relationship between all these variables. The study could be biased by a high attrition rate. Efforts to address potential sources of bias were controlled by

confounders and limiting the performance bias by selecting the participants who attended the intervention visits.

Potential applications of study are designing personalized nutrition interventions, aiming at improving the gut microbiota to increase the success of the intervention.

Conclusions

In conclusion, specific baseline microbiota signatures may predict the improvement of BMI and metabolic health after an intervention. The abundance of *Faecalibacterium* may be a good predictor of the effectiveness of an intervention in children with obesity. Further research is needed to holistically combine the lifestyle, the physiological parameters, and the microbiota signatures to improve the personalized treatments of children and adults with obesity.

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Supplementary material for

Children's gut microbiota predicts the efficacy of the obesity treatment

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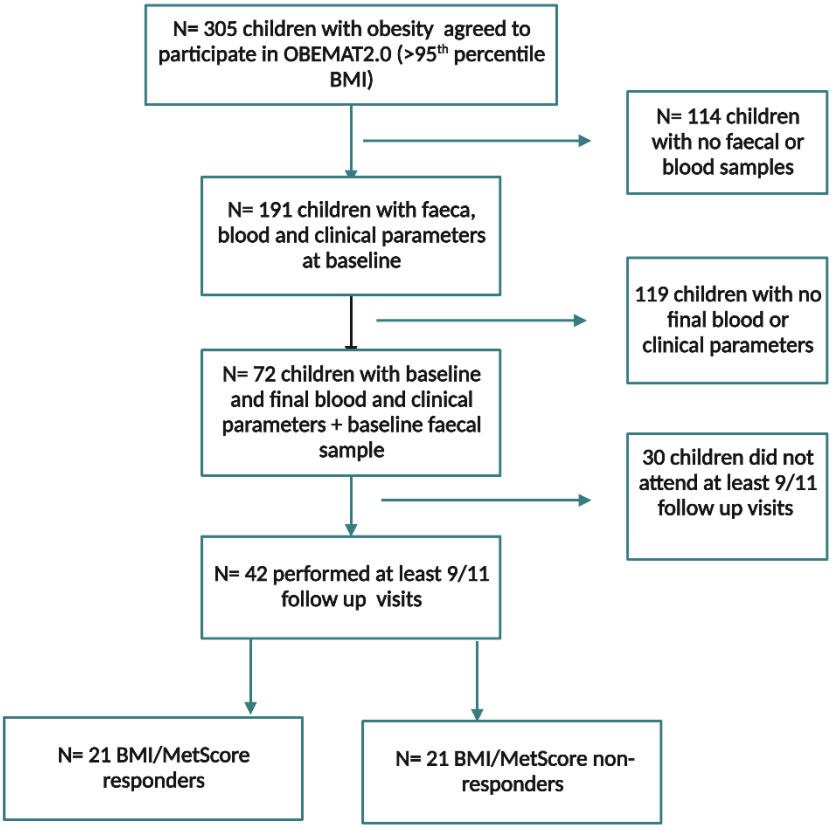

S1

Figure S1. **Flowchart of participants included in this study.** BMI Body mass index, MetScore: Metabolic risk score. Created with BioRender.com.

Supplementary Table 1. Characteristic of the study cohort by the response to the intervention

	BMI Response			MetScore Response		
	Responders	Non-responders	P-value	Responders	Non-responders	P-value
N	21	21		21	21	
Sex(boy/girl)	12/9	12/9		15/6	9/12	
Baseline						
Age (y)	10.0 [10.0;12.0]	11.0 [9.00;12.0]	0,918	10.0 [9.00;11.0]	11.0 [9.00;12.0]	0.224
BMI z-score	2.50 [2.21;2.91]	2.60 [2.12;2.72]	0,86	2.32 [2.08;2.91]	2.61 [2.42;2.70]	0,365
Waist z-score	1.86 [1.63;2.39]	1.88 [1.35;2.15]	0,443	1.86 [1.35;2.39]	1.95 [1.60;2.16]	1
Tryglicerides z-score	0.29 [-0.29;0.47]	0.04 [-0.51;0.75]	0,91	-0.22 [-0.31;0.40]	0.44 [-0.20;0.75]	0,314
HDLc z-score	-0.45 [-0.93;0.21]	-0.39 [-0.93;0.21]	0,94	-0.15 [-1.01;0.38]	-0.58 [-0.90;-0.07]	0,365
HOMA-IR z-score	0.62 [0.47;1.43]	0.50 [-0.11;1.17]	0,099	0.48 [0.10;0.98]	0.90 [0.37;1.45]	0,232
Systolic Blood pressure z-score	0.48 [-0.44;1.28]	0.54 [-0.10;1.32]	0,93	0.48 [-0.44;0.82]	0.54 [-0.10;1.32]	0,563
Diastolic blood pressure z-score	-0.18 [-0.71;0.31]	-0.04 [-0.37;0.62]	0,327	-0.08 [-0.58;0.29]	-0.11 [-0.40;0.66]	0,505
MetScore	4.59 [1.28;6.11]	3.79 [2.04;5.03]	0,458	3.46 [1.22;5.48]	4.64 [3.37;5.75]	0,178
Final assessment						
Age (y)	11.0 [11.0;13.0]	12.0 [10.0;13.0]	0,98	11.0 [10.0;12.0]	12.0 [10.0;13.0]	0,205
BMI z-score	2.09 [1.68;2.45]	2.26 [1.92;2.67]	0,538	1.76 [1.45;2.09]	2.49 [2.17;2.70]	0,001
Waist z-score	1.38 [1.02;2.04]	1.63 [1.07;2.30]	0,302	1.20 [0.92;1.63]	1.76 [1.41;2.52]	0,004
Tryglicerides z-score	-0.35 [-0.79;-0.02]	0.17 [-0.11;1.61]	0,008	-0.22 [-0.54;0.04]	0.17 [-0.11;0.62]	0,08
HDLc z-score	-0.13 [-0.60;0.68]	-0.56 [-0.97;-0.17]	0,131	-0.06 [-0.60;0.68]	-0.59 [-0.97;-0.17]	0,116
HOMA-IR z-score	0.14 [-0.58;0.61]	0.27 [-0.27;0.63]	0,346	-0.20 [-0.62;0.34]	0.29 [0.14;0.67]	0,061
Systolic Blood pressure z-score	-0.04 [-1.01;0.42]	0.76 [0.01;1.34]	0,011	0.13 [-0.25;0.50]	0.46 [-0.38;1.53]	0,314
Diastolic blood pressure z-score	-0.06 [-0.87;0.66]	0.58 [0.40;1.03]	0,004	0.21 [-0.87;0.84]	0.41 [0.19;0.84]	0,279
MetScore	1.27 [-0.79;3.49]	4.57 [2.58;6.43]	0,001	1.70 [0.69;3.49]	4.57 [2.26;5.76]	0,012
MetScore reduction	-2.72 [-4.11;-1.48]	0.86 [0.40;1.98]	<0.001	-1.72 [-4.03;-0.07]	0.40 [-1.21;1.20]	0,03
BMI z-score reduction	-0.47 [-0.55;-0.30]	-0.16 [-0.57;-0.01]	0,068	-0.57 [-0.80;-0.53]	-0.09 [-0.25;0.03]	<0.001

Supplementary Table BMI: Body mass index; HDLc: High-density lipoprotein cholesterol; MetScore: Metabolic risk score. P-value calculated by paired samples Wilcoxon test. [a]BMI reduction = Final BMI z-score – Baseline BMI z-score. [b]MetScore reduction= Final MetScore – Baseline MetScore. A greater negative value indicates a greater reduction in both, BMI and MetScore

Supplementary Table 2. Multiple linear regression models of the baseline alpha diversity indices and the response to the intervention

Dependent variable	Difference of MetScore					
	CHAO1 model		Baseline Shannon		Baseline Simpson	
Predictors	Estimates	p	Estimates	p	Estimates	p
(Intercept)	1.15 (-10.42 – 12.73)	0.841	6.11 (-5.37 – 17.60)	0.287	2.35 (-7.70 – 12.40)	0.638
dif BMI	1.70 (-0.91 – 4.31)	0.195	1.76 (-0.67 – 4.19)	0.151	1.76 (-0.67 – 4.18)	0.150
B BMI	1.16 (-1.22 – 3.55)	0.329	1.11 (-1.12 – 3.33)	0.320	0.67 (-1.56 – 2.90)	0.545
B MetScore	-0.40 (-0.75 – -0.05)	**0.026**	-0.35 (-0.67 – -0.03)	**0.035**	-0.33 (-0.65 – -0.01)	**0.044**
Sex [girl]	-1.75 (-3.44 – -0.07)	**0.042**	-1.46 (-3.00 – 0.08)	0.063	-1.79 (-3.33 – -0.24)	**0.025**
Age	0.15 (-0.45 – 0.74)	0.619	0.04 (-0.52 – 0.60)	0.884	0.03 (-0.53 – 0.59)	0.917
B CHAO1	-0.03 (-0.09 – 0.02)	0.171				
B Shannon			-2.60 (-4.54 – -0.66)	**0.010**		
B Simpson					-0.24 (-0.41 – -0.07)	**0.008**
Observations	42		42		42	
R^2 / R^2 adjusted	0.252 / 0.124		0.349 / 0.237		0.356 / 0.245	

BMI: Body mass index; B_BMI: Baseline BMI; B_MetScore: Baseline metabolic risk score; B_CHAO1: Baseline CHAO1 index; B_Shannon: Baseline Shannon's index; B_Simpson = Baseline Simpson's index. [a]Difference of MetScore = Final MetScore – Baseline MetScore MetScore; [b]Difference of BMI = Final BMI z-score – Baseline BMI z-score. A greater negative value indicates a greater reduction in MetScore

Supplementary Table 2 continuation. Multiple linear regression models of the baseline alpha diversity indices and the response to the intervention

Dependent variable	Difference of BMI					
	CHAO1 model		Baseline Shannon		Baseline Simpson	
Predictors	Estimates	p	Estimates	p	Estimates	p
(Intercept)	-1.20 (-2.56 – 0.16)	0.082	-1.29 (-2.70 – 0.11)	0.070	-1.29 (-2.48 – -0.10)	**0.035**
dif BMI						
B BMI	0.11 (-0.15 – 0.37)	0.393	0.11 (-0.15 – 0.37)	0.392	0.11 (-0.15 – 0.38)	0.392
B MetScore						
Sex [girl]	-0.09 (-0.30 – 0.12)	0.410	-0.08 (-0.29 – 0.13)	0.424	-0.08 (-0.29 – 0.13)	0.431
Age	0.06 (-0.00 – 0.13)	0.065	0.06 (-0.00 – 0.13)	0.061	0.06 (-0.00 – 0.13)	0.061
B CHAO1	-0.00 (-0.01 – 0.01)	0.831				
B Shannon			0.00 (-0.26 – 0.27)	0.979		
B Simpson					0.00 (-0.02 – 0.02)	0.978
Observations	42		42		42	
R^2 / R^2 adjusted	0.109 / 0.013		0.108 / 0.012		0.108 / 0.012	

BMI: Body mass index; B_BMI: Baseline BMI; B_MetScore: Baseline metabolic risk score; B_CHAO1: Baseline CHAO1 index; B_Shannon: Baseline Shannon's index; B_Simpson = Baseline Simpson's index. [a]Difference of MetScore = Final MetScore – Baseline MetScore MetScore; [b]Difference of BMI = Final BMI z-score – Baseline BMI z-score. A greater negative value indicates a greater reduction in MetScore

General results & discussion

Results summary

One hundred ninety-one participants of the Obemat 2.0 were classified by their metabolic status as MHO and MUO and included in this thesis's first study. A subsample of forty-two participants that attended at least 9 of the 11 intervention visits and performed a final assessment and a final blood extraction were classified as responders and non-responders to the intervention based on their BMI and metabolic risk score (MetScore) improvements. This subsample was used to evaluate the associations between gut microbiota and the response to the intervention in the second paper of this thesis.

In summary, this thesis found that the alpha diversity of the gut microbiota is associated with a better metabolic profile and a higher response to the intervention. Moreover, specific bacteria were related to the metabolic health of the children with obesity and the efficacy of the intervention.

We have observed that participants with MHO presented higher alpha diversity, represented as Chao1 ($p=0.021$), ACE ($p=0.020$) and Simpson's ($p=0.045$) indices. However, we did not observe significant differences between responders and non-responders regarding alpha diversity indices. No significant differences were found in the overall baseline microbiota β-diversity between responders and non-responders, neither between MHO nor MUO.

At the phylum level, we have found that MHO presented higher Firmicutes ($p=0.002$) and Verrucomicrobiota ($p=0.001$) phylum abundances in comparison with MUO, which presented higher abundances of *Bacteroides* ($p=0.002$). MHO presented an enrichment of the *Akkermansia and*

Christensenellaceae r-7 group, while *Bacteroides* were in higher abundance in the MUO group. Regarding the response to the intervention, we have found that participants that significantly improved their MetScore (MetScore-R) presented higher abundances of the *Faecalibacterium* and *Eubacterium coprostanoligenes group.* The participants that presented a higher reduction of BMI (BMI-R) presented lower abundances of *Sutterella* (p=0.019) and higher abundances of *Veilonella* (p=0.041) than BMI-NR.

Significant associations were observed between CHAO1 and the baseline MetScore (Rho=-0.15; p=0.03). Moreover, Shannon's and Simpson's indexes were associated with improvements in MetScore after the intervention (Rho=-0.37p=0.01; Rho = -0.42; p=0.005). These associations remain significant after the adjustment by specific parameters.

In the second work, linear regression models showed that baseline *Bilophyla* abundances were significantly associated with less BMI improvement by the intervention (p=0.005). We have found that baseline *Faecalibacterium* and *Eubacterium coprostanoligenes group* were significantly associated with MetScore improvement (p=0.001 and p=0.005, respectively). However, *Escherichia shigella* and *Bacteroides* were associated with lower MetScore improvement.

Logistic regression models revealed that *Akkeramansia* (Odds ratio, 95%IC: 0.86[0.74-0.97], p=0.033) and *Christensenellaceae R7 group* (Odds ratio, 95%IC: 0.86 [0.75-0.98], p=0.031) were associated with a lower risk of present cardiometabolic alterations. However, *Bacteroides* increase the risk of MUO (Odds ratio, 95%IC: 1.03 [1.01-1.05], p=0.001). Logistic regression models in the second work revealed that higher abundances of the *Faecalibacterium* genus at baseline increased the odds of responding successfully to the intervention (Odds ratio, 95%IC: 1.47[1.14-2.07], p=0.008).

General Discussion

With this thesis, the hypothesis that the gut microbiota was associated with cardiovascular risk factors, and that the composition of gut microbiota determined the efficacy of the dietary intervention was confirmed in children and adolescents with obesity through two publications.

Trying to cover some of the gaps still open in this field, this thesis focused on the role of gut microbiota on the cardiovascular health of children and adolescents with obesity. To attempt this, we first characterized and compared the microbial diversity and the community composition between children with obesity and some cardiometabolic alteration and children with healthy obesity. Secondly, we analysed the selected taxonomic groups previously identified as markers of healthy or unhealthy obesity. Furthermore, we analysed if the gut microbiota profile could predict BMI and metabolic improvements after a multi-component (diet and physical activity) intervention.

The relationship between gut microbiota and human health is gaining particular interest nowadays. However, there is still a lack of knowledge to apply all of these findings to clinical practices for the prevention or treatment of obesity. One of the biggest challenges nowadays in understanding the symbiotic relationship between the host and the gut microbiome is to characterize the healthy microbiota and its interplay as either a cause or a consequence of health and disease. However, intra- and inter-individual differences are considerable and make the assignment challenging.

Based on the first objective of our work, we would like to remark on the difficulties of establishing a “healthy” or “unhealthy” gut microbiota pattern

linked to cardiovascular health. To our knowledge, two previous works (249,262) have compared gut microbiota composition between children with unhealthy obesity and children with healthy obesity. Our results show that MHO presented higher abundances of *Christensenellaceae R7 group* and *Akkermansia* and lower abundances of *Bacteroides* genus than the MHO group. Yuan X et al. (262) analysed the gut microbiota of 89 Chinese children with obesity aged between 5 and 15 years. They observed that the MHO children presented higher Tenericutes, Christensenellaceae, Rikenellaceae and Ruminococcaceae than children with MUO. Gallardo-Becerra et al. (249) characterized the gut microbiome of a group of Mexican children composed of 10 children with normal weight, 10 with obesity and 10 with metabolic alteration, aged between 7 and 10 years. They concluded that those with metabolic alteration presented increased abundances of Coriobacteria up to the genus *Collinsella*. These differences showed considerable variability between populations, even if they were more or less the same age. We want to remark that further studies on children should be carried out to establish a specific profile that leads to the early detection and prevention of the development of comorbidities in children with obesity.

Our results showed that gut microbiota diversity should be considered a key factor in metabolic alterations and may predict an intervention's success. We observed that a profile characterized by low alpha diversity indices could be considered an indicator of worse cardiometabolic health and is associated with lower metabolic improvement after the intervention, independently of the metabolic status before starting the treatment. To date, a reduction of the alpha diversity is a common pattern in adults with cardiovascular alterations such as high blood pressure (233,263), dyslipidaemia or insulin resistance. (147) For instance, Cuesta-Zuluaga et al. (264) observed, in a cohort of 441 adult participants, that high microbiota diversity was significantly associated with less gut permeability and translocation of LPS, less adiposity and

improved cardiometabolic health. However, two works have reported inverse association in Mexican populations. The first one in Mexican women (265) and the second one on Mexican children aged between 7-10 years (249). Both were composed of healthy participants, participants with obesity and participants with obesity and metabolic alteration. They concluded that participants with obesity and/or metabolic alteration presented higher diversity than participants with normal weight. In agreement with our findings, in a sample of 123 non-obese and 169 Danish individuals with obesity, those participants with lower richness gained significantly more weight than those who presented higher richness after nine years of follow-up (67). However, Stanislawki et al. (216) did not find associations between the already existing diversity and the body weight loss, nor for the waist circumference after a weight loss intervention on 59 adults with obesity.

Several factors could influence gut microbiota diversity; for instance, inactive lifestyle (208), high-fat diets or Western (266) diets are associated with a decrease in alpha diversity. Stanislawski et al. (216) observed that alpha diversity indices increased significantly over the first three months of the intervention; however, they cannot describe the mechanism that led to this increase. Another interventional study by Hanna et al. (267) observed that a fermented food diet could increase microbiota diversity coincident with a decrease in several markers of inflammation.

To date, the mechanistic process under these associations is still not elucidated. Olaf et al. (268) tried to show that the gut microbiome is a redundant system and different bacteria are doing similar functions. Therefore the higher diversity, the higher efficiency and stability against alteration.

Beyond diversity, our results showed that the *Bacteroides* genus is involved in the cardiometabolic health of children with obesity. A limitation of our work

is that we cannot establish causality. However, we observed that in children with obesity, *Bacteroides* abundance was associated with a worse metabolic profile before the intervention. Moreover, the enrichment of *Bacteroides* was associated with less improvement of the metabolic profile, independently of the severity of the alterations before the intervention. The study of this genus is very complex due to its high diversity. Different *Bacteroides*-belonging species show opposite functions and associations with human health. For instance, *Bacteroides erggerthy* has been associated with higher body fat and obesity while *B. plebeius* is a characteristic of normal-weight participants (68). Therefore, the diversity and composition inside the *Bacteroides* genus could be a field of investigation. We did not find significant associations between specific species of the *Bacteroides* genus and the cardiovascular alterations or the response to the intervention.

Published works on the role of *Bacteroides* on cardiometabolic health are still controversial. *Bacteroides* have been usually associated with healthier conditions, as shown by imbalances with Firmicutes in children with obesity. Previous reports in children with obesity reported that *Bacteroides* were negatively associated with TG (269). However, in agreement with the direction of our results, it was recently suggested that the *Bacteroides* enterotype might be an independent risk factor for T2D in adults (270). The authors suggested that this enterotype was associated with higher intestinal permeability. Therefore, higher bacterial translocation causes a low-grade inflammatory profile associated with alterations in insulin sensitivity (270). In this line, work on children with obesity (271) found that the abundance of *Bacteroides* was associated with intercellular adhesion molecule 1 (ICAM-1), a marker of endothelial dysfunction. Studies showed this genus is one of the most abundant genera in the human intestine. It plays a role in several mechanisms to adapt and persist in modifiable environments (272). However, further

investigations are needed in children to elucidate the mechanisms by which the different species of this genus interconnect with cardiovascular health.

The children in our study are children with obesity, and their gut microbiota composition is characterized by higher abundances of Firmicutes and lower abundances of *Bacteroides* compared to the general population (66,273). Interestingly, comparing children with MUO and children with MHO, we have observed that there was a higher abundance of Bacteroidetes and a lower abundance of Firmicutes in MUO than in MHO. However, previous works in children (72,249) showed a trend to lower Firmicutes in children without metabolic alteration but without founding significant differences. Different studies (203,274) have shown that diet may increase SCFA-producing bacteria and SCFA levels. As Firmicutes include several well-known SCFA producers, we hypothesize that the increase of *Firmicutes* abundance in our samples may be associated with higher abundances of SCFA producers such as *Christensenllaceae*, and this increase may have protective effects against cardiometabolic alterations. However, further work is needed to confirm this hypothesis.

From our first publication, it is worth highlighting that significantly lower abundances of Verrucomicrobiota and its well-known genus, *Akkermansia,* were observed in children with MUO. This finding has been previously reported in children and adults with obesity (246,248,275,276) and adds pieces of evidence to the consideration of *Akkermansia* as a health-related bacteria. Within *Akkermansia*, the major focus of study to date is *A.mucinihpla*, the most abundant specie belonging to this genus. *A.muciniphyla* is a mucin-degrader bacteria that converts dietary fibres into acetate and propionate and stimulates the proliferation of butyrate-bacteria producers such as *F.prausnitzii* (277). Even though we have not observed any association between *Akkermansia* and the effectiveness of the intervention,

previous works reported that the enrichment of *A. muciniphyla* was associated with metabolic improvement and glucose tolerance and blood lipid profile after a caloric restriction period (247). In mice, the administration of *A. muciniphyla* reversed high-fat diet-induced metabolic disorders, including fat-mass gain, adipose tissue inflammation or insulin resistance (278). All this evidence leads the researchers to suggest A.*muciniphyla* as a possible bacterium to use in therapeutic interventions. Our results add pieces of evidence that this bacteria might be an open window of opportunity for treatment, not only in adults but also in children.

Regarding our second work, few studies have investigated if specific microbiota composition predicts the response to a dietary intervention. Our results evaluated whether the host's gut microbiota predicted the response on BMI and MetScore improvement. To our knowledge, no previous works on children are analysing the potential role of the host's microbiota in response to interventions. In adults, gut microbiota composition, as well as specific bacteria such as Coriobacteriaceae, *Akkermansia* or Eubacterium *ruminantium*, has been suggested as predictors of waist circumference reduction (216), weight loss (247), and cholesterol decrease (216,247,279). There were high differences between studies, according to outcomes and methods used, but also different confounding factors such as the host's genetics, age, long-term dietary and lifestyle habits, and geographic area (280).

Regarding the prediction of success, we would like to highlight the role of *Faecalibacterium.* In our work, the presence of higher abundances of *Faecalibacterium and Eubacterium coprostanoligenes* predicted the success of the intervention. F.*prausnitzii* is the most well-known specie within the *Faecalibacterium* genus. Recently it has been receiving particular interest as a health-related biomarker due to its capacity to produce anti-inflammatory

metabolites such as butyrate (281). Interestingly, members of the genus *Eubacterium* are also associated with beneficial health effects. We found that higher abundance *Eubacterium coprostanoligenes group* were associated with more significant MetScore improvement. *E.coprostanoligenes* can transform cholesterol into coprostanol, which can not be easily absorbed. Li et al. (282,283) reported that the supplementation with *E.coprostanoligenes* might have hypocholesterolemic effects by decreasing the absorption of dietary cholesterol and degrading it to coprostanol in animal models. However, to the best of our knowledge, there is no clinical trial assessing this kind of intervention in human populations. We did not find associations between *E.coprostanoligenes* and cholesterol levels; however, it was associated with a more remarkable improvement of HOMA-IR and SBP after the nutritional intervention. Our results support that both *Faecalibacterium and E. coprostanoligenes groups* could be considered in future intervention studies to treat childhood obesity and its comorbidities.

The findings of this thesis add shreds of evidence to the fact that gut microbiota is involved in the cardiometabolic health of children with obesity and to the importance of considering the gut microbiota as an essential factor in the first steps of nutritional treatment. Further work is needed to analyse the potential transference of this knowledge to clinical practice.

This thesis presents some strengths and limitations that will be commented on below.

Our work could have been limited by the young age of the participants and the low prevalence of cardiometabolic alterations expected in this age; however, the fact that our findings were consistent with our hypotheses and previous works conducted in adults provides relevance to our results.

One of the limitations of our second publication is the small sample size that followed the intervention *Per Protocol* and participated in the faecal sample collection. Although it could have limited our results, our work was consistent with previous works in adults, providing robustness to our findings.

Another limitation of our work is that we analysed our samples with the 16S rRNA method. This method is generally limited to identifying bacteria at the genus level. Therefore, we could not detect specific strains associated with beneficial effects, such as *A. muciniphyla* or *C. minuta.*

One of the most relevant strengths of our work is the relatively big cohort of children with obesity. Finally, one of the most important questions to be answered in the field of gut microbiota and health is that it is not clear when and how it acts either as a cause or as a consequence of the health status. Although we could not establish a cause-effect relationship between the gut microbiota and health alterations, we were able to support the hypothesis that the gut microbiota present in the host before intervention may influence the outcome. This fact claims for research on possible supplementation to improve the success of interventions.

Conclusions

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Conclusions

1. The gut microbiota of children with unhealthy obesity differs from the composition of children with healthy obesity
2. Poor gut microbiota diversity, high *Bacteroides* abundance, low *Akkermansia* and low *Christensenellaceae R7 group* abundances are associated with a worse metabolic profile in children with obesity
3. The host's baseline gut microbiota composition predicted the efficacy of lifestyle intervention. Specifically, the higher presence of *Bilophyla* may help to predict the success in BMI loss after a nutritional intervention
4. The host's baseline gut microbiota diversity predicted the efficacy of a lifestyle intervention on metabolic health
5. A gut microbiota profile enriched in *Faecalibacterium* and *Eubacterium coprostanoligenes group* and with low *Bacteroides* may predict the improvement of metabolic health after a nutritional

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Future perspectives

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Future perspectives

Several factors may influence the modulation of gut microbiota, such as dietary patterns or physical activity, resulting in an improvement in metabolic health. However, as is shown in Figure 13, one of the biggest challenges nowadays is to elucidate whether gut microbiota composition is the cause or the consequence of the development of obesity and cardiometabolic alterations.

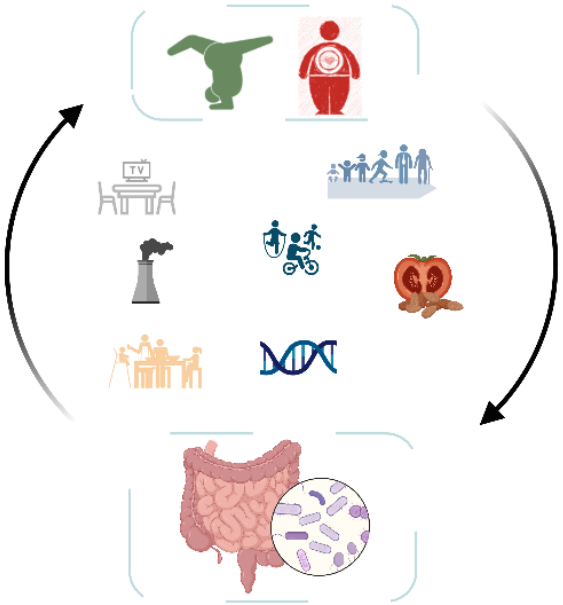

Figure 13. **Gut microbiota, cause, or consequence?** Created with BioRender.com

Further research is needed to transfer these results to clinical practice and to understand how they could be used to prevent and treat childhood obesity and its comorbidities. One of our future steps will be to investigate whether a motivational intervention based on lifestyle changes and nutritional education may shift a harmful profile of the gut microbiota to a beneficial one and link it

to the achievement of beneficial effects associated with “healthy” bacteria. This work will integrate dietary patterns and physical activity with gut microbiota composition, products such as SCFA and cardiometabolic health factors such as cytokines.

An exciting work we would like to carry out would be an intervention to improve the baseline gut microbiota before starting a nutritional intervention leading to an increase in the efficacy of such intervention.

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