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COMPOSITION AND REMODELING OF THE GUT MICROBIOTA DURING PREGNANCY IN PATIENTS WITH GESTATIONAL DIABETES MELLITUS AND THEIR OFFSPRING

PhD Thesis in Medical Physiopathology

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1 BACKGROUND OF THE STUDY

Significant metabolic, immunological and hormonal changes occur during pregnancy in order to support fetal growth and development.(1) The complex changes that are associated with the gestational period are similar to those that occur in the metabolic syndrome such as insulin resistance, glucose intolerance, elevated fasting blood-glucose levels, weight gain, low grade inflammation, and variations in metabolic hormone levels.(1–4) In the same time with the metabolic and immunological modifications, there are also noticeable alterations in the gut microbiota during pregnancy.

Some recent studies have provided evidence that the gut microbiota composition could be different between lean and obese individuals, and between patients with and without type 2 diabetes mellitus.(5–8) This evidence has led to the study of a fascinating potential link between gut microbiota and metabolism, suggesting that impaired balance in the gut microbiota or the so called dysbiotic microbiota could contribute to the development of different metabolic diseases and furthermore may impact gestational metabolism and the development of GDM. Dysbiotic microbiota is also reported to be involved in the diffusion of gut bacterial endotoxin into the systemic circulation inducing a low-grade inflammatory response, which together with insulin resistance could represent the hallmark pathway to the development of the cardiometabolic complications of pregnancy such as gestational diabetes mellitus (GDM) and gestational hypertension.(6,9–12) This remodeling of the gut microbiota during pregnancy in women with gestational diabetes mellitus and their offspring are the focus of my research.

This study is of the utmost significance because the number of women being diagnosed with gestational diabetes is constantly increasing in the world, so finding a simple and efficient way such as understanding and possibly manipulating the gut microbiota in order to prevent women developing gestational diabetes mellitus could be very important. The results of my research are already published in two separate articles:

1. Changes in the gut microbiota composition during pregnancy in patients with gestational diabetes mellitus (GDM). (13)

2. The microbiota composition of the offspring of patients with gestational diabetes mellitus (GDM). (14)

2 GESTATIONAL DIABETES MELLITUS

2.1 Definition and epidemiology

Gestational Diabetes Mellitus (GDM) is considered one of the most common medical complications of pregnancy.(15) GDM is defined as diabetes diagnosed in the second or third trimester of gestation that is not clearly either type 1 or type 2 diabetes mellitus.(16) Women diagnosed with diabetes in the first trimester of gestation are classified as having preexisting pregestational diabetes that is mostly type 2 diabetes mellitus or very rarely type 1 diabetes mellitus.
Most of the women do not receive screening for diabetes mellitus before pregnancy and sometimes that can cause difficulties in distinguishing GDM form preexisting diabetes.
It is reported that the GDM occurs in about 7% of all pregnancies, this range is highly variable between 1%-14%, depending mostly on the population and the diagnostic test used in the studies.(17) The prevalence of GDM has been progressively increasing and it reflects the increasing prevalence of obesity and T2DM in general population.(18,19) This happens most likely due to the increased prevalence of the common risk factors for T2DM and GDM such as obesity, sedentary lifestyle and older maternal age.(20)

According to the national prevalence data reported by the Ministry of Health every year in Italy >40.000 pregnancies are complicated by the diagnosis of GDM. Moreover, it is predicted that in the next years there's going to be a further increase of the diabetes in pregnancy due to the increased incidence of DMT2 in women in fertile age and the increased immigration from countries with high prevalence of diabetes. (21)

2.2 Diagnoses and risk factors associated with GDM

The diagnosis and screening of GDM are subject of significant controversy in the literature especially their cost-effectiveness. There is still no universally accepted consensus on the criteria for the diagnosis of GDM. Different screening strategies are recommended by different organizations. A one- or two-step approach can be used for the diagnosis of GDM. It is suggested that the institutions should choose only one approach for consistency among patients.
It is also important to remember that hyperglycemia first detected during pregnancy beside GDM can also be classified as pre-existing diabetes mellitus or overt diabetes and it be diagnosed if one or more of the following WHO criteria are met:

- fasting plasma glucose (FPG) ≥ 7.0 mmol/l (126 mg/ dl)
- 2-hour plasma glucose ≥ 11.1 mmol/l (200 mg/dl) following a 75g OGTT
- random plasma glucose (RPG) ≥ 11.1 mmol/l (200 mg/ dl) in the presence of symptoms.

Despite the different approaches on the screening and diagnosis of GDM it is widely accepted that pregnant women should be screened for GDM at 24-28 weeks of gestation with a laboratory-based test using blood glucose levels. Earlier testing may be indicated for women at high risk.

Firstly some of the countries have adopted two-step approach proposed by the American College of Obstetricians and Gynecologists (ACOG) committee, based on a glucose challenge (GCT) test (50g-1h) and later an oral glucose tolerance test (100g-3h) if the result of the GCT is greater than the cut-off value considered. In the evaluation of the oral glucose tolerance test (OGTT), two or more abnormal values had been considered diagnostic for gestational diabetes.
This two-step approach was later on modified by the National Diabetes Data Group (NDDG)(22) and Carpenter and Coustan (23), that proposed different diagnostic cut-off values for the diagnosis of GDM, that were widely used in different countries, until some years ago when the International Association of Diabetes and Pregnancy Study Group (IADPSG) reevaluating the results of the Hyperglycemia and Adverse Pregnancy Outcome (HAPO) study, proposed new diagnostic criteria based on one-step approach. At the beginning it was proposed evaluating the first trimester fasting glycemia to exclude cases of pre-existing diabetes or overt diabetes mellitus (Fasting plasma glucose (FPG)≥126mg/dl or ≥7 mmol/L), and then IADPSG and American Diabetes Association (ADA) suggested that all pregnant women between 24 and 28 weeks of gestation should undergo 75g-2h oral glucose tolerance test (OGTT) after overnight fast considering a diagnosis of GDM if there is presence of ≥ 1 of any of the following criteria: (24)

- fasting plasma glucose ≥ 92 mg/dL (5.1 mmol/L)
- 1-hour plasma glucose ≥ 180 mg/dL (10 mmol/L)
- 2-hour plasma glucose ≥ 153 mg/dL (8.5 mmol/L)

These IADPSG/ADA diagnostic criteria that are the most commonly used criteria worldwide, were also accepted by the Italian Study Group on Diabetes in Pregnancy with the agreement of two scientific societies: Italian Association of Diabetologist (AMD) and Italian Diabetes Society (SID) and implemented in most Italian medical centers. (25) These criteria were further re-evaluated only few months later when the Italian Institute of Health in the Guidelines of Physiological Pregnancy, suggested that only the pregnant women with a defined risk factor for GDM should undergo an OGTT between 24 and 28 weeks of gestation.(26,27) Critics of these international criteria state that it causes over diagnosis of GDM and unnecessary interventions. That's why the suggestion of the Italian Institute of Health that the gestational diabetes diagnosis could be made much more cost effective by screening only those patients who have at least one risk factor for GDM. However, the decision of whether the screening for GDM should be performed in all pregnant women or selectively in women that present risk factors for GDM is still a topic of debate on an international level.

There are several risk factors related to the development of GDM including obesity and overweight, advanced maternal age (≥35 years), personal history of prior GDM or glucose intolerance, strong family history of type 2 diabetes mellitus, excessive weight gain during pregnancy, macrosomia in previous and/or in index pregnancy, polycystic ovarian syndrome(PCOS) and some ethnicities (Latino, South Asian (India, Pakistan or Bangladesh), Middle Eastern (Saudi Arabia, United Arab Emirates, Iraq, Jordan, Syria, Oman, Qatar, Kuwait, Lebanon or Egypt), Native Americans, African Americans, Pacific Islanders). It is reported that previous adverse pregnancy outcome, multiple gestation, polyhydramnios and gestational hypertension may also increase the risk of GDM.

Some authors have found a significant relative risk for the development of GDM with the increased intake of the Western diet, high glycemic load and a low cereal-fiber diet.(28–30)
Micronutrients have also been found to influence glucose tolerance, women with lower daily intake of vitamin C and vitamin D were found to be associated with an increased risk for GDM compared with those women with higher intakes.(31,32)
Among all risk factors associated with GDM, increased maternal weight is the most evaluated modifiable risk factor. Several studies have stated that the GDM prevalence is higher in the categories of overweight (BMI 25-29.9 kg/m2) and obese women (BMI 30-34.9 kg/m2) compared with pregnant women with normal weight (BMI 18.5-24.9 kg/m2). (33,34)

Table 1. Glucose threshold values for the diagnosis of GDM according to the IADPSG/ADA diagnostic criteria

Measure of glycemia	Glucose threshold	
	mg/dL	mmol/L
Fasting plasma glucoses	≥92 mg/dL	≥5.1 mmol/L
1-hour post 75g OGTT glucose	≥ 180 mg/dL	≥10 mmol/L
2-hour post 75g OGTT glucose	≥ 153 mg/dL	≥8.5 mmol/L

2.3 GDM associated complications

Even though in most of the cases after delivery there is a restoration of the glucose tolerance and the insulin sensitivity, numerous studies in the literature have demonstrated that diabetes diagnosed during pregnancy is associated to a greater risk for adverse health outcomes and complications for both the mother and the offspring. These can further be classified into short and long-term maternal and fetal adverse outcomes. Consequently, there are two different generations at risk.

2.3.1 Maternal complications

GDM has both short and long-term risks for the mother and the infant. It is associated with higher incidence of maternal morbidity including hypertensive disorders such as preeclampsia, eclampsia and gestational hypertension, polyhydramnios, birth trauma especially 3rd -degree and 4th -degree lacerations after a vaginal birth, post-partum hemorrhage, Cesarean delivery with all the complications that brings an operative delivery (infection, bleeding, thrombosis, wound dehiscence) and preterm birth.(35)
Additionally, women with previous history of GDM are at an increased risk of long-term adverse health outcome such as developing T2DM further in life, GDM in a subsequent pregnancy and an increased risk of future cardiovascular diseases (CVD). One recent systematic review has demonstrated that women with gestational diabetes had 7.43 times the likelihood of developing type 2 diabetes compared with those who had a normoglycemic pregnancy (RR 7.43, 95% CI

4.79–11.51). Incidence of diabetes mellitus among women with a history of GDM has been shown to range from 6% to 62%.(36) The predominant sources of difference in the reported incidence rates are most probably attributed to the ethnic variation, length of follow-up, diversity in selection and diagnostic criteria for GDM and diabetes mellitus.(37) Carr et al. studied a population of women with a family history of type 2 diabetes and they found that those women that were previously diagnosed with GDM were associated with an increased prevalence of CVD at a significantly younger age in comparison with women without GDM. (38) Moreover, the risk of the metabolic syndrome is increased 3-fold in women with prior GDM. (39)

2.3.2 Fetal and infant complications

There is an increasing body of evidence supporting the hypothesis that the abnormal metabolic environment of the mother with GDM may affect some developing fetal tissues, organs, and control systems, leading to short and long-term consequences for the infant. The fetal tissues most likely to be affected are the pancreatic beta cells, adipocytes, muscle cells, and neural cells. It has been shown that maternal hyperglycemia leads to fetal hyperglycemia which stimulates fetal pancreatic islet cells to produce more insulin, so it eventually leads to fetal hyperinsulinemia.

The most common short-term adverse outcome for the offspring in pregnancy complicated with GDM seems to be the macrosomia defined as birth weight greater than 4000-4500 g or in other words any fetus/infant weighing >90th percentile. The macrosomia may lead to obstetric and neonatal complications directly related to the size of the baby, including shoulder dystocia which can lead to neonatal musculoskeletal and brachial plexus injury.(40,41) Untreated maternal hyperglycemia during pregnancy also increases the risk for congenital malformations, stillbirth, neonatal complications such as hypoglycemia, hypocalcemia and hyperbilirubinemia, respiratory distress syndrome (RSD) and admission to the neonatal intensive care unit. (42)
Moreover, offspring of mothers with GDM are at risk of future chronic complications with greater clinical relevance such as development of obesity and impaired glucose metabolism and diabetes during childhood, adolescence, and adulthood. (42,43)

It is now clear that an abnormal intrauterine environment such as the intrauterine exposure to hyperglycemia can have consequences on the offspring later life through the so call "fetal programming". However, the exact mechanisms by which having diabetes during pregnancy may lead to these long-term complications in the offspring are still not fully understood.(44)
Children born by women with GDM had an eight times increased risk of developing diabetes or prediabetes when they are 19-27 years old compared with children born from women without GDM.(42,45)

2.4 Treatment of GDM

Early identification of women with GDM is of great importance because it is demonstrated that prompt treatment could reduce both fetal and maternal adverse health outcomes. Once gestational diabetes is diagnosed the recommended treatment consists of medical nutrition therapy (MNT) and weight management, physical exercise, self-monitoring of blood glucose and pharmacological therapy if required. (42)

After the diagnosis of GDM, the women should initiate immediately with non-pharmacological approach including MNT and physical activity. In addition, frequent self-monitoring of blood glucose is required in order to monitor the glycemic control of the pregnant woman and to determine whether it is adequately achieved or there is necessity of initiating a pharmacological therapy. In order to establish an appropriate dietary intake for women with GDM, several studies were conducted to compare different types of diet. Even if diet is the cornerstone of the treatment, by now there is no evidence for optimal medical nutrition therapy and weight gain for women diagnosed with GDM. Exercise on the other hand is associated with improved insulin sensitivity which might improve both fasting and postprandial glucose levels avoiding the use of insulin in some women with GDM.(46)
The recommended glycemic targets for maternal capillary glucose after self-monitoring of the blood glucose are:

- Fasting blood glucose ≤ 95 mg/dL (5.3 mmol/L) and either of
- 1-hour postprandial ≤ 140 mg/dL (7.8 mmol/L)
- 2-hour postprandial ≤ 120 mg/dL (6.7 mmol/L)

Women with GDM who fail to maintain the glycemic targets with nutritional therapy and exercise should initiate pharmacological treatment. Human insulin is the preferred pharmacological treatment for GDM and is considered the first choice, while in some cases insulin analogues and certain oral agents such as metformin and glyburide may also be used. Both metformin and glyburide may be considered as second-line pharmacological treatment. However, the long-term safety of using oral agents in GDM remains obscure.(47)
It has been demonstrated that 70–85% of women diagnosed with GDM can control GDM only by lifestyle modification.(48)
Different studies suggest that treatment of gestational diabetes is effective in reducing macrosomia, large for gestational age, shoulder dystocia, hypertensive disorders in pregnancy and cesarean section.(49,50)

After delivery, most of the women return to their previous pre-gestational glycemic levels. However, some of them may continue to have high glucose levels, so the current American Diabetes Association guidelines recommend that women that were diagnosed with GDM should undertake postpartum glucose testing using 75-g 2-hour oral glucose tolerance test and nonpregnancy diagnostic criteria at 6–8 weeks postpartum and every 3 years afterwards.

2.5 Pathophysiology of GDM

Although gestational diabetes mellitus is one of the most common metabolic complications of pregnancy and it represents an important public health concern, its pathophysiology is still not fully understood. Pregnancy is a condition characterized with significant and complex metabolic, immunological and hormonal changes. All these physiological changes occur in order to support fetal growth and development.
Even though the pathophysiological mechanisms that can lead to development of GDM are not yet completely explained, it is insulin resistance, low-grade chronic inflammation and pancreatic β-cell dysfunction that are considered the major aspects responsible for its development. They are also reported to be the key pathogenetic mechanisms of obesity and other metabolic disorders such as T2DM.

The first trimester of pregnancy is characterized by increased insulin sensitivity, which promotes the accumulation of adipose tissue in early pregnancy. During this period women are at risk of hypoglycemia especially if accompanied by nausea and vomiting that often happens at the beginning of pregnancy. This situation changes completely with the progression of pregnancy, so in the second and third trimester there is a decrease in insulin sensitivity and an increase of the insulin resistance in order to ensure an adequate supply of nutrients to the growing fetus. All these physiological changes during pregnancy serve to meet the increasing metabolic demands of the fetus, which requires 80% of its' energy as glucose, in the same time maintaining euglycemia in the mother. (51)
During pregnancy in order glucose levels of the mother to be maintained normal, it is necessary that maternal insulin secretion increases sufficiently to counteract this physiological decrease of insulin sensitivity and consequently increase of the insulin resistance. The gestational diabetes mellitus happens when there is insufficient insulin secretion to counteract and overcome the pregnancy related increase in the insulin resistance. In the literature there are different mechanisms that explain this increase of the insulin resistance that can be mediated by number of different factors. It is thought that the production by the placenta of various hormones with insulin-antagonistic effect could play a role in the induction of the insulin resistance. These are the so called diabetogenic placental hormones that include human placental lactogen, prolactin and increasing levels of progesterone. Various studies in the literature using animal models provide evidence for the role of placental hormones in the induction of insulin resistance in pregnant rats. According to them the increasing levels of progesterone, prolactin and human placental lactogen play a causal role in the insulin resistance throughout pregnancy.(52,53)

Chronic low-grade inflammation seems also to contribute to the pathogenesis of GDM. Obesity and overweight are frequent findings among women diagnosed with GDM. Obesity is considered a state of low-grade chronic inflammation characterized by both inflammatory cells and biochemical markers of inflammation that are produced in excess into the systemic circulation. Pro-inflammatory cytokines have been discovered to both impair insulin signaling and inhibit insulin release from β-cells. These inflammatory mediators are reported to influence alterations in the post-receptor insulin signaling resulting in increased insulin resistance.(54)

Pregnancy and GDM are also characterized by an altered inflammatory profile compared to the non-pregnant state. In normal pregnancy there is physiological regulation of the innate immune system in order to prevent the rejection of the growing fetus. All these inflammatory conditions are characterized by accretion of adipose tissue that is considered an endocrine organ that produces an array of adipokines such as leptin, adiponectin, resistin and different cytokines often collectively referred to as adipocytokines. Adipokines are involved in the regulation of maternal energy metabolism and insulin resistance. Different animal and human studies have demonstrated that obesity induces macrophage infiltration of adipose tissue which produce various cytokines including TNF-α, IL-6, and IL-1β, MCP-1. These studies demonstrated for the first time that there is a close association between the immunocompetent cells and the adipocytes in an endocrine organ. (55,56) The inflammatory mediators act locally to provoke inflammation in adipose tissue and increase peripheral insulin resistance. Obesity, T2DM and GDM are associated with an increased number of resident adipose tissue macrophages that produce these pro-inflammatory cytokines, like TNF-α, IL-6, and IL-1β.(57)
Among the various pro-inflammatory cytokines, tumor necrosis factor-alpha (TNF-α) is reported to have the major role in the development of T2DM.(58) TNF-α impairs insulin signaling and beta cell function, which may directly contribute to GDM. Interleukin-6 (IL-6) in another inflammatory marker found to be significantly higher in women diagnosed with GDM, compared with women without GDM, independent of body mass index.(59–61) Different cross-sectional studies have described increased circulating concentrations of leptin in women with GDM, which might contribute to GDM pathophysiology by suppressing insulin secretion from pancreatic beta cells.(62–64) Various studies described increased circulating concentrations of pro-inflammatory adipocytokines in GDM. (59,62,65) However, data regarding the pattern of adipocytokines secretion in normal and pregnancies complicated with GDM are very contradictory.(65–67)

There is an emerging evidence in the current literature that gut microbiota might play an important role in the pathophysiology of different metabolic diseases, including GDM. Most probably the gut microbiota is the missing piece of the puzzle that connects the major aspects believed to be responsible for the development of GDM such as insulin resistance and low-grade chronic inflammation. But how does gut microbiota affect our metabolism and influence our health, contributing to a range of different diseases including GDM, is going to be widely discussed in the next section.

3 GUT MICROBIOTA

The human body is home to a unique and complex microbial community defined as “microbiota” including different bacteria, archaea, fungi, and viruses. On our body surfaces such as skin (skin microbiota), gut (gut microbiota), vagina (vaginal microbiota), placenta (placental microbiota), lungs (lung microbiome) reside a lot of different microorganisms. The human gastrointestinal tract is the main site which host the majority of these microorganisms (mostly bacteria) widely recognized as “gut microbiota” or “gut flora”. (68)
The gut microbiota is estimated to be composed of about 100 trillion microorganisms which comprises 10 times more bacterial cells than the number of human cells in the body.

(69,70)That's why the microbiota is now considered a virtual organ of the human body and together with the host they are referred to as "the superorganism".(69,71,72) The number and variety of bacteria increase exponentially from the upper gastrointestinal tract to the colon where the bacterial count reaches around 10^{12} colony-forming units (CFU) per gram.(73)
The collective genome of the gut microbiota known by the term microbiome, encodes over 3 million genes producing thousands of metabolites, which is about 100 times more genes than our human genome consisting of approximately 23,000 genes.(69,74,75)

Every individual has a unique gut microbiota profile, just like our fingerprints. (76) Taxonomically, bacteria are classified into phyla, classes, orders, families, genera, and species. The two dominant bacterial phyla representing about 90% of gut microbiota are Firmicutes and Bacteroidetes.(74) The other bacterial phyla such as Actinobacteria, Proteobacteria and Verrucomicrobia are less abundant in the gut, but are reported to have a great impact on the host health.(10) The Firmicutes phylum is composed of more than 200 different genera such as *Lactobacillus*, *Faecalibacterium, Bacillus, Clostridium, Roseburia, Enterococcus, Ruminicoccus* ecc. *Clostridium* genera represent 95% of the Firmicutes phyla. Bacteroidetes consists of predominant genera such as *Bacteroides, Alistipes* and *Prevotella*. It is reported that species from the genus *Bacteroides* alone constitute about 30% of all bacteria in the gut, suggesting that this genus is especially important in the functioning of the human organism.(77) The Actinobacteria phylum is mainly represented by the *Bifidobacterium* genus.(78)

There is an emerging evidence that the gut microbiome plays a crucial role in maintaining gut homeostasis through interactions with the host immune system and metabolism. The host and their microbiota have a symbiotic relationship, so these interactions are bilateral, affecting both the host and the microbiome.
The gut microbiota has many specific functions in the human organism such as regulation of some metabolic processes, maintenance of the structural integrity of the gut mucosal barrier, immunomodulation, and protection of the host against pathogens. (79) Some of the metabolic functions of the gut microbiota are well known, such as the fermenting of nondigestible plant carbohydrates (starch and fibers) into short-chain fatty acids (SCFAs), such as acetic acid, propionic, and butyric acid, which are then absorbed and used as energy source by the host. SCFAs promote the growth of intestinal epithelial cells and control their proliferation and differentiation. It has also been known that intestinal bacteria play a role in producing vitamin K, vitamin B, as well as metabolizing bile acids, sterols, and xenobiotics. (79–81)

The true diversity of the gut microbiota and its functions are being revealed every day due to the rapidly advancing field of high-throughput sequencing technologies and bioinformatics. The traditional culturing methods are very limited in identifying and characterization of the gut microbiota, in fact they can only detect around 30% of the total intestinal bacteria.(82) However with the advances of the novel culture-independent methods such as metagenomics and next-generation sequencing (NGS) technologies, an important progress has been made in the study of the gut microbiota. Moreover, functional metagenomics and metabolomics can help identifying novel functional genes, microbial pathways, antibiotic resistance genes, metabolites present in the sample and can determine interactions and co-evolution between microbiota and the host,

yet there are still some limitations.(83) Consequently, the analysis of the gut microbiota in the last few years has emerged as one of the most dynamic and exciting area of research in medicine.

The gut microbiota of every person is shaped in early life. The development of the human microbiota is a dynamic process that starts right after birth and is reported to obtain stability, resembling the microbiota of a healthy adult by 2-3 years of age.(84,85) The establishment of the human microbiota goes through different life stages characterized by notable differences in terms of diversity and variation. The neonatal gut microbiota is characterized by low diversity and a relative dominance of the phyla Proteobacteria and Actinobacteria, subsequently there is a gradual increase in the phylogenetic diversity of the microbiota over time with the emergence of the dominance of the phyla Firmicutes and Bacteroidetes, that characterizes the adult microbiota. (68,85)

There are some factors that can influence the initially developing microbiota during early life such as: gestational age (pre-term or full-term birth), mode of delivery (vaginal delivery or cesarean section), types of feeding (breast-fed or formula fed), weaning period (cessation of breastfeeding and introduction to solids), infant hospitalization and some environmental factors (e.g. antibiotic use).

Starting from the birth of the infant, the mode of delivery strongly affects the composition of the microbiota. In the case of caesarean delivery (C-section), newborns are exposed and acquire bacteria deriving from the hospital environment and the mother's skin (*Staphylococcus, Corynebacterium, Propionibacterium* spices) which form the basis for the initially developing microbiota instead the exposure of the vaginal and fecal bacteria from the mother in case of vaginally born infants.(86,87) Moreover, the intestinal microbiota of neonates delivered by cesarean delivery are less diverse in terms of bacteria species than the microbiota of vaginally delivered infants.(88) It is reported that the microbiota of breast-fed infants is dominated by *Bifidobacteria* whereas the microbiota of exclusively formula-fed infants have a greater abundance of *Escherichia coli*, *Clostridium difficile, Bacteroides fragilis and Lactobacilli.*(89)

The effect of gestational age on the gut microbiota development is shown by the results of a few studies reporting that the microbiota of hospitalized, preterm infants differs from that of healthy, full-term offspring. (90–92) Moreover, hospitalization and prematurity were associated with higher prevalence of C*lostridium difficile*, which on the other side might explain the increased incidence of gastrointestinal problems in premature infants.(91)

The weaning period or the introduction of solid food to the infants is a period where important changes of the gut microbiota happens and is reported that in this period the microbiota of breastfed infants and the microbiota of formula-fed infants become closer to each other and is characterize by predominance of the genera *Bifidobacterium, Clostridium coccoides, and Bacteroides*.(93)

In a healthy individual later in adult life the core gut microbiota remains relatively stable, still in this period there are some factors that can also influence the variations in our gut microbiota. These factors include diet, body mass index (BMI),age, genetics, physical activity and antibiotic use. (79,85) The dietary and cultural habits seem an important determinant in shaping the microbiota composition, diversity and richness throughout adulthood. It is now well known that healthier dietary pattern and intake of diet rich in fruits, vegetables and fibers is associated with

a higher richness and diversity of the gut microbiota.(94,95) On the other hand high fat diet is found to be associated with increased intestinal permeability (caused by high levels of Lipopolysaccharide-LPS), endotoxemia and low-grade inflammation.(96) Previous findings have demonstrated that the gut microbiota composition is being more similar in individuals that are genetically related, that belong to the same age group, or with a common dietary and cultural habits.(97–99)
We have seen that wide range of factors contribute to the diversity of the microbiota and can cause shifts in the composition of the core gut microbiota which is known as dysbiosis. The dysbiosis is associated with long-term consequences on human health and can lead to different diseases.

3.1 Gut Microbiota in Disease

While some of the changes that occur in the gut microbiota composition throughout host life promote good health and survival and therefore are considered beneficial, others cause an imbalance in this complex microbial system and are associated with numerous diseases. With the introduction and the advances of the Next generation sequencing techniques there is a considerable increase in the amount of evidence on the role of gut microbiota in different disease states such as obesity, diabetes (T1DM, T2DM and GDM), metabolic syndrome, inflammatory bowel disease (IBD), nonalcoholic steatohepatitis, asthma, allergies, Crohn's disease, ulcerative colitis, celiac disease and cardiovascular disease.(71,100–104)
Although, there is still not a clear definition of a healthy gut microbiota, it is evident now that the composition of the gut microbiota in healthy individuals is highly diverse than the composition of the gut microbiota in individuals with pathologic conditions.
Some recent studies have provided evidence that the gut microbiota composition is different between lean and obese individuals and also between patients with and without diabetes mellitus.(5,7,105) This evidence has led to the study of a fascinating potential link between the gut microbiota and metabolism, suggesting that impaired balance in the gut microbiota could contribute to the development of different metabolic diseases like obesity, diabetes and metabolic syndrome. There are different mechanisms related to the gut dysbiosis that are hypothesized to play a role in the pathogenesis of these metabolic conditions such as alteration of the intestinal permeability, modification of the host intestinal immunity, aberrant production of SCFA, increased energy harvest from the diet, abnormal gut peptide secretion, dysregulation of the endocannabinoid (eCB) system, increased lipopolysaccharide (LPS) uptake through the Toll-Like receptor 4 (TLR-4) and induced endotoxemia and low-grade chronic inflammation.(106,107)

Accumulating data in both animal and human models showed an increase in the relative abundance of Firmicutes and decrease in the relative abundance of Bacteroidetes in both obese mice and individuals.(108,109) Other studies instead have failed to reproduce these findings and showed a divergent results with respect to the changes in the gut microbiota in obese individuals.(110–113) For instance Santacruz et al have detected a reduced numbers of

Bifidobacterium and *Bacteroides* and increased numbers of *Staphylococcus, Enterobacteriaceae* and *Escherichia coli* in overweight compared with normal-weight pregnant women.(111)

Because of the anatomical, histological, genetical and physiological similarities between the gastrointestinal tract of mice and humans, the murine models have provided crucial insight in the role of the gut microbiota in health and disease. Turnbaugh et al. have demonstrated that a transfer of the gut microbiota from obese mice to germ-free mice resulted in significant increase in weight in the recipient mice, suggesting that the "obese" microbiota has an increased capacity to harvest energy from the diet and is in some way implicated in obesity. (114,115) In addition increased weight gain was also observed when gut microbiota from obese people was transferred to germ-free mice.(116,117) These observations have paved the path for the introduction of the Fecal microbiota transplant (FMT) as a novel therapeutic method in the treatment of different pathologic conditions. FMT has emerged as a new and promising therapeutic approach for *Clostridium difficile* infection.(118,119)

Obesity is one of the main risk factors for metabolic syndrome, and together with insulin resistance predisposes patients to the development of T2DM and cardiovascular disease. There are recent studies that associate specific alterations in the gut microbiota not only to obesity but also to insulin resistance and T2DM.(120,121) The results of one experimental study showed that fecal microbiota transfer from lean, healthy donors to obese recipients with metabolic syndrome resulted in a significant increase in insulin sensitivity in the obese recipients after the fecal microbiota transplantation.(122)

There are multiple reports providing evidence to support the concept that obesity and diabetes, as metabolic alterations are both associated with a state of low-grade inflammation. According to recent studies it is emerging that the gut microbiota plays a critical role in this low-grade inflammation and is involved in the development of the metabolic disorders. It is hypothesized that the gut microbiota is promoting this inflammatory state by distinct mechanisms.

First the lipopolysaccharide (LPS) which is a component of the Gram-negative bacteria wall has been suggested to cross the mucosal barrier of the intestine on newly synthesized chylomicrons by the epithelial intestinal cells, into the bloodstream. (105,121,123) The LPS is considered one of the most potent and well-studied inducers of inflammation. Increased plasma levels of LPS is defined as metabolic endotoxemia. (105) Another mechanism that was hypothesized to be involved in the onset of inflammatory state in the metabolic alterations is the increased permeability of the intestinal mucosa. Some studies have demonstrated that the gut microbiota associated with T2DM produces lower levels of short-chain fatty acids (SCFAs) which are known to support the integrity of the intestinal mucosa.(124,125) SCFA are produced by bacterial fermentation of nondigestible plant carbohydrates, and it is reported that Bacteroidetes provide most of the acetate and propionate, whereas Firmicutes are believed to be the primary producers of butyrate.(126,127) Inadequate production of SCFA leads to an increase in the permeability of the intestinal mucosa, allowing gut bacteria (LPS) to traverse and enter the circulation. LPS which is pathogen-associated molecular patterns (PAMPs), then binds to a specific molecules called pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) expressed by cells of the innate immune system of the host and elicit pro-inflammatory cytokine production, resulting in a chronic low-grade inflammation state. (105,128)

Consistently with this hypothesis, one study has reported significantly increased circulating levels of LPS in patients with T2DM when compared with matched controls.(129) Cani et al. have demonstrated that mice chronically exposed to low levels of LPS over a short period of time, gained weight and became resistant to insulin. (11)
However, more large-scale human studies are needed in order to disclose the exact mechanism that stays behind the low-grade inflammation and its involvement in the development of the metabolic disorders like obesity, diabetes (T2DM, GDM) and metabolic syndrome.

3.2 Gut Microbiota and Pregnancy

Over the course of normal, physiological pregnancy significant metabolic, immunological and hormonal changes take place in order to support fetal growth and development.(130) During gestation there is an increase in the levels of some hormones such as progesterone, estrogens, human placental lactogen, prolactin and some complex immune changes occur. On one hand especially at the beginning of pregnancy there is a moderate immune suppression needed for the mother's body to accept the growing fetus and on the other hand there is a modulation in the immune system in order to protect both of them from pathogens.(131)
Towards the third trimester of pregnancy many of the metabolic changes that occur including maternal weight gain, elevated fasting blood-glucose levels, decreased insulin sensitivity and increased insulin resistance, augmented circulating pro-inflammatory cytokines and low-grade inflammation are reported to be very similar to those that occur in the metabolic syndrome.(132)
Nowadays there is an increasing amount of evidence that together with these changes there are also profound alterations in the gut microbiota composition from the first to the third trimester of pregnancy.
Studies on the gut microbiota composition during gestation have reported highly divergent results. Some of the studies have reported that the gut microbiota remains relatively stable during pregnancy others instead have demonstrated that there is a dramatic change in the gut microbiota composition from the first to the third trimester of pregnancy with a decrease in the bacterial richness and the within subject (α) diversity, increase in the between-subject (β) diversity, an increase in Proteobacteria and Actinobacteria and a decline in butyrate-producing bacteria such as *Faecalibacterium prausnizii* at the end of pregnancy.(132,133) Authors reported that together with the increased adiposity in the third trimester of pregnancy they found higher levels of circulating glucose, leptin, insulin, cholesterol, pro-inflammatory cytokines TNFα, IFNγ IL2, IL6, and also significant changes in the HOMA index associated with a greater inflammatory response and insulin resistance. Moreover, Koren et al. also used an animal model which showed that the transfer of the gut microbiota from the third trimester of pregnancy to a germ-free mice induced greater adipose tissue accumulation and greater insulin resistance when compared to the first trimester gut microbiota transferred to a germ-free mice.(132) These observations appear similar to those observed in obese individuals.

It has been hypothesized that the composition of the maternal gut microbiota may in some way be related and influenced by pre-pregnancy weight and weight gain during pregnancy. Overweight pregnant women were found to have significantly higher levels of *Bacteroides, Clostridium* and *Staphylococcus* when compared to women with normal weight.(134)
Excessive weight gain over the course of pregnancy was associated with high *Bacteroides* concentrations, increased abundance of *Enterobacteriaceae* and *Escherichia coli*, reduced abundance of *Bifidobacterium* and *Akkermansia muciniphila* and decreased within subject (α) diversity in comparison to normal weight gain during pregnancy.(111,134,135)

Besides the body weight and the weight gain during pregnancy different metabolic biomarkers were correlated to specific taxa from the gut microbiota such as increased relative abundance of *Staphylococcus* was correlated with increased plasma cholesterol values, while increased *Bacteroides* abundance was related to increased HDL-cholesterol and reduced levels of triglycerides. (111)
Gomez-Arango et al. have also suggested that the modifications in the metabolic hormone levels in overweight and obese pregnant women during pregnancy are associated with alterations in the maternal gut microbiota composition. Their results have shown a positive correlation of the genus *Collinsella* (phylum Actinobacteria) with insulin, C-peptide, and HOMA-IR and triglycerides, from the adipokines leptin levels were strongly correlated with *Ruminococcaceae* and *Lachnospiraceae*, that are described as dominant families in the host energy metabolism.(136)
Based on these findings, it seems plausible that the gut microbiota is implicated in the regulation of the glucose and lipid metabolism during pregnancy and scientists are now trying to unravel the complex mechanisms behind these observations.

3.3 Gut Microbiota in women with GDM and their offspring

The increased insulin resistance observed towards the end of normal, physiological pregnancy and considered beneficial for the developing fetus, when combined to an inadequate insulin secretion required to maintain euglycemia, in certain women lead to the development of gestational diabetes mellitus. GDM was found to be associated with specific changes in the gut microbiota composition. (137–139)
Our knowledge of the composition of the gut microbiota in women with metabolic complications of pregnancy such as GDM is still based on few studies with very contradictory results. Some of the studies have reported that there was no difference in the gut microbial composition between women with GDM when compared to healthy pregnant women, although women with GDM tended to have the most depleted microbial richness in the first trimester of pregnancy.(132) Others have reported decreased placental abundance of Bacteroidetes, Firmicutes and *Acinetobacter* genus and increased placental Proteobacteria in women with GDM in comparison with normoglycemic mothers.(133,140) Crusell et al. instead have demonstrated that women with GDM has an altered gut microbiota at various levels, including phylum and genus levels when compared with normoglycemic pregnant women. GDM women showed an increased abundance of the phylum Actinobacteria and the genera *Collinsella, Bacteroides, Rothia*, and

Desulfovibrio.(137) They have also demonstrated that after about 8 months postpartum, the gut microbiota of women with previously diagnosed GDM is still different from women who had a normal pregnancy and that the gut microbiota composition of women with GDM, both during and after pregnancy, resembles the aberrant microbiota composition of non-pregnant individuals with type 2 diabetes and metabolic syndrome. (137)
Recently Kuang et al. have compared the gut microbial composition between GDM patients and healthy pregnant women via whole-metagenome shotgun sequencing of their fecal samples, trying to elucidate the associations between GDM and the composition of different microbial taxonomic units and functional genes.(141) This study indicated that some species such as *Parabacteroides distasonis, Klebsiella variicola* were enriched in GDM patients, whereas *Methanobrevibacter smithii, Alistipes, Bifidobacterium* and *Eubacterium* were enriched in the normoglycemic controls. Moreover, they have demonstrated differences in the distribution of metagenome linkage groups (MLGs) in GDM patients, as well as differences in the functional analysis showing a greater abundance of membrane transport, energy metabolism pathways, lipopolysaccharide, and phosphotransferase systems in the microbiome of GDM patients when compared to the control group. (141) The cross-sectional design of most of these studies did not allow to draw conclusions about the causal relationships of the associations found.

Whether the dysbiosis of the gut microbiota is just a consequence and a reflection of the disease or a cause and a driving factor resulting in a disease is still a controversial and debated topic in the literature nowadays. However, the finding that a different microbial pattern precedes the onset of GDM leads to the hypothesis that microbiota alterations might have a role in the pathogenesis of GDM.(132,142)

Interestingly, different studies have reported that specific microbial taxa associated with GDM can be transmitted to the offspring and can differentiate their gut microbiota from that of the offspring of normoglycemic women.(138,143–145) It is now well known that the maternal environment affects the offspring health. The newborn gut microbiota was thought to be sterile, but recent studies show that specific bacteria or bacterial components are already present in the gut in utero before birth. The most important period for the colonization and development of the offspring gut microbiota is the early life which is strongly influenced by maternal health factors and pregnancy conditions and participates in the development programming of the newborn and the maturation of their immune system.(146–148) The dysbiosis of the infant's microbiota in early life has been associated with many inflammatory, immune-mediated, allergic, and dysmetabolic diseases in later life.(146,149,150) Children's obesity, asthma, non-alcoholic hepatic liver diseases, aberrant cardiac growth are, among others, the conditions that have been associated with maternal/newborn dysbiosis.(146,151,152) However, uncertainty still exists about the microbiota offspring colonization, and both the modality and the timing of the microbial exposure for the fetus/newborn are controversial.(153–156)
GDM has been associated with increased risk for the offspring of developing dysmetabolic diseases, such as obesity and diabetes mellitus.(157) As previously mentioned, according to the results of some recent studies, the gut microbiota in newborns from mothers with GDM was significantly different from the microbiota of the control newborns, but only few data are available about the associations between maternal characteristics and newborn microbiota

pattern. Maternal fasting glucose concentrations were found to be correlated positively with the relative abundance of phylum Actinobacteria and genus *Acinetobacter*, and negatively with the phylum Bacteroidetes and the genus *Prevotella*.(145)

We hypothesized that some of the maternal metabolic characteristics may impact on the offspring's gut microbiota. The possibility of early modulation of the offspring gut microbiota by acting on specific maternal factors and/or characteristics is a topic of great interest.
Bearing in mind the highly divergent results deriving mostly from cross-sectional studies, we designed a prospective observational study aimed to evaluate the dynamic changes of the microbiota occurring throughout pregnancy in women with GDM and the characterization of the gut microbiota of their offspring.

4 AIMS AND OBJECTIVES OF THE STUDY

The objectives of the *first (1st) phase of this study* were evaluating:

1) whether the within-patient gut microbiota composition varied from the second to the third trimester of pregnancy.
2) whether patients with greater adherence to dietary recommendations presented a different microbial pattern than the less adherent ones.
3) whether changes in the gut microbiota composition were associated with variations in some anthropometric and metabolic variables.
4) whether specific microbiota oligotypes were implicated in these associations.

Instead the aims and objectives of the *second (2nd) phase of the study* were evaluating:

1) whether maternal metabolic variables and pregnancy outcomes of GDM patients are associated with the gut microbiota composition of their offspring.
2) And whether there are differences the gut microbiota composition of the offspring of mother with GDM when compared to the gut microbiota of the offspring of healthy normoglycemic women.

5 EXPERIMENTAL PART OF THE STUDY (1st PHASE)

5.1 Methods

5.1.1 Recruitment of patients

This is a prospective cohort study including 50 pregnant women diagnosed with GDM, consecutively recruited from the "Città della Salute e della Scienza" Hospital of Turin from April 2016. Each participant gave her written informed consent to participate in the study. The study

protocol was approved by the Ethics Committee of the "Città della Salute e della Scienza" Hospital of Turin (approval 707/2016). All research was performed in accordance with relevant guidelines/regulations.

In order to be included in the present study the participants had to meet the following inclusion criteria: gestational age between 24th and 30th weeks, Caucasian race and GDM diagnosed with Oral Glucose Tolerance Test (OGTT with 75g glucose). Instead women who met the following criteria were excluded from the study: twin pregnancy, use of prebiotics/probiotics, antibiotics or any drug during pregnancy, history of known, underlying disease before or during gestation (overt diabetes mellitus, hypertension, cardiovascular, pulmonary, autoimmune, joint, liver or kidney diseases, thyroid dysfunction, cancer, any other disease/condition), and no compliance to the study protocol. All women were taking folic acid supplementation.

GDM was diagnosed by OGTT performed at 24–28 gestational weeks in the morning, after at least 8h-overnight fast, when the fasting plasma glucose was ≥92 mg/dL and/or 1 h post-OGTT glycemia ≥180 mg/dL and/or 2 h post-OGTT glycemia ≥153 mg/dL, according to international criteria.(158) In our cohort, all the patients with GDM routinely received dietary counselling and nutritional recommendations in line with the current guidelines (carbohydrates 45% of total energy, rapidly absorbed sugars <10% of total energy, proteins of 18–20% total energy, fats 35% of total energy, at least 20–25 g/day fiber intake, no alcohol).(158) Additionally, 30-min daily moderate exercise was recommended (i.e. brisk walking).
Patients were instructed to self-monitor finger-prick capillary blood glucose at least 4 times per day. Insulin treatment was prescribed by the physicians in the presence of hyperglycemia, in accordance with guidelines.(25,159)

5.1.2 Collection of samples, anthropometric measurements and dietary information

Questionnaires, anthropometric measurements, fasting blood samples and stool samples were collected for all participants both at 24–28 weeks of gestational age at the time of GDM diagnosis (enrolment), and at 38 weeks, or before delivery, in the case of preterm delivery (study end). There was a continuous contact with the patients, through weekly telephone contact, in order to be aware of the progress of the pregnancy.

Stool samples were self-collected by the patients.(160) They were instructed on how to self-collect the samples, and all materials were provided in a convenient, refrigerated, specimen collection kit. Patients were also provided with sterile containers to collect the feces (VWR, Milan, Italy). The fecal samples were collected at home and transferred to the sterile sampling containers using a polypropylene spoon (3 spoons of about 10 g) and immediately stored at 4 °C. The specimens were transported to the laboratory within 12 hours of collection at a refrigerated temperature. At the arrival to the laboratory the containers were immediately stored at −80 °C for DNA extraction. No storage medium was used.

The study participants completed a 3-day food record (2 weekdays and 1 weekend day) and the Minnesota-Leisure-Time-Physical Activity Questionnaire at enrolment and at the study end.(161)

Detailed information on how to record food and drink consumed by using common household measures was provided to all participants. Two dieticians checked all questionnaires for completeness, internal coherence and plausibility.

Data relative to pre-pregnancy weight was self-reported. Instead, weight, height, and arterial blood pressure (BP) were measured at time of enrolment, and weight and BP were also remeasured at the study end. Body weight was measured to the nearest 0.1 kg, and height was measured to the nearest 0.1 cm with a stadiometer (SECA model 711, Hamburg, Germany), with the participants wearing light clothes and no shoes. Arterial BP was measured from the left arm, in a sitting position, after at least 10 min of rest, with a mercury sphygmomanometer with appropriate cuff sizes (ERKA Perfect-Aneroid, Germany). Two measurements were taken by trained personnel with arm supported at heart level and the values reported were the means of the two. Glucose levels were self-measured by the patients by the BGSTAR® glucometer (Sanofi-Deutscland GmbH, Frankfurt, Germany). The average of the values measured 1-hour after each meal during the third trimester has been reported.

Babies were classed as large for gestational age (LGA) if their birthweight was >90th percentile, considering neonatal anthropometric standards for Northern Italy.(162)

5.1.3 Blood analyses

Serum glucose was measured by the glucose oxidase method (Sentinel Ch., Milan) with an intra-assay CV of 1.1% and an inter-assay CV of 2.3%. HbA1c levels were determined by a latex-based method (Sentinel, Milan, Italy). The intra-assay e inter-assay CVs were respectively 1.1–1.5% and 1.1–1.6%. Triglycerides and cholesterol were assayed by enzymatic colorimetric assays (Sentinel, Milan) with an intra-assay CV of 3.0% and an inter-assay CV of 3.5% for triglycerides and with an intra-assay CV of 2.2% and an inter-assay CV of 3.4% for cholesterol. HDL-cholesterol was determined by enzymatic colorimetric assay after precipitation of LDL and VLDL fractions using heparin-MnCl2 solution and centrifugation at 4 °C and it had an intra-assay variation CV of 2.5% and an inter-assay CV of 4.1%. Insulin was measured by a biotin labelled antibody-based sandwich enzyme immunoassay (LDN, Germany). The kit had a sensitivity of less than 1.8 U/mL and a range of 0–100 U/mL. The intra-assay and inter-assay CVs were respectively 1.8–2.6% and 2.9–6.0%. Serum CRP values were determined using a high-sensitivity-latex agglutination assay on HITACHI 911 Analyzer (Sentinel, Milan). The intra-assay and inter-assay CVs were 0.8–1.3% and 1.0–1.5%, respectively. All laboratory measurements were centralized.

Body Mass Index (BMI) was calculated as weight divided for the square of height. The Homeostatic Model Assessment of Insulin Resistance (HOMA-IR) was calculated according to the published algorithm.(163) Adherence to the given dietary recommendations was considered in the presence of all the following criteria: consuming at least 20 g/day fiber (or increasing fiber intake more than 50% than enrolment) and reducing sugars <10% of total energy and abolishing alcohol intake.

5.1.4 Fecal DNA extraction

Nucleic acid was extracted from the feces samples collected. Total DNA from the samples was extracted using the RNeasy Power Microbiome KIT (Qiagen, Milan, Italy) following the manufacturer's instructions. One microliter of RNase (Illumina Inc. San Diego. CA) was added to digest RNA in the DNA samples, with an incubation of 1 h at 37 °C. DNA was quantified using the QUBIT dsDNA Assay kit (Life Technologies, Milan, Italy) and standardized at 5 ng/µL.

5.1.5 16S rRNA amplicon target sequencing

Culture-independent approach or High-throughput amplicon target sequencing (HTS) was used for the analysis of the composition of the maternal gut microbiota. More precisely, DNA directly extracted from the fecal samples was used to assess the microbiota by the amplification of the V3-V4 region of the 16S rRNA gene using the primers and protocols described by Klindworth et al.(164) PCR amplicons were cleaned using Agencourt AMPure kit (Beckman Coulter, Milan, Italy) and the resulting products were tagged by using the Nextera XT Index Kit (Illumina Inc. San Diego. CA) according to the manufacturer's instructions. After the 2nd purification step, amplicons products were quantified using a QUBIT dsDNA Assay kit (Life Technologies). Subsequently, equal amounts of amplicons from different samples were pooled. The pooled sample was run on an Experion workstation (Biorad, Milan, Italy) for quality analysis prior to sequencing. The sample pool (4 nM) was denatured with 0.2 N NaOH, diluted to 12 pM, and combined with 20% (vol/vol) denatured 12 pM PhiX, prepared according to Illumina guidelines. The sequencing was performed with a MiSeq Illumina instrument (Illumina) with V3 chemistry and generated 250 bp paired-end reads according to the manufacturer's instructions.

5.1.6 Bioinformatics analysis

Paired-end reads were first assembled using FLASH software with default parameters.(165) Joint reads were further quality filtered (at Phred <Q20) using QIIME 1.9.0 software and short reads (<250 bp) were discarded through Prinseq. (166,167)Chimera filtering was performed through USEARCH software version 8.163. Operational Taxonomic Units (OTUs) were picked at 97% of similarity threshold by UCLUST algorithms and centroids sequences of each cluster were matched to the Greengenes 16S rRNA gene database version 2013.(168) After sequencing, a total of 2,100,009 raw reads (2 × 250 bp) were obtained. After joining, a total of 1,919,311 reads passed the filters applied with QIIME, with an average value of 23,406 ± 31,535 reads/sample and a sequence length of 457 bp. The rarefaction analysis and Good's coverage, expressed as percentages, indicated that there was satisfactory coverage for all the samples (Good's coverage average, 92%). In order to avoid biases due to the different sequencing depth, OTU tables were rarefied to the lowest number of sequences per sample (4078 reads/sample). The OTU table displays the higher taxonomy resolution that was reached, for example when the taxonomy assignment was not able to reach the genus, family name was displayed. Phylogenetic

Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) was used to predict abundances of KEGG orthologs (KO) based on 16S-based structure of the microbiota.(169) The KO abundance table was then collapsed at level 3 of the KEGG annotations in order to display the inferred metabolic pathways and the table was imported in gage Bioconductor package in order to carry out pathway enrichment analysis to identify biological pathways overrepresented or underrepresented between samples.(170)

5.1.7 Oligotyping analysis

In order to identify sub-OTUs populations, reads assigned to genera within *Ruminococcaceae* and *Lachnospiraceae* were extracted and entropy analysis and oligotyping were carried out.(171) Briefly, the extracted reads were then used to identify nucleotide positions that will explain the maximum amount of biological diversity across the samples utilizing Shannon entropy in order to identify positional variation to facilitate the identification of nucleotide positions of interest.(171) Only *Blautia* and *Roseburia* oligotypes showed a higher level of entropy and were the only two taxa able to be differentiated in sub-OTUs. After the first round of oligotyping, high entropy positions were chosen (-C option) 8, 9, 12, 223, 225, 247, 261, 282, 432, 433 for *Blautia*; while position 8, 9, 12, 245, 432, 433 and 434 were chosen for *Roseburia*. To reduce the noise, each oligotype was required to appear in at least 10 samples, occur in more than 1.0% of the reads for at least one sample, represent a minimum of 500 reads in all samples combined, and have a most abundant unique sequence with a minimum abundance of 100. BLASTn was used to query the representative oligotype sequences against the NCBI nr database, and the top hit was considered for taxonomic assignment.

5.1.8 Statistical analysis

Gut microbiota α-diversity was assessed by Chao1 index, estimating the number of different taxa, and by Shannon diversity index, evaluating the taxa richness and evenness calculated using the diversity function of the vegan package in R environment (http://www.r-project.org).(172)
OTU table was used to build a principal-component analysis (PCA) as a function of the sampling time by using the made4 package of R. ADONIS and ANOSIM statistical test was used to detect significant differences in the overall microbial community by using the Weighted UniFrac distance matrices and the OTU table.

Not-normally distributed variables were presented as median (range interquartile). The individual differences (deltas) between end of the study values minus baseline values were calculated. The delta median values were reported. Within-participant differences in bacterial richness and in the variables at enrolment compared with values at the pregnancy-end were evaluated by paired-sample t-test, or Wilcoxon matched pairs test, as appropriate. Differences between categorical variables were computed by chi-square test.

Box plots represented the interquartile range between the first and the third quartile, with the error bars showing the lowest and the highest value. Pairwise Spearman's non-parametric correlations were used to study the relationships between the relative abundance of microbial taxa or oligotypes and metabolic variables, and between gut microbiota and inferred metabolic pathways. The correlation plots were visualized in R using the corrplot package of R.

The associations between blood pressure and laboratory variables (dependent variables) with OTUs were calculated using a multiple regression model, after adjusting for age and weight (at baseline) or adjusting for age, weight change, and adherence to the given dietary recommendations (at the pregnancy end) (Statistica, ver. 7.0; StatSoft Inc., Tulsa, OK, USA).

Bonferroni's correction for multiple comparisons was applied, a P value of 0.002 or lower was considered as statistically significant.

5.2 Results

5.2.1 Characteristics of the participants

Data of 41 patients were analyzed, because nine women did not return the stool samples and were therefore lost to follow-up. The clinical characteristics of the study participants did not differ from those of the 9 women who dropped out (data not shown).

Seven women (17.1%) gave birth before the 38th week of gestation. These patients provided the fecal and blood samples and the food questionnaire about a week before all the others (37th week); they did not differ regarding nutritional, anthropometric, or metabolic characteristics when compared to the others.

Most of the study participants were overweight women, with excessive fat intake and lower than recommended fiber consumption. From enrolment (24–28 weeks of gestational age) to the study end (38 weeks), weight and Body Mass Index (BMI) increased, and metabolic and inflammatory pattern of the participants worsened, as usually occurs during the third trimester of pregnancy (Table 2).

Table 2. Characteristics of the participants at enrolment and at the study end.

	At enrolment	Study end	P*
Number of study participants	41	41	
Age	37.1±4.2		
Pre-pregnancy weight (kg)	69.3±14.6		
Pre-pregnancy BMI (kg/m2)	25.8±5.9		
Nulliparous	58.5		
Education (%)			
Primary school	17.1		
Secondary school	41.5		
University degree	41.5		
Anthropometric and Blood measurements			
METS (h/week)	27.0 (36.4)	27.0 (26.5)	0.74**
Weight (kg)	75.8±12.9	79.0±13.3	**<0.001**
BMI (kg/m2)	28.2±5.3	29.4±5.4	**<0.001**
Systolic BP (mmHg)	110.8±11.7	116.1±11.6	0.02
Diastolic BP (mmHg)	72.9±7.5	75.8±9.1	0.07
Fasting glucose (mg/dL)	97.9±19.2	96.6±19.1	0.57
Fasting insulin (µU/mL)	10.1 (8.4)	11.6 (10.0)	0.02**
HOMA-IR (mmol/L*µU/mL)	2.3 (1.9)	2.8 (2.7)	0.15**
Total cholesterol (mg/dL)	234.1±32.4	257.0±48.6	**<0.001**
HDL-cholesterol (mg/dL)	65.8±13.4	67.0±15.6	0.54
Triglycerides (mg/dL)	173.3±53.0	259.2±70.5	**<0.001**
CRP (mg/L)	4.1 (4.2)	4.5 (7.5)	0.007**
Pregnancy outcomes			
Insulin treatment (%)		9.8	
Cesarean section (%)		24.4	
Gestational age at delivery (weeks)		39.2±1.2	
LGA newborns (%)		9.8	
Male newborns (%)		53.7	

BMI=body mass index, METS=metabolic equivalent of activity, BP=blood pressure, HOMA-IR=Homeostasis Model Assessment-Insulin Resistance, HDL=high density lipoprotein, LDL=low-density lipoprotein, CRP=C-reactive protein. Values are expressed as mean±standard deviation or median (interquartile range) *paired-sample t-test, **Wilcoxon matched pairs test

5.2.2 Adherence to the dietary recommendations

After the dietary counselling, 34.1% of the participants declared to be adherent to the given dietary recommendations. Characteristics at enrolment did not significantly differ between adherents and non-adherents, even if adherents showed increased values of weight and BMI

(Table 3). Adherents had a better metabolic and inflammatory pattern, with a significantly greater reduction in fasting glucose and Homeostasis Model Assessment-Insulin Resistance (HOMA-IR) levels at the end of the study.

Table 3. Characteristics of the participants by adherence to the lifestyle recommendations and median changes from enrolment (deltas).

	Baseline			Study End			Delta		
	Adherent	**Non adherent**	***p***	**Adherent**	**Non adherent**	***p***	**Adherent**	**Non adherent**	***P****
Number	14	27		14	27		14	27	
Age	35.5 ± 3.8	38.0 ± 4.3	0.08						
Pre-pregnancy weight (kg)	73.1 ± 18.0	67.4 ± 12.5	0.24						
Pre-pregnancy BMI (kg/m2)	28.0 ± 8.0	24.7 ± 4.3	0.09						
Nulliparous (%)	64.3	55.6	0.27						
Education (%)									
Secondary school	42.9	40.7							
University degree	42.9	40.7	0.94						
METS (h/week)	32.3 (37.0)	24.5 (38.0)	0.39*	27.9 (31.5)	23.3 (32.3)	0.08*	0.0	0.0	0.17
Weight (kg)	78.6 ± 16.7	74.3 ± 10.4	0.31	80.9 ± 17.0	77.9 ± 11.2	0.50	+ 2.0	+ 3.0	0.10
BMI (kg/m2)	30.1 ± 7.4	27.2 ± 3.6	0.10	30.9 ± 7.4	28.6 ± 3.8	0.18	+ 0.7	+ 1.2	0.10
Systolic BP (mmHg)	111.7 ± 12.8	110.3 ± 11.4	0.73	117.1 ± 12.2	115.6 ± 11.5	0.70	+ 7.0	+ 4.0	0.61
Diastolic BP (mmHg)	71.8 ± 9.0	73.5 ± 6.6	0.49	77.9 ± 10.2	74.7 ± 8.5	0.29	+ 7.0	0.0	0.21
Fasting glucose (mg/dL)	99.8 ± 29.3	96.9 ± 11.4	0.65	88.9 ± 25.3	100.6 ± 13.8	0.06	−6.0	+ 1.0	**<0.001**
Post-prandial glucose (mg/dL)				106.3 ± 8.3	118.1 ± 12.9	0.004			
HbA1c (%)	4.8 ± 0.9	4.6 ± 0.8	0.42	4.8 ± 0.8	5.0 ± 0.8	0.40	+ 0.1	+ 0.5	0.19
Fasting insulin (μU/mL)	11.3 (11.3)	9.0 (6.1)	0.83*	11.4 (10.8)	11.6 (12.4)	0.66*	−0.20	+ 2.0	0.003
HOMA-IR (mmol/L*μU/mL)	2.7 (2.8)	2.1 (1.3)	0.19*	2.4 (3.0)	3.1 (2.5)	0.38*	−0.45	+ 0.47	**<0.001**
Total cholesterol (mg/dL)	227.1 ± 33.1	237.8 ± 32.1	0.32	246.3 ± 41.5	262.6 ± 51.7	0.32	+ 20.0	+ 27.0	0.82
HDL-cholesterol (mg/dL)	68.0 ± 11.5	64.6 ± 14.4	0.45	68.9 ± 12.4	66.0 ± 17.1	0.58	+ 1.0	+ 1.0	0.73
Triglycerides (mg/dL)	159.1 ± 57.4	180.6 ± 50.1	0.22	246.5 ± 81.7	265.9 ± 64.5	0.41	+ 76.5	+ 91.0	0.65
CRP (mg/L)	3.2 (5.2)	4.3 (4.4)	0.76*	3.2 (3.1)	8.4 (8.3)	0.008*	−0.02	+ 2.5	0.003
Pregnancy outcomes									
Insulin treatment (%)				7.1	11.1	0.68**			
Cesarean section (%)				21.4	25.9	0.75**			
Gestational age at delivery				39.2 ± 1.3	39.2 ± 1.2	0.91			
LGA newborns (%)				7.1	11.1	0.68**			
Male newborns (%)				50.0	55.6	0.74**			

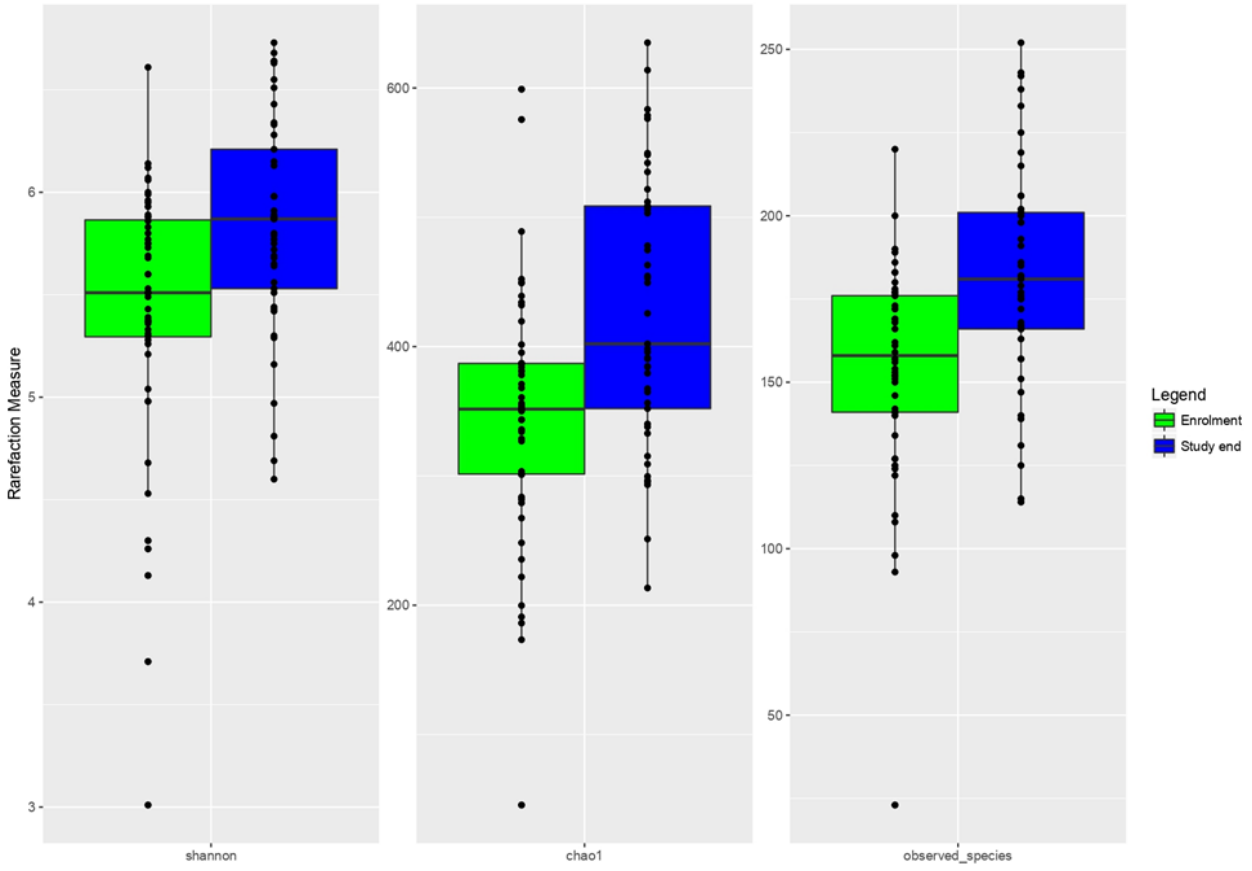
BMI = body mass index, METS = metabolic equivalent of activity, BP = blood pressure, HbA1c = glycated hemoglobin, HOMA-IR = Homeostasis Model Assessment-Insulin Resistance, HDL = high density lipoprotein, LDL = low-density lipoprotein, CRP = C-reactive protein, LGA = large-for-gestational age. Values are expressed as mean ± standard deviation or median (interquartile range); deltas = median values of the following difference: (end-of the study values minus baseline values). P-values were calculated by t-student test or chi-square test; *P-values by Mann-Whitney test; **P-values by Chi-square test.

5.2.3 Microbiota composition at enrolment and at the study end

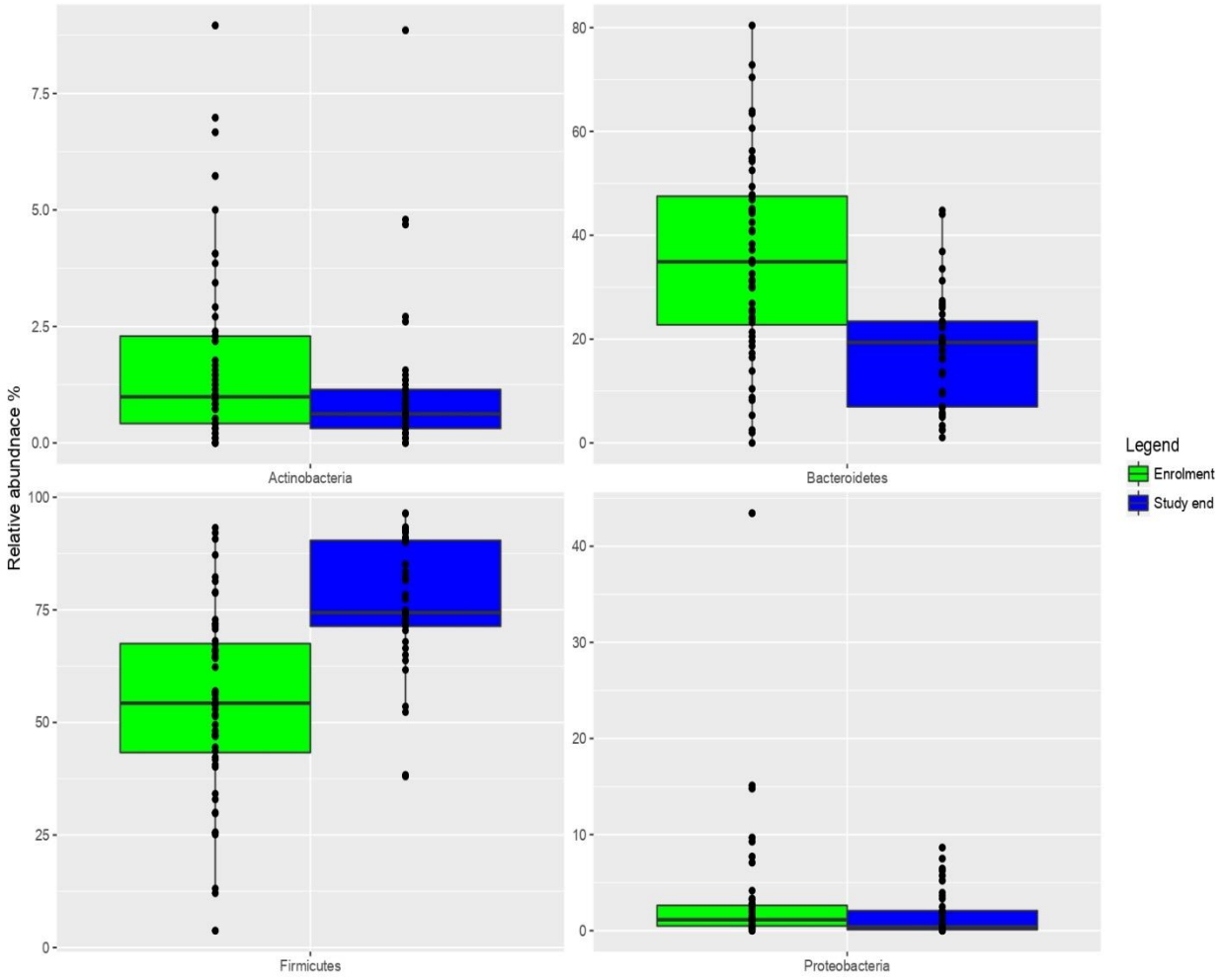
The microbiota α-diversity values were significantly different between subjects at enrolment-2nd trimester of pregnancy when compared to subjects at the end of the study-3rd trimester of

pregnancy ($P < 0.001$). In particular, species richness, number of different species and the Shannon index were significantly higher at the end of the study ($P < 0.001$) (Fig. 1). The analysis of the microbial taxa abundance at phylum level showed an increase of Firmicutes at the study end, and a reduction of the phyla Actinobacteria and Bacteroidetes (Fig. 2). Going more deeply in the microbial composition, the level of diversity of the subjects based on the structure of their microbiota was clearly different across time (Fig. 3). Moreover, Principal Component Analysis (PCA) based on microbiota composition (Fig. 4) revealed a significant relationship between the microbiota composition on genus-level and sampling time confirmed by ADONIS and ANOSIM statistical test ($P < 0.001$). Boxplot at genus level (Fig. 5) showed a significant reduction in the abundance of *Bacteroides, Collinsella and Rikenellaceae*, and a significant increase of the genera *Blautia, Butyricicoccus, Clostridium, Coprococcus, Dorea, Faecalibacterium, L−Ruminococcus* (*Ruminococcus* genus assigned to *Lachnospiraceae* family), and the *Lachnospiraceae* family at the end of the study when compared to enrolment (Fig. 5).

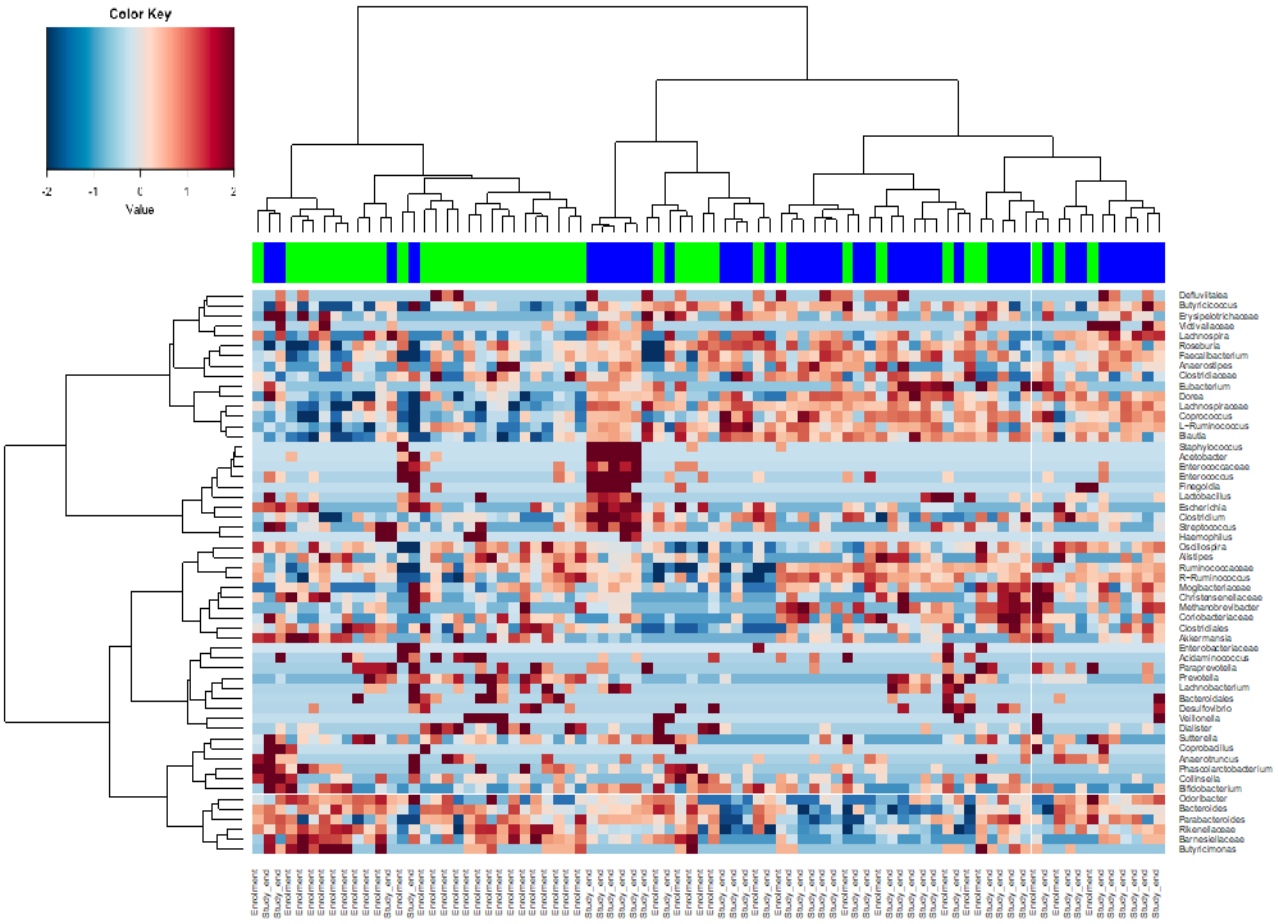
<u>Figure 1.</u> Boxplots to describe α-diversity measures of fecal microbiota of GDM patients at enrolment (green bars) and study end (blue bars). Individual points and brackets represent the richness estimate and the theoretical standard error range, respectively.



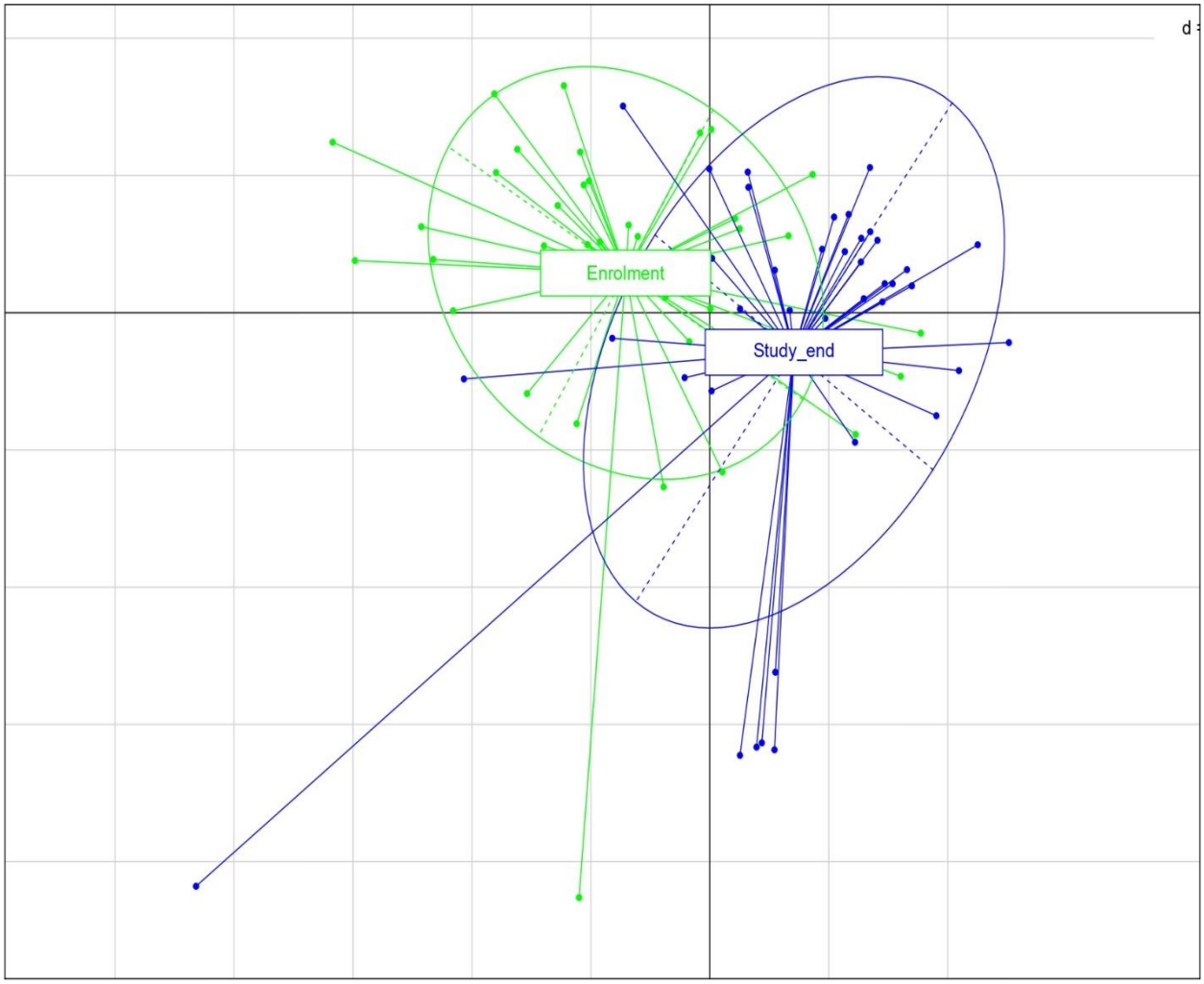
<u>Figure 2.</u> Boxplots showing the relative abundance of Actinobacteria, Bacteroidetes, Proteobacteria and Firmicutes phyla in fecal samples of GDM patients at enrolment (green bars) and study end (blue bars). Boxes represent the interquartile range (IQR) between the first and third quartiles, and the line inside represents the median (2nd quartile). Whiskers denote the lowest and the highest values within 1.56 IQR from the first and third quartiles, respectively. Circles represent outliers beyond the whiskers.



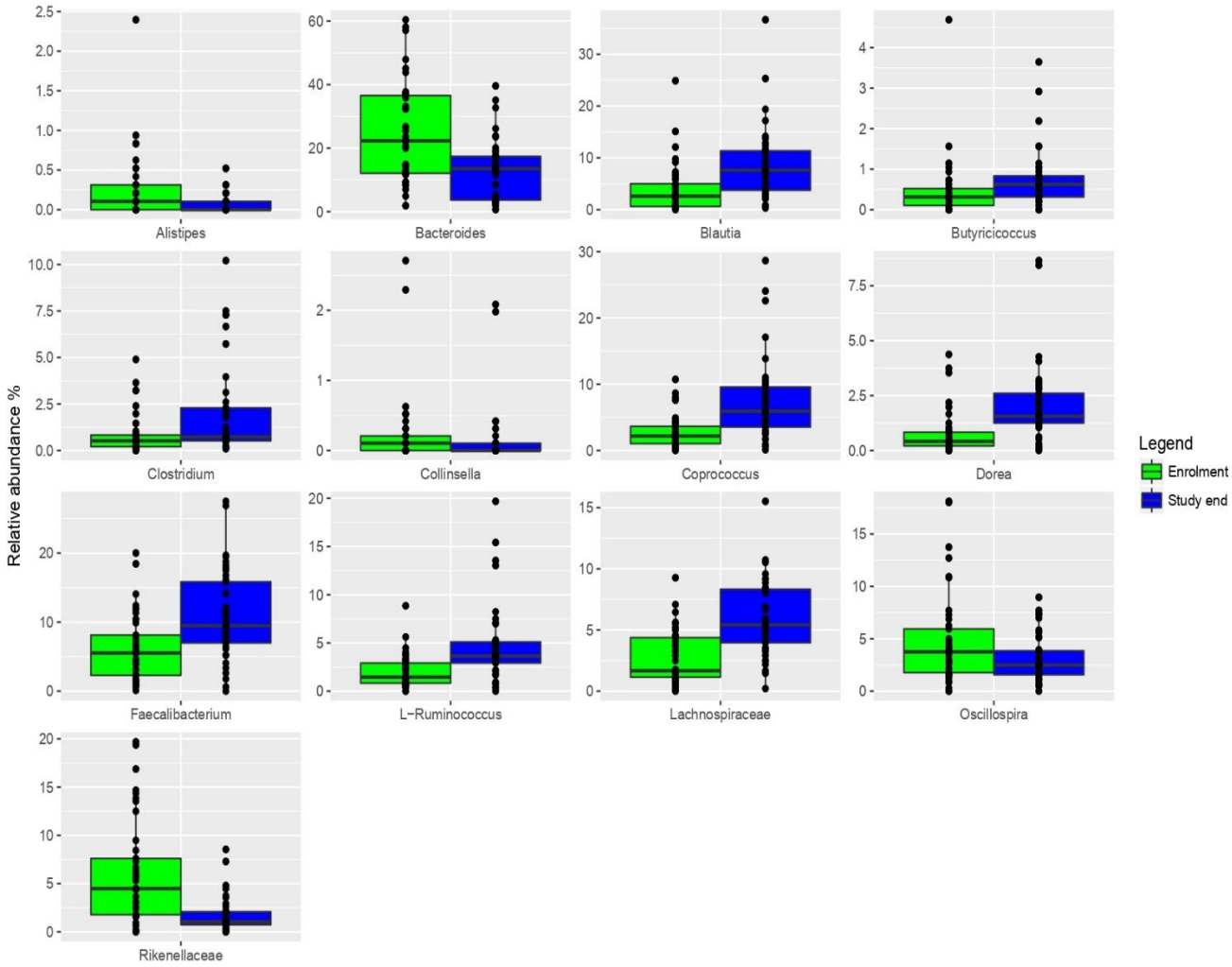
<u>Figure 3.</u> Average-linkage clustering based on the Spearman distance of fecal samples of GDM patients at enrolment (green bars) and at the study end (blue bars). Rows and columns are clustered by Ward linkage hierarchical clustering.



<u>Figure 4.</u> Principal Component Analysis (PCA) based on OTUs relative abundance of GDM patients at enrolment (green) and study end (blue). The first component (horizontal) accounts for the 22.9% of the variance and the second component (vertical) accounts for the 23.5%.



<u>Figure 5.</u> Boxplots showing the relative abundance at genus or family level of the OTUs differentially abundant based on Wilcoxon matched pairs test (P ≤ 0.002) in fecal samples between: GDM patients at enrolment (green bars) and at the study end (blue bars);



5.2.4 Associations between microbiota and metabolic variables

Several different associations between metabolic variables and microbiota could be detected both at enrolment and at the study end (Fig. 6).

At enrolment, glycated hemoglobin (HbA1c) levels were found to be positively correlated with both *Bacteroidales* (β = 1.43; 95% CI 0.67 2.19; P < 0.001) and *Prevotella* (β = 0.11; 95% CI 0.06 0.16; P < 0.001).

At the end of the study, many associations among specific microbiota relative abundance and metabolic and inflammatory variables and their changes across pregnancy were detected in multiple regression analyses, after adjusting for age, weight change, and adherence to the given recommendations (Table 4). Among the associations, *Faecalibacterium* resulted inversely correlated with fasting glucose. *Collinsella* and *Blautia* were respectively directly and inversely associated with insulin and HOMA-IR values. Moreover, *Blautia* was inversely correlated with HbA1c levels, while *Sutterella* positively associated with the CRP values (Table 4). Results did not change significantly, after adjusting for pre-pregnancy BMI, educational level and exercise (data not shown).

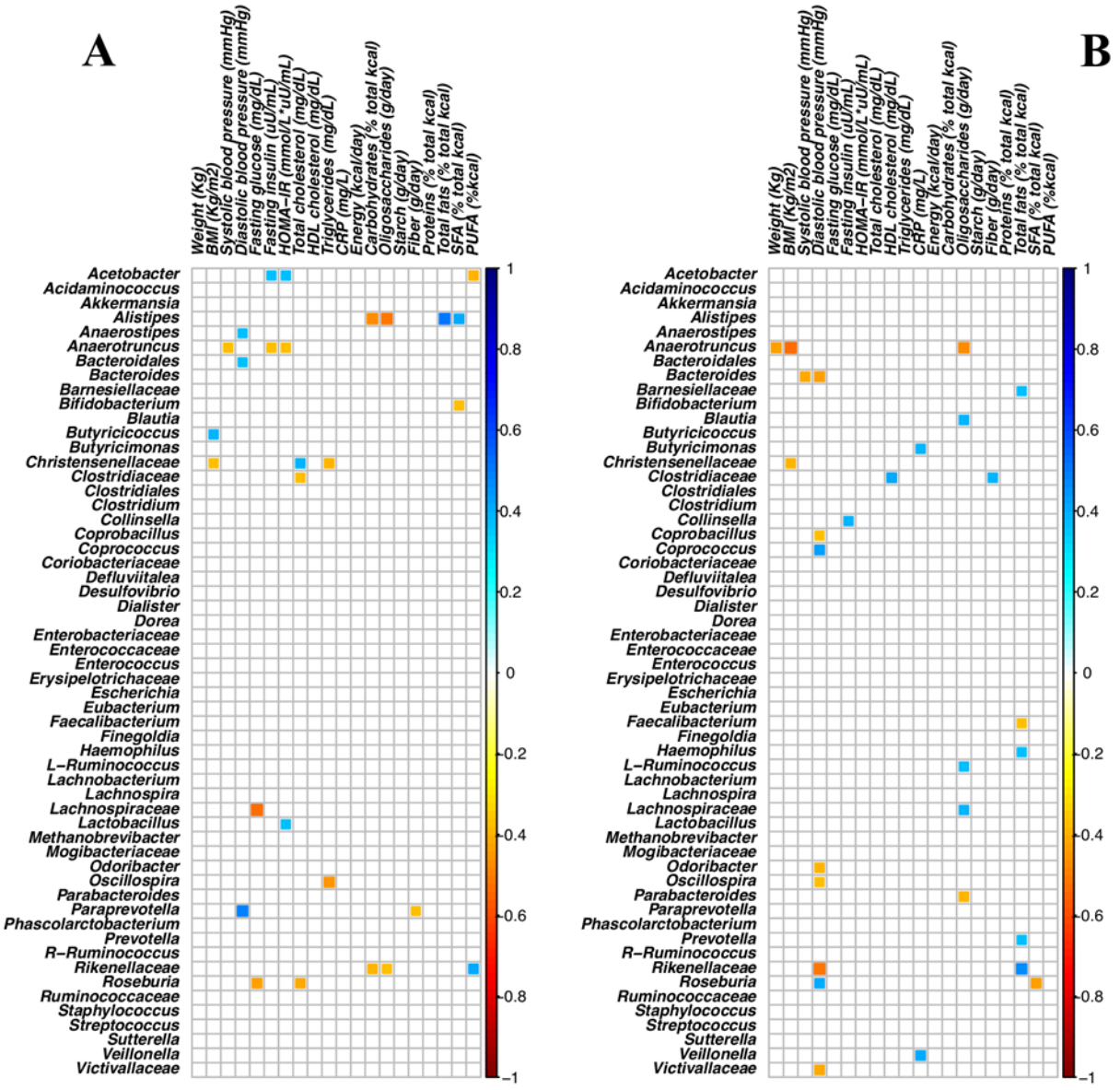
Table 4. Statistically significant associations between microbiota composition at the study end and dietary and metabolic variable by Spearman's correlations (left) and multiple regression analyses (right).

		Rho	Beta	95% CI	P
Metabolic variables*					
Diastolic BP (mmHg)					
Oscillospira		−0.44	−2.01	−3.11 −0.91	**<0.001**
Rikenecellaceae		−0.51	−2.74	−3.97 −1.51	**<0.001**
Delta fasting glucose (mg/dL)					
Faecalibacterium		−0.54	−1.28	−1.71 −0.85	**<0.001**
Delta glycated hemoglobin (%)					
Blautia		−0.51	−0.06	−0.10 −0.03	**0.001**
Delta fasting insulin (µU/mL)					
Blautia		−0.35	−0.42	−0.67 −0.17	**0.001**
Butyricimonas		0.41	36.1	14.7 57.5	**0.002**
Collinsella		0.45	8.69	6.00 11.4	**<0.001**
Coprobacillus		0.39	6.52	3.29 9.75	**<0.001**
Delta HOMA-IR (mmol/L*µU/mL)					
Blautia		−0.36	−0.11	−0.17 −0.05	**0.002**
Butyricimonas		0.51	11.2	6.50 15.9	**<0.001**
Collinsella		0.45	2.37	1.80 2.94	**<0.001**
Erysipelotrichia		0.37	1.87	1.09 2.65	**<0.001**
Delta CRP (mg/L)					
Sutterella		0.62	7.57	5.02 10.1	**<0.001**

BP = blood pressure.

*Multiple regression model evaluating the association between BP and metabolic laboratory variables (dependent variables) and bacteria (independent variables) after adjusting for age, weight change, and adherence to the given recommendations. Each row is a model.

Figure 6. **Spearman's rank correlation matrix of OTUs with > 0.2% abundance in at least 10 fecal samples, dietary information and blood variables. Strong correlations are indicated by large squares, whereas weak correlations are indicated by small squares. The colors of the scale bar denote the nature of the correlation, with 1 indicating a perfectly positive correlation (dark blue) and -1 indicating a perfectly negative correlation (dark red) between the two datasets. Only significant correlations (P <0.01) are shown. GDM patients at enrolment (plot A) or at study end (Plot B).**



5.2.5 Gut microbiota signature at sub-genus level

The oligotyping approach was suggested as a powerful tool allowing resolution at species level and below and taking into account the possible different associations and responses that species or strains belonging to the same genus may have. Therefore, In order to explore the possible effects at sub-genus level, we carried out oligotyping on sequences of *Blautia* and *Roseburia* since these were the only genera changing over time that showed a Shannon entropy index sufficient to identify all nucleotide positions that would resolve the oligotypes. Specific *Blautia* oligotypes (identified as *Blautia wexlerae* by BLASTn match): B1, B2, B4, B9, B11, B18, B27, B32, B36 B42, B49, B51 and B53, identified as *Blautia luti* were more abundant at the study end, while B41 and B59 decreased with the advancement of pregnancy (Fig. 7). When plotting the correlation between those oligotypes and different metabolic and inflammatory variables (Fig. 8), various correlations were found. In a multiple regression model, B42 was associated directly with total cholesterol (β = 16.6; 95% CI 7.10 26.1; P = 0.002), and B42 inversely with diastolic blood pressure (β = −3.06; 95% CI −4.75 −1.37; P = 0.001).

Roseburia oligotypes R10, R24, R50, and R70 increased at the study end when compared to enrolment (data not shown). Among the different correlations found by Spearman's nonparametric correlations, none resulted significantly different in the regression model analysis.

<u>Figure 7.</u> Boxplots of differentially abundant *Blautia* oligotypes in fecal samples between GDM patients at enrolment (green bars) and at the study end (blue bars). Only oligotypes that significantly differed between enrolment and study-end fecal samples are displayed (P <0.01).

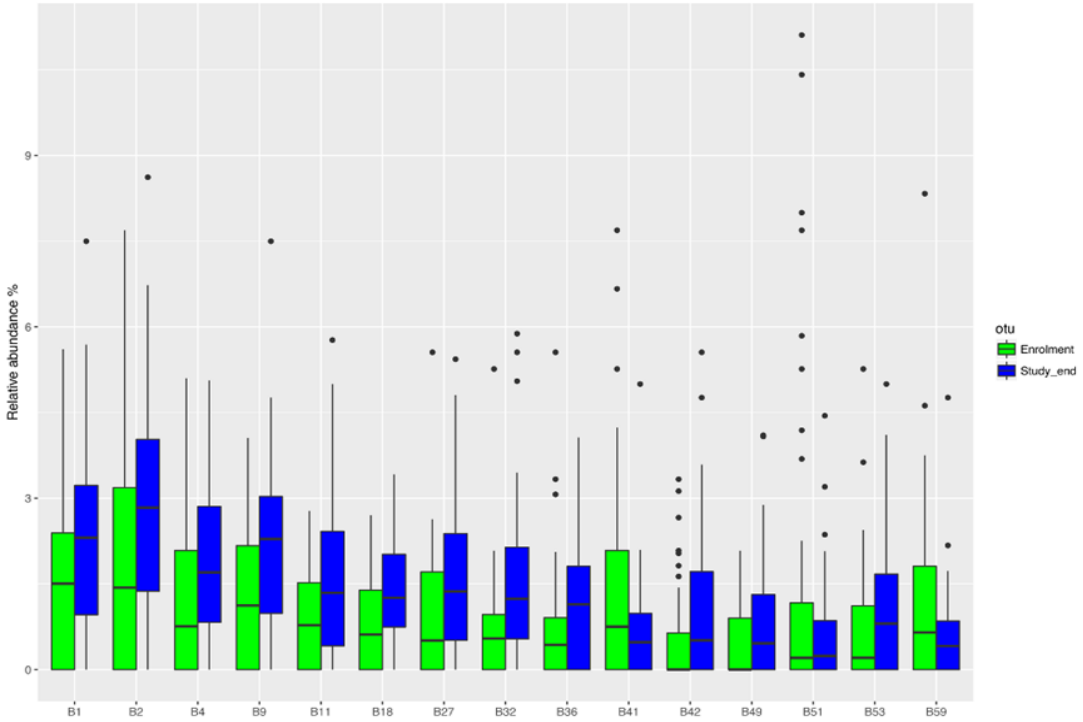
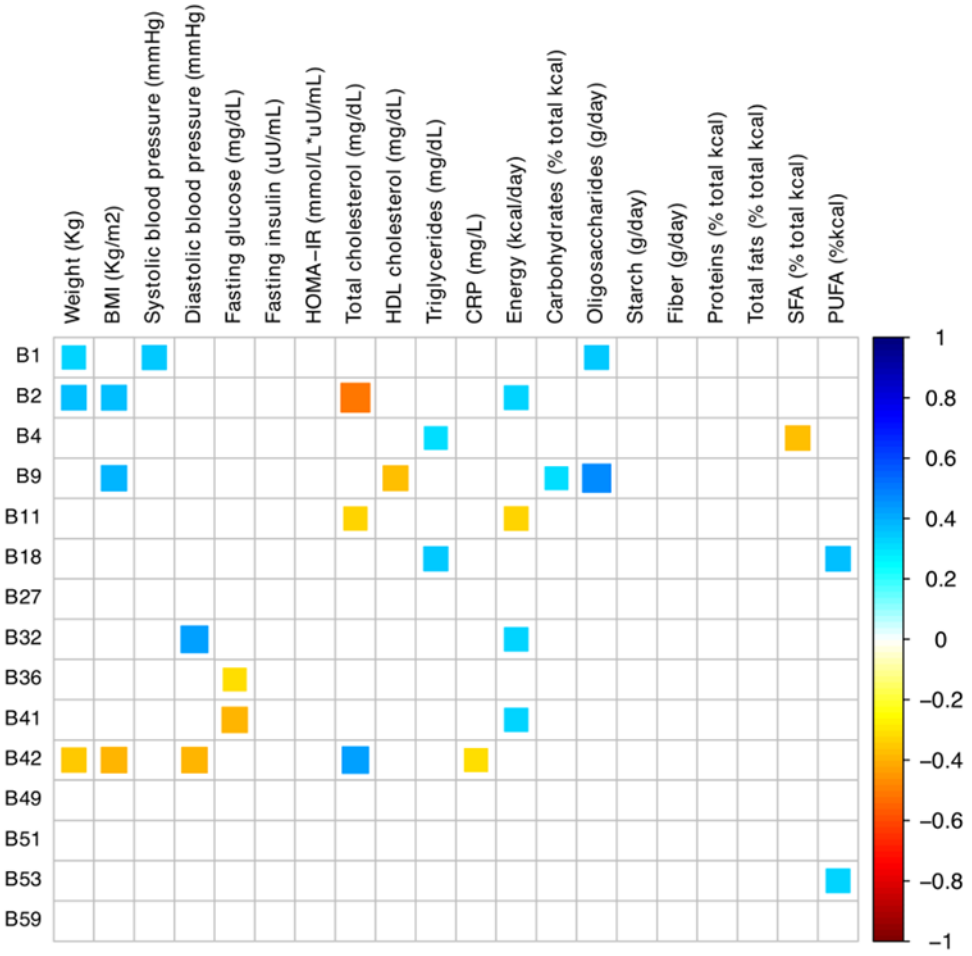


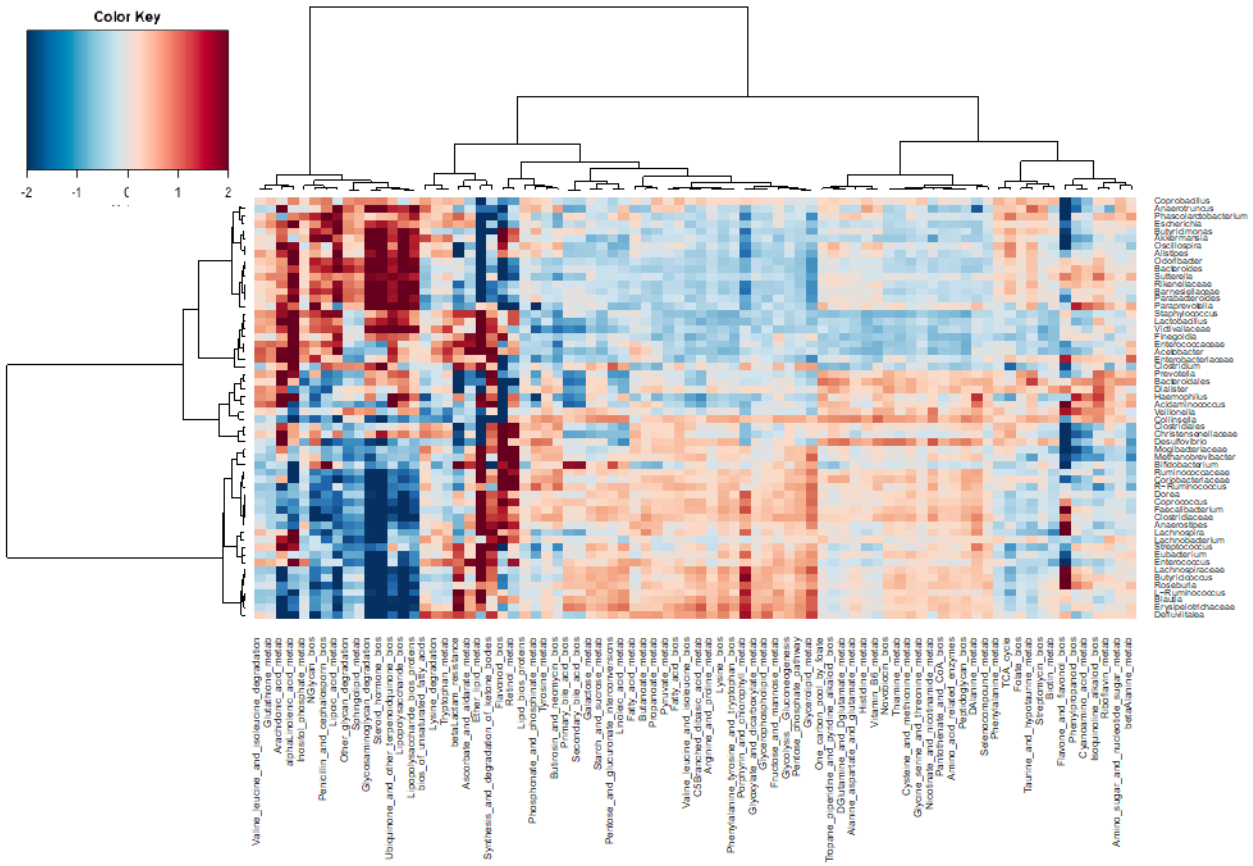
Figure 8. Spearman's rank correlation matrix of *Blautia* oligotypes abundance, nutrients, and blood variables. Strong correlations are indicated by large squares, whereas weak correlations are indicated by small squares. The colors of the scale bar denote the nature of the correlation, with 1 indicating a perfectly positive correlation (dark blue) and -1 indicating a perfectly negative correlation (dark red) between the two datasets. Only significant correlations (P <0.01) are shown.



5.2.6 Shift in predicted metagenomes

The pathway enrichment analysis of the predicted metagenomes showed an enrichment of KEGG orthologues, at study end when compared with enrolment, of glycolysis/gluconeogenesis (ko00010), fructose and mannose metabolism (ko00051), galactose metabolism (ko00052), starch and sucrose metabolism (ko005009), biosynthesis of amino acids (ko01230), and a reduction of fatty acid metabolism (ko01212), biotin metabolism (ko00780) and folate biosynthesis (ko00790). When plotting the correlations between OTUs and inferred metabolic pathways, we observed a positive correlation between Lipopolysaccharide (LPS) biosynthesis (ko00540) with *Sutterella, Bacteroides* and *Phascolartobacterium* (Fig. 9).

Figure 9. **Heat plot showing Spearman's correlations between OTUs occurring at 0.2% in at least 10 samples and predicted metabolic pathways, filtered for KO gene sample presence >10 in at least 10 samples, related to amino acid, lipid, cofactors and vitamins, energy and carbohydrate metabolism. Rows and columns are clustered by Ward linkage hierarchical clustering. The intensity of the colors represents the degree of correlations between the OTUs and KO as measured by the Spearman's correlations.**



5.3 Discussion

The results of the study have demonstrated that there is a significant alteration in the gut microbiota over the course of pregnancy, from the second to the third trimester of pregnancy in women with gestational diabetes mellitus. In particular we have observed a higher α-diversity, increase in the relative abundance of the Firmicutes phylum, and a reduction of the relative abundance of the phyla Bacteroidetes and Actinobacteria. Furthermore, we have detected associations between specific bacterial abundance and different metabolic variables.

The reduced insulin sensitivity and increased insulin resistance of late pregnancy is considered beneficial to support the growing fetus and the increased nutrient absorption, even if it is associated with metabolic impairment and inflammation.(173) Women who developed GDM have greater reduction in insulin sensitivity and their insulin secretion is not sufficient to maintain euglycemia, leading to glucose intolerance. (174) This can be counterintuitive due to the progressive weight gain and increase in circulating levels of insulin, lipids, and inflammatory markers in the patients over the course of pregnancy and the well-known association of low bacterial richness and diversity with adiposity, insulin resistance, dyslipidemia, and inflammatory phenotypes.(175)

Going more deeply in the microbiota composition of our patients, we observed that the higher bacterial richness was related to the phylum Firmicutes. In one Finnish study using the(METSIM) cohort, it has been reported that glycated hemoglobin (HbA1c) values were positively associated with the microbiota richness.(176) Other study using animal models have demonstrated that mice transplanted with feces from obese and lean individuals showed a positive correlation of the OTUs richness with both fasting insulin and HOMA-IR level.(177)

The increase in Firmicutes abundance (and the reduction in Bacteroidetes) can be explained by the patients' gestational weight gain, not different to what happens in obese and overweight patients. (120,178) Accordingly, the inferred metagenome profiles showed an increase in pathways involved in the carbohydrate metabolism (in particular, glycolysis/gluconeogenesis, fructose and mannose metabolism, galactose metabolism, and starch and sucrose metabolism) with the release of simple sugars due to the higher abundance of Firmicutes that harvested more energy from the diet.(114,179) It can be hypothesized that those enriched function could be related with the progressive weight gain and could be a feature in hyperglycemic phenomena.

Literature data related to weight gain during pregnancy are controversial: in normoglycemic pregnancy, some studies have reported that weight gain is associated with an increase of *Escherichia coli* or *Bacteroides* abundance and others found both an increase in the phyla Proteobacteria and Actinobacteria and a decline in butyrate-producing bacteria (such as *Faecalibacterium*).(173,180,181)

A higher Bacteroides-to-Firmicutes ratio has been found to correlate with elevated plasma glucose levels.(182) In our patients, however, the most relevant change was the weight gain at the end of pregnancy (Table 2). We detected a reduction in Bacteroidetes across pregnancy, but we found a significant direct association between *Bacteroidales* and *Prevotella* and glycated hemoglobin (HbA1c) levels at enrolment-during the 2nd trimester of pregnancy when GDM is normally being diagnosed.

Indeed, the comparison between studies is difficult due to the different ethnicity and food habits of the analyzed cohorts leading to inter-individual variations in the gut microbiota composition and the various methods used to analyze the microbiota, both causing sometimes contradictory results.(160)

Additional aspects of previous studies make the comparison with our results difficult, such as the fact that both normoglycemic and GDM women were combined together, participants that were

taking probiotics or antibiotics were not excluded from the study, dietary intakes did not change during pregnancy or only the early pregnancy period was evaluated.(173,183,184)

Most of the study participants (about 2/3) did not change substantially their dietary habits after having received nutritional recommendations and showed a worse metabolic and inflammatory pattern than the adherent women. Even if arbitrary, the subdivision by dietary adherence distinguished women with greater increments of fasting glucose, insulin resistance and CRP values. During normal pregnancy a low grade of inflammation develops and GDM is considered a pro-inflammatory state.(185) Accordingly, we observed higher values of CRP during the third trimester, at the end of pregnancy. An imbalance of pro- and anti-inflammatory bacterial species have been proposed to trigger low-grade inflammation and insulin resistance in human.(175)
In particular, *Faecalibacterium*, an anti-inflammatory commensal bacterium, significantly increased with the pregnancy progression, and is consistently reported to be more prevalent in individuals with higher bacterial richness.(175) It could be hypothesized that this increase could be a compensatory mechanism to counterbalance the pro-inflammatory state, potentially harmful for the fetus. Indeed, we found a strong inverse relationship between *Faecalibacterium* abundance and fasting glucose values, supporting the well-known association between inflammation and dysmetabolism. Accordingly, *Faecalibacterium prausnitzii* resulted highly discriminant for the diagnosis of type 2 diabetes in metagenomic analyses.(186,187) Furthermore, these butyrate-producer bacteria have been found inversely linked to diabetes in different human studies investigating the composition of gut microbiota.(188–191)

In this study, we observed a negative association between diastolic blood pressure and *Rikenellaceae* and *Oscillospira*. *Rikenellaceae* is a butyrate producer, while *Oscillospira* is considered an enigmatic bacterial genus that has never been cultured, probably producing butyrate. Few available data support a beneficial role on human health.(192) Other studies found a protective role of *Odoribacter* (Bacteroidetes phylum) on blood pressure in overweight pregnant women, and its capability to produce butyrate was mainly implicated in the maintenance of normal blood pressure.(193)

The strong direct associations that we found between the genus *Collinsella* and insulin/HOMA-IR values were mostly in line with other studies during pregnancy and also outside pregnancy the higher abundance of the lactate-producing *Collinsella* was found in patients with type 2 diabetes mellitus.(183,194) These bacteria are reported to play pro-inflammatory effects and to affect the metabolism by altering intestinal cholesterol absorption, decreasing glycogenesis in the liver and increasing triglyceride synthesis.(194)

Insulin resistance was associated positively with Erysipelotrichia and negatively with *Blautia* in our patients. Very few human data are available on Erysipelotrichia, suggesting a relationship with inflammatory diseases for this class, which seems in a close relationship with the class of Mollicutes, which is in turn associated with many pathological conditions in human like endotoxemia, obesity and insulin resistance. (182,195)

In our GDM patients, the butyric acid-producing genus *Butyricimonas* was directly associated with insulin resistance. The possible role of this taxa in human diseases awaits further investigation, even if a positive association with mean arterial pressure has been detected.(196)

Changes in CRP values during the third trimester of pregnancy resulted directly associated with the genus *Sutterella*. Even if we did not detect an overall increase in Proteobacteria during pregnancy, as other authors observed, we found that *Sutterella*, a proteobacteria with known pro-inflammatory capacity, was associated with an increased value of CRP over the course of pregnancy.(173) Consistent with this, the predicted metagenomes showed a correlation between *Sutterella* and KEGG genes associated with LPS biosynthesis. Gram-negative bacteria could produce inflammatory LPS triggering a pro-inflammatory state, a condition characterizing both type 2 diabetes and obesity.(8,197)
We also observed a correlation between LPS inferred KEGG genes and *Bacteroides*. In diabetic patients, LPS from a species belonging to *Bacteroides* (*B. fragilis*) was reported to play a major pathogenic role.(198) In addition, the metagenomic content of GDM patient was reported to be enriched of genes involved in LPS biosynthesis and in the regulation of blood glucose levels.(199)

At genus level, strong inverse relationships between *Blautia* and Hba1c and insulin resistance were observed. At sub-genus level, we observed a higher number of oligotypes belonging to the same species, even if only a few of them changed during the progression of the pregnancy. Moreover, some controversial associations between *Blautia* and blood pressure and cholesterol values were found. Indeed, a controversial role of Blautia in the human gut is already reported. Several studies showed a direct association between *Blautia* and hyperglycemia, but other studies reported that increased abundance of this genus indicates a healthy gut, reduced inflammation and blood pressure values, diminished risk for type 1 diabetes and obesity, and increased survival. (200–203) Our results suggest a possible different strain-dependent effect on the host metabolism. The diversity at sub-genus-level is indeed well known to play a key role in establishing the interconnection between gut microbiome and host responses.(204) As recently observed by De Filippis and colleagues, different oligotypes belonging to the same species showed different relative abundance and different correlation patterns with metabolomic data. Those authors suggested that different putative strains could have different impact on the host.(205)

The interactions between the host and the gut microbiota during pregnancy and the its metabolic complications such as GDM, remains to be fully understood. The knowledge of the gut bacterial composition might allow the identification of subsets of women with different metabolic risks, because of its role in the gestational pro-inflammatory status, potentially contributing to the increased insulin resistance in pregnancy. This is a topic of great interest, also taking into consideration the possible benefit of probiotic supplementation in the reduction of inflammation in women with GDM, a condition well-known for exposing to an increased risk for chronic health conditions not only the mother but also the offspring.(206)

This study has some limitations, one of it is the small sample size. Nevertheless, the power of our study to detect differences in alpha diversity was 0.84 with $\alpha = 0.01$. Another limitation is that

the fecal samples were used as proxies for the microbial content of the entire gastrointestinal tract, it is however reasonable to consider that mouth and skin microbiota could vary too. Owing to the observational design of this study, the presence of unmeasured confounding factors cannot be excluded. Microbiota assessment through amplicon-based sequencing has also several biases due to the PCR amplification step, while shotgun metagenomic sequencing can identify significantly more bacterial species per read than the 16S method.(207) Correlations were performed by considering individual groups of bacteria independently from each other, therefore it was not possible to establish neither the causality nor the biological relevance of the reported relationships. Finally, the predictive metagenomic profiling was obtained from the bacterial abundance and was therefore a derived result.

In conclusion, the results of this study demonstrate that in our GDM patients, a shift in the microbiota composition with higher α-diversity, and numerous associations between the metabolic/inflammatory pattern and specific bacterial abundance were detected. If confirmed by further studies in larger sample, these results suggest that the development of strategies to modulate the gut microbiota might be the next step in order to impact on the maternal and possibly fetal health and their future risk for metabolic diseases.

However, one part of the puzzle is still missing and that's the complex interactions and the possible effect of maternal metabolic health and dysbiosis during pregnancy on the microbiota of the offspring. This is one of the main aspects of the 2nd phase of the study that will be presented in detail in the next section.

6 EXPERIMENTAL PART OF THE STUDY (2nd PHASE)

6.1 Methods

6.1.1 Patients recruitment

All pregnant women routinely performed an oral glucose tolerance test at 24–28 gestational weeks at the "Città della Salute e della Scienza" Hospital of Turin. As previously explained in the 1st phase of the study, the first 50 patients who met both the criteria for GDM according to international guidelines (fasting plasma glucose ≥92 mg/dL and/or 1h post-test glycemia ≥180 mg/dL and/or 2h post-test glycemia ≥153 mg/dL) and the inclusion criteria were enrolled. Out of them, 41 women participated in an observational study aiming to evaluate the changes in gut microbiota composition across pregnancy, the other 9 women were lost to follow-up. The fecal samples of their offspring were collected in the first week of life. Indeed, as the mode of collection and/or storage of the offspring stool samples by 12 mothers resulted inappropriate, data of only 29 infants (70.7%) could be analyzed. As mentioned before the inclusion criteria were: GDM diagnosed by a 75g oral glucose tolerance test (OGTT) between 24–28 weeks gestational age and European origin with both parents born in Europe. Women with twin

pregnancy, any pathological conditions before or during pregnancy (known diabetes mellitus, hypertension, cardiovascular, pulmonary, autoimmune, joint, liver or kidney diseases, thyroid dysfunction, cancer, any other disease/condition), no compliance to the study protocol, or use of prebiotics/probiotics, antibiotics or any drug during pregnancy were excluded.
All the GDM patients received dietary counselling and the recommendation of performing 30-min daily moderate exercise (i.e. brisk walking) and were instructed to self-monitor finger-prick capillary blood glucose at least 4 times per day. Finally, all mothers were suggested to breastfeed their children. However, only 10 of them followed this suggestion. All the others declared to have used formulas without added probiotic/prebiotic compounds.

In order to compare our offspring to infants from normoglycemic women, we have used the recently released 16S rRNA gene sequence from 19 Italian infants collected in the first week of life (NCBI SRA, under BioProject ID PRJNA378341).(208) All infants from healthy women were vaginally delivered and exclusively breastfed. Neither infants nor their mothers had received any antibiotics or probiotics during pregnancy and postpartum. We used as comparison the results of the 4th day of life.

6.1.2 Ethical aspects

The present study conforms to the principles outlined in the Declaration of Helsinki. The study protocol was approved by the Ethics Committee of the "Città della Salute e della Scienza" hospital of Turin (approval 707/2016). All patients provided written informed consent prior to participation in the study protocol.

6.1.3 Data and samples collections

Anthropometric values, 3-days food record questionnaires, fasting blood samples (for the determination of metabolic variables and C-reactive protein levels) and stool samples of the mothers were collected both at the time of GDM diagnosis, at 24–28 weeks of gestational age, and at 38 weeks, or before delivery, in the case of preterm delivery. (209) Fecal samples of the offspring were collected between the 3rd and the 5th day of life, after the meconium expulsion. Data relative to the type of delivery, birth weight, gestational age and the type of feeding were extracted from the medical records.
Insulin therapy was added to diet when fasting blood glucose levels were consistently ≥90 mg/dL, 1-hour levels consistently ≥130 mg/dL, or 2-hour levels ≥120 mg/dL, according to guidelines.(210) Mothers were instructed to collect the stool samples and all materials were provided in a convenient, refrigerated, specimen collection kit (VWR, Milan, Italy). The fecal samples were transferred to the sterile sampling containers using a polypropylene spoon (3 spoons of about 10 g) and immediately stored at 4 °C. The specimens were transported to the laboratory within 12 hours of collection at a refrigerated temperature. Containers were immediately stored at −80 °C for DNA extraction. No storage medium was used.
In the case of infants from normoglycemic mothers, fecal samples have been reported to be collected from diapers using standard sterile collection tubes.(208)

6.1.4 Fecal DNA extraction and sequencing

Total DNA from the feces collected was extracted using the RNeasy Power Microbiome KIT (Qiagen, Milan, Italy) following the manufacturer's instructions. One microliter of RNase (Illumina Inc. San Diego. CA) was added to digest RNA in the DNA samples, with an incubation of 1 h at 37°C. DNA was quantified using the QUBIT dsDNA Assay kit (Life Technologies, Milan, Italy) and standardized at 5 ng/µL. DNA was used as a template in a PCR reaction in order to amplify the V3-V4 region of the 16S rRNA gene.(211) Library preparation and sequencing (2X250bp) was performed as previously explained in the methods section in the 1st phase of the study.

6.1.5 Bioinformatics analyses

Paired-end reads were first assembled using FLASH software and quality filtering using QIIME 1.9.0 software and the pipeline already explained.(209,212) OTUs picked at 97% of similarity were rarefied to the lowest number of sequences per sample. The OTU table obtained through QIIME displays the higher taxonomy resolution that was reached. When the taxonomy assignment was not able to reach the genus, family name was displayed.

In order to identify sub-OTUs populations in the offspring cohort, reads assigned to genera *Blautia, Bacteroides* and *Bifidobacterium* were extracted and entropy analysis and oligotyping were carried out since these were the only OTUs showing higher level of entropy able to obtain sub-OTUs.(171) After the first round of oligotyping, high entropy positions were chosen (-C option): 8, 12, 113, 223, 224, 247, 432, 433 for *Blautia*; position 8, 12, 40, 74, 75, 116, 117, 124, 129, 130, 390, 397, 452 and 453 were chosen for *Bacteroides*; while position 8, 12, 14, 81, 114, 115, 158, 159, 160, 328, 329, 389, 437, 438, 439, 440, 441, 442, 443 and 444 were chosen for *Bifidobacterium*. In order to reduce the noise, each oligotype should appear in at least 10 samples, occur in more than 1.0% of the reads for at least ten samples, represent a minimum of 750 reads in all samples combined, and have a most abundant unique sequence with a minimum abundance of 50. A cladogram of representative sequences was generated using the ANVIOs software.(213)

6.1.6 Statistical analyses

Gut microbiota α-diversity was calculated by the diversity function of the vegan package in R environment (http://www.r-project.org).(214) ADONIS and ANOSIM statistical test were used to detect significant differences in the overall microbial community by using the unweighted UniFrac distance matrices or OTU table. Offspring variables were compared with maternal variables by paired-sample t-test, or Wilcoxon matched pairs test, as appropriate. Differences in gut microbiota between the GDM offspring and healthy-women offspring were calculated by t-Student test or Mann-Whitney test. Pairwise Spearman's non-parametric correlations were used to study the relationships between the infant relative abundance of microbial taxa and maternal metabolic variables. The degree of agreement (concordance) among maternal oligotypes at enrolment, maternal oligotypes at the study-end and infant oligotypes was assessed by the Friedman ANOVA & Kendall's test.

Multiple regression analyses were performed to evaluate the associations between infant microbial taxa abundance (dependent variable) and maternal metabolic variables, after adjusting for gestational weight change, breastfeeding and Cesarean section (Statistica, ver. 7.0; StatSoft Inc., Tulsa, OK, USA).

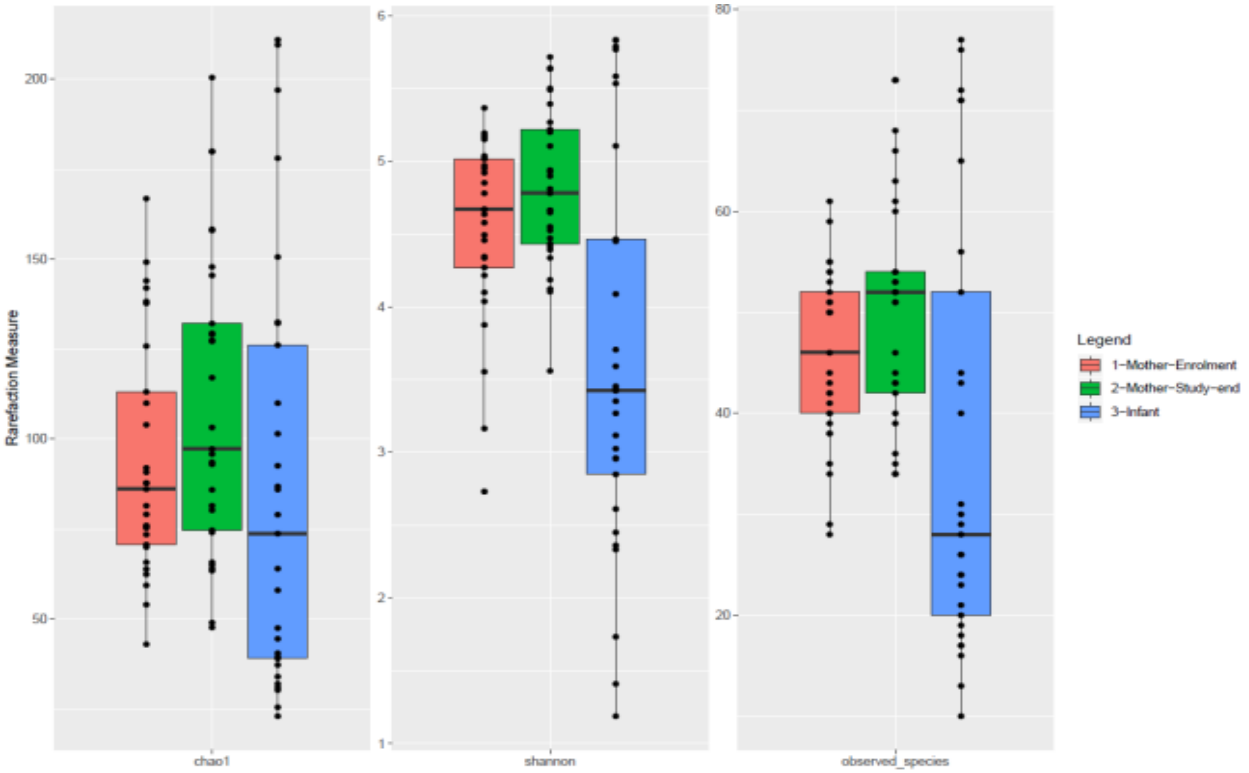
The post-hoc power estimated on partial R2 according to the multivariate linear regression model adjusted for maternal weight change, breastfeeding and Cesarean section was 0.80 with $\alpha = 0.05$. A P value of 0.002 or lower was considered as statistically significant.

6.2 Results

6.2.1 Maternal and infant gut microbiota composition

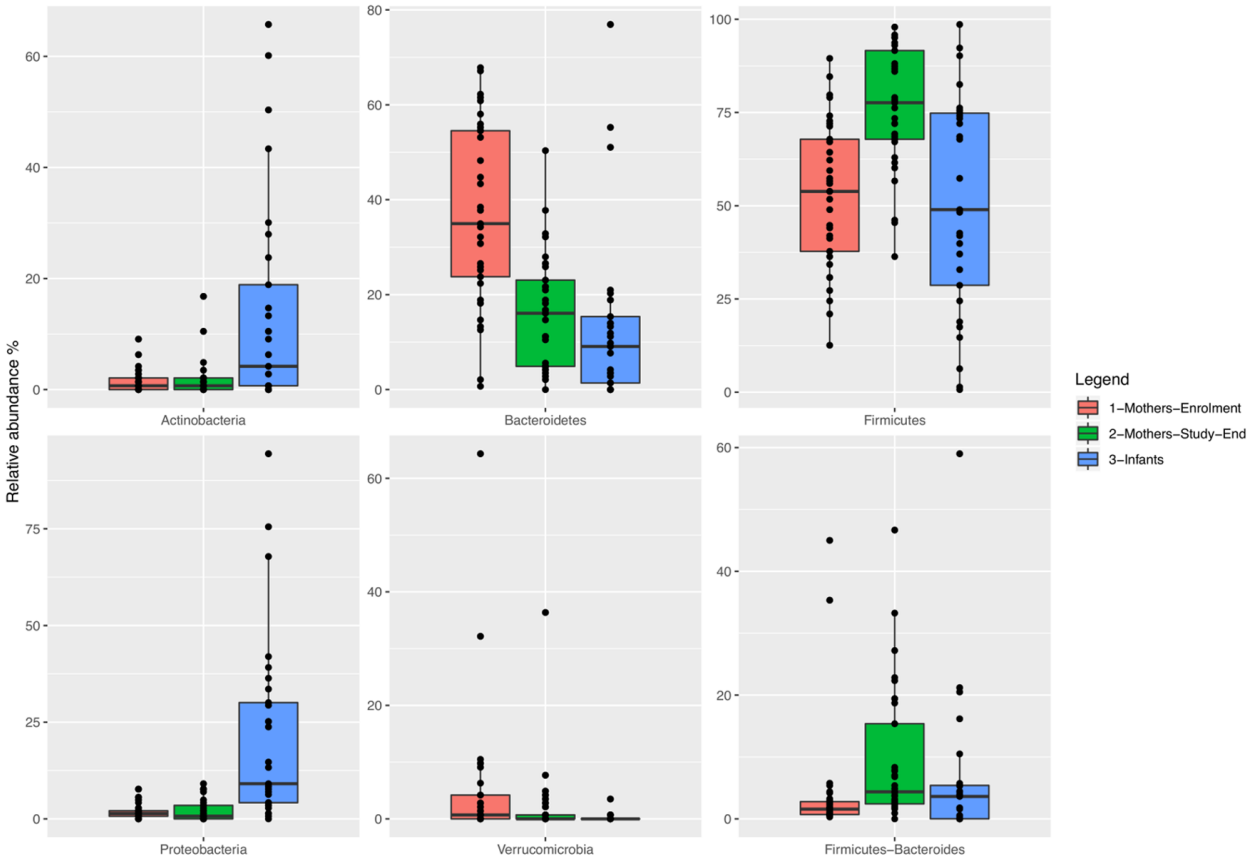
The microbial richness (alpha diversity index) was lower in infant stool samples than in the corresponding maternal samples, and also fewer taxa were present in the samples of the offspring (Fig 10). An increase in the rarefaction measure across pregnancy was evident in GDM patients. The number of observed species was significantly different between mother samples (both at enrolment and at the study-end) and the corresponding offspring samples (for the Chao-1 diversity index, P for paired data <0.001). The decrease in the complexity and the number of taxa observed in offspring samples was not a consequence of the lower number of infant sequences because the analysis was performed by using a rarefied down-sampling operational taxonomic unit (OTU) table.

<u>Figure 10.</u> α-diversity measures of fecal microbiota.
Boxplots describe α-diversity measures of fecal microbiota of GDM patients at enrolment (red bars), of GDM patients at the study end (green bars) and of their offspring (blue bars). Individual points and brackets represent the richness estimate and the theoretical standard error range, respectively.



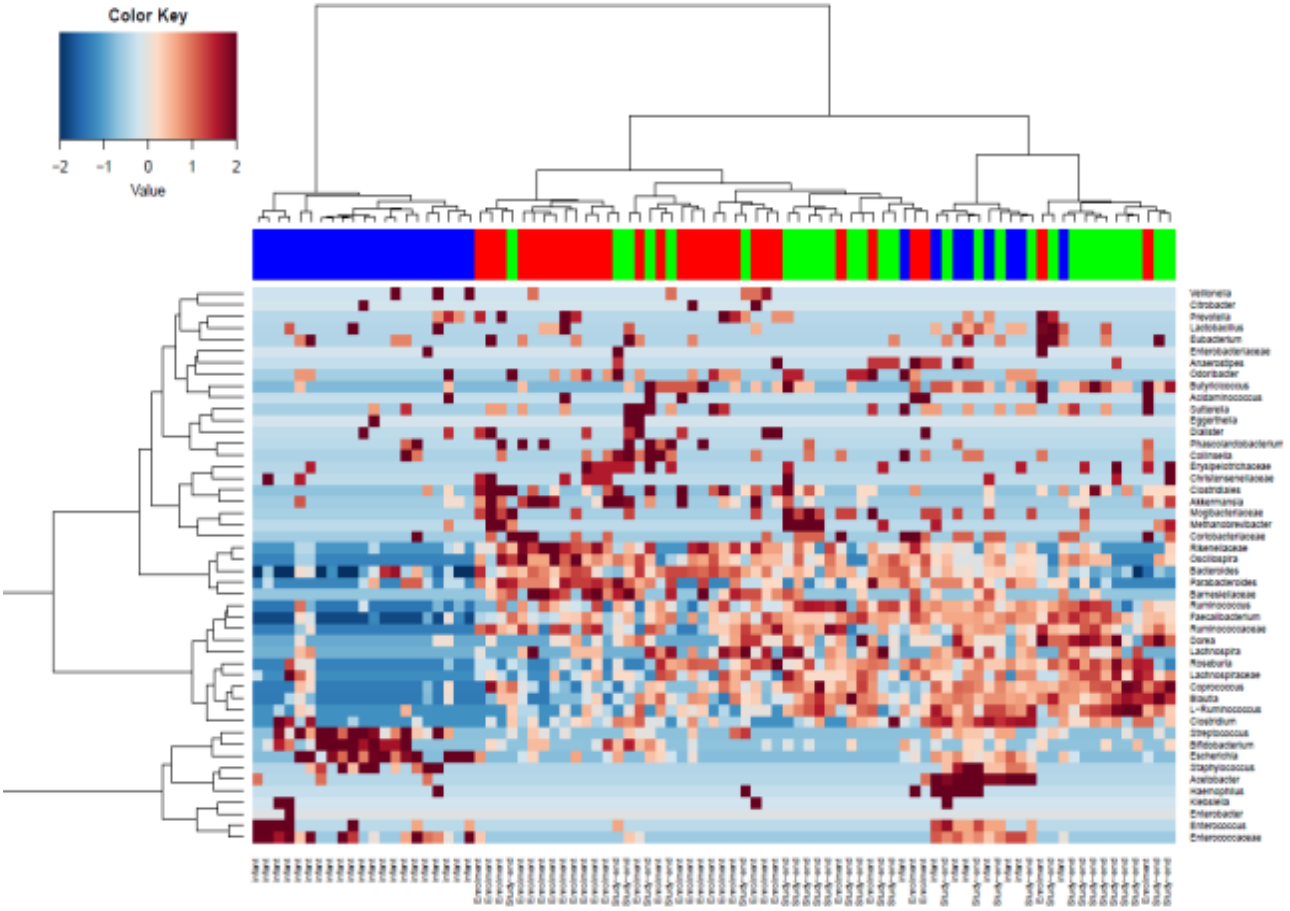
While mothers with GDM showed a predominance of Firmicutes (increasing during the gestational period) and Bacteroidetes (whose relative abundance decreased), the gut microbiota of the offspring was characterized by an increased relative abundance of the phyla Actinobacteria and Proteobacteria (Fig 11).

<u>Figure 11.</u> Phylum-level abundance profiles.
Boxplots describe the relative abundance of phyla in GDM patients at enrolment (red bars), in GDM patients at the study end (green bars) and in the infants (blue bars).



Hierarchical clustering analysis at genus level (Fig. 12) showed a separation between the infant microbiota and the microbiota of the mother (anosim P<0.01). Infants displayed a higher abundance *of Bifidobacterium, Streptococcus, Escherichia, Staphylococcus* and *Enterococcaceae* while mothers had a more complex microbiota composition (Fig. 12).

Figure 12. Heat-plot of the abundances of OTUs at genus level.
Heatplot showing OTUs of GDM patients at enrolment (red bars), study end (green bars) and their offspring (blue bars). Rows and columns are clustered by means of Ward linkage hierarchical clustering. The intensity of the colors represents the degree of correlation between the samples and OTUs as measured by Spearman's correlations.

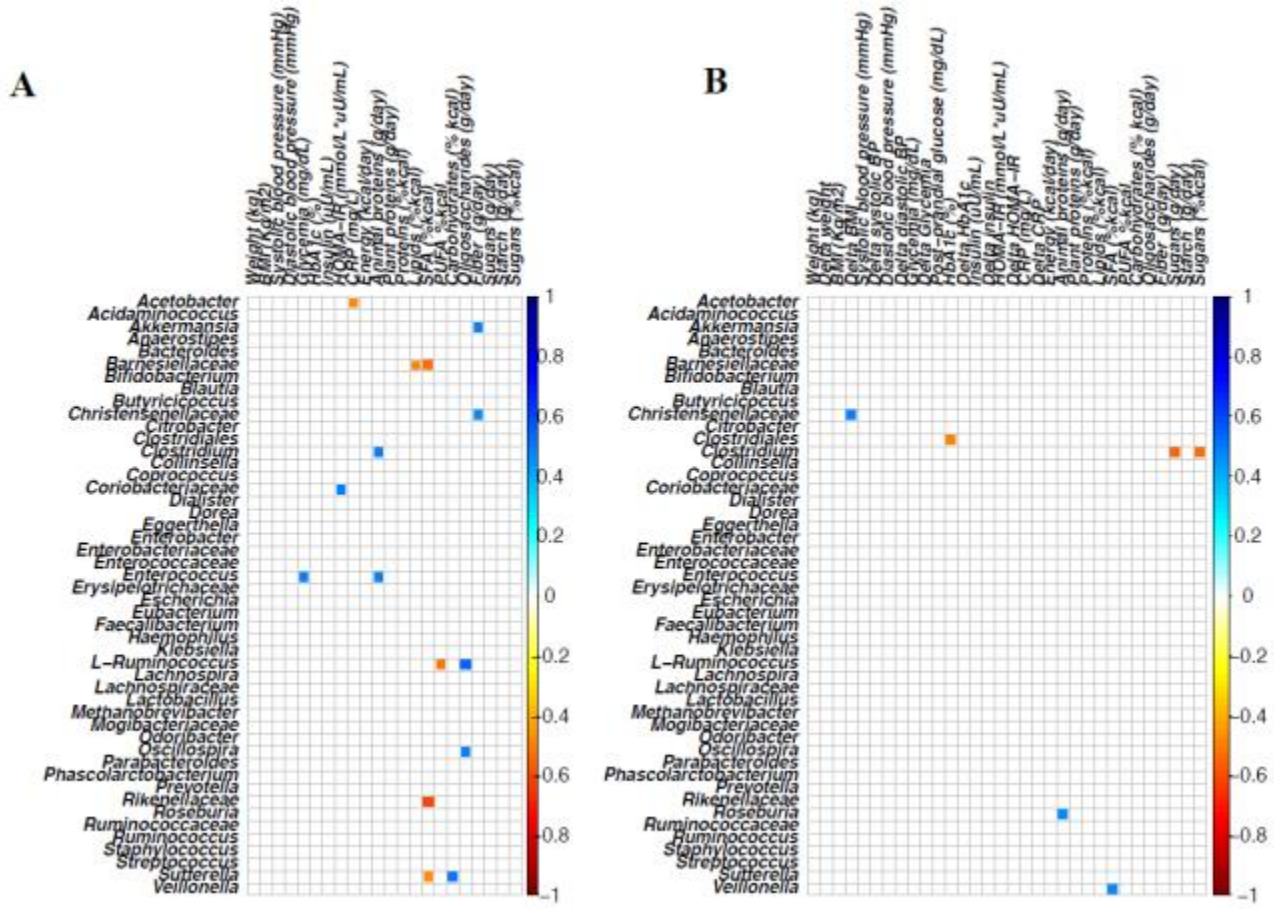


6.2.2 Infant gut microbiota and maternal metabolic variables and delivery outcomes

The correlation plot between maternal metabolic variables and infant gut microbiota showed a higher number of significant associations with maternal variables at enrolment than at the study-end (Fig 13).

<u>Figure 13.</u> Spearman's rank correlation of OTUs dietary information and blood variables.

Spearman's rank correlation matrix of OTUs with > 0.2% abundance in at least 10 fecal samples, dietary information and blood variables. The colors of the scale bar denote the nature of the correlation, with 1 indicating a perfectly positive correlation (dark blue) and -1 indicating a perfectly negative correlation (dark red) between the two datasets. Only correlations with P-values <0.002 are shown. Data at enrolment (plot A) or at study-end (Plot B).



The direct association between maternal fasting glucose at enrolment and *Enterococcus* (Rho = 0.49) was no longer confirmed in the multivariate regression model, while the inverse association between *Clostridiales* and maternal changes across pregnancy (delta) in glycated hemoglobin (HbA1c) levels was statistically significant (Table 5). No significant association between maternal anthropometric or metabolic variables and the gestational changes in these variables and infant gut microbiota composition was found. Two out of 29 women (6.9%) were treated with insulin; none received metformin (Table 6). After adjusting for insulin treatment, the results of the multiple regression analyses did not change.

Out of the 29 women 27.6% delivered with a C-Section. (Table 6). No significant associations between infant gut microbiota and mode of delivery (Cesarean section vs vaginal delivery) was found.

Table 5. Statistically significant associations between infant microbiota composition and maternal variables by Spearman's correlations for continuous variables (left) and multiple regression analyses (right).

	Rho	**Beta**	**95% CI**	**P**
Maternal outcomes and laboratory variables				
Delta HbA1c				
Clostridiales	-0.49	-0.33	-0.48 -0.18	**<0.001**
Delivery week				
Roseburia	-0.43	-1.28	-1.95 -0.61	**0.001**

Model adjusted for maternal weight change, breastfeeding, and Cesarean section.

Table 6. Enrolment characteristics and pregnancy outcomes of the GDM women

Number of study participants	29
Age	37.1±4.5
Pre-pregnancy weight (kg)	71.2±15.1
Pre-pregnancy BMI (kg/m2)	26.4±5.9
Nulliparous	58.6
Weight (kg)	77.0±13.4
BMI (kg/m2)	28.5±5.1
Fasting glucose (mg/dL)	95.9±13.7
Systolic blood pressure (mmHg)	109.0±11.7
Diastolic blood pressure (mmHg)	73.2±7.9
Glycated heamoglobin (%)	4.7±0.8
Fasting insulin (µU/mL)	10.1 (8.2)
HOMA-IR (mmol/L*µU/mL)	2.3 (1.6)
Total cholesterol (mg/dL)	237.7±30.2
HDL-cholesterol (mg/dL)	66.3±11.3
Triglycerides (mg/dL)	172.0±52.1
C-reactive protein (mg/L)	3.8 (4.4)
Pregnancy outcomes	
Insulin treatment (%)	6.9
Cesarean section (%)	27.6
Gestational age at delivery (weeks)	39.1±1.3
LGA newborns (%)	6.9
Male newborns (%)	51.7
Breastfed newborns (%)	34.5

BMI=body mass index, HOMA-IR=Homeostasis Model Assessment-Insulin Resistance, HDL=high density lipoprotein, Values are expressed as mean ± standard deviation or median (interquartile range)

6.2.3 Gut microbiota signature at sub-genus level

In order to explore the possible mother-to-infant gut microbiota transmission at sub-OTUs level, we carried out oligotyping on sequences of *Blautia*, *Bacteroides* and *Bifidobacterium* since these were the only genera showing a Shannon entropy index sufficient to identify all nucleotide positions that would resolve the oligotypes. We considered the dominant oligotypes shared by mothers and their offspring only to explore possible mother-to-child oligotype transmission. The oligotypes which were present in at least 5 mother-to-child pairs were selected. We found 18 oligotypes for *Bacteroides*, 30 for *Blautia*, and 81 for *Bifidobacterium*. For *Bifidobacterium*, only the oligotypes of the offspring were taken in consideration, since resolving *Bifidobacterium* in mothers was not possible. A high inter-individual diversity in the *Bifidobacterium* oligotypes was observed in the offspring samples (Fig 14). We observed that the relative abundance of the *Blautia* oligotypes increased during pregnancy and were lower in the infants (Fig 15 A). A high degree of mother-offspring concordance was found for B11 ($P = 0.008$) and B42 ($P<0.001$); both were identified as *Blautia wexlerae* by best BLASTn match. A reduction in the relative abundance of *Bacteroides* oligotypes was observed across pregnancy while few oligotypes were observed to be dominant in the offspring samples (Fig 15 B).

<u>Figure 14. (S3 Fig).</u> Distribution of representative *Bifidobacterium* oligotypes in the offspring samples. Inner black bars indicate the presence of an oligotype in each sample.

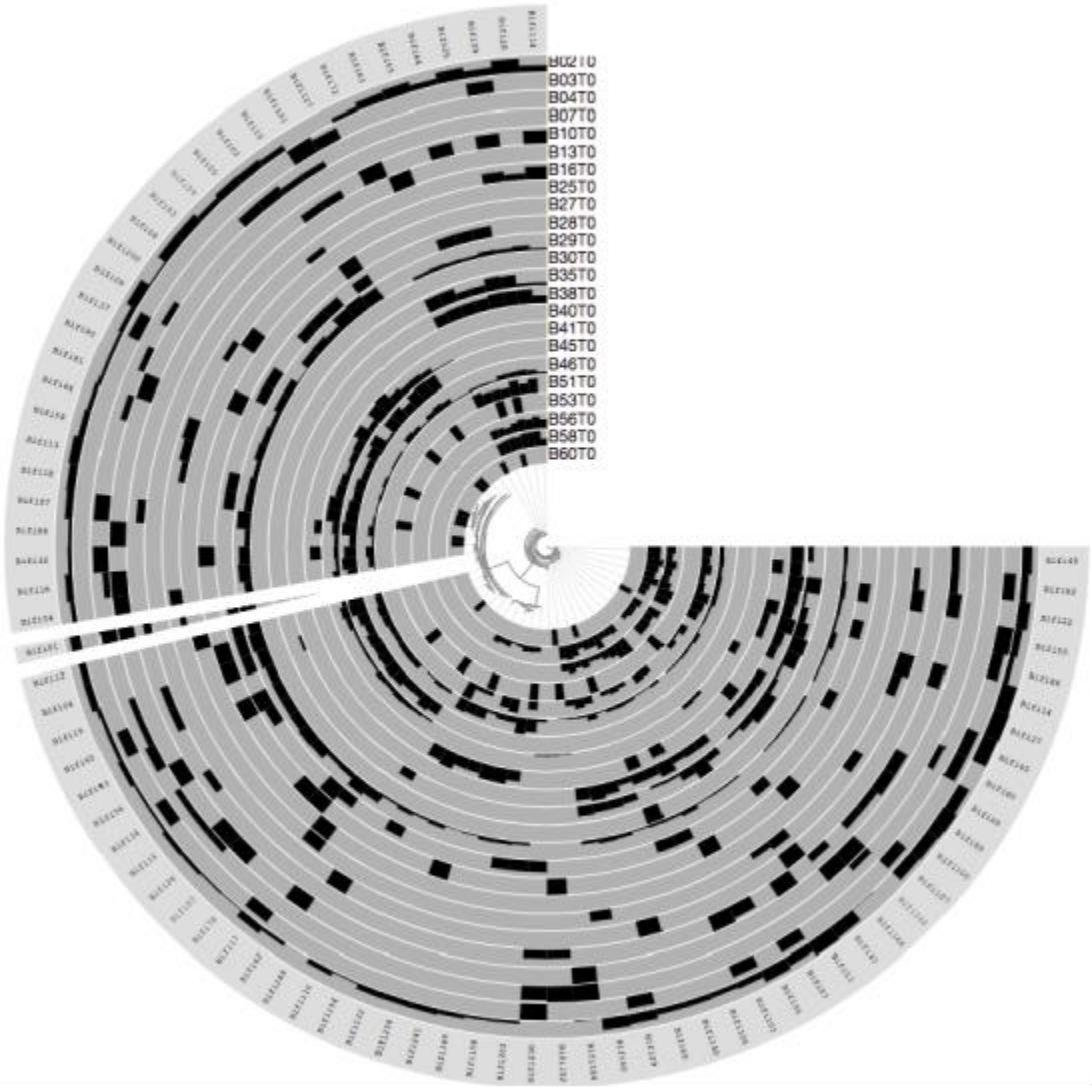
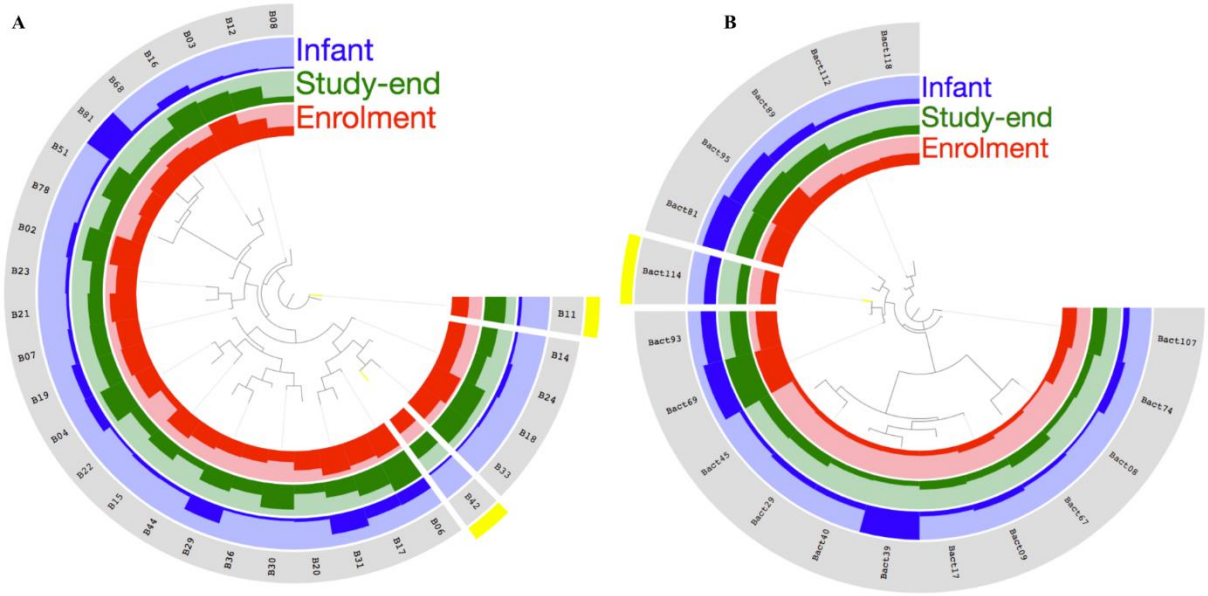


Figure 15. Distribution in representative *Blautia* (plot A) and *Bacteroides* (plot B) oligotypes. Plot showing the sequence distribution in GDM patients at enrolment (red bars), study end (green bars) and their offspring (blue bars). Inner bars indicate the presence of an oligotype in a given sample. Outer circle, if colored, denotes oligotype abundance with high degree of mother-offspring concordance.



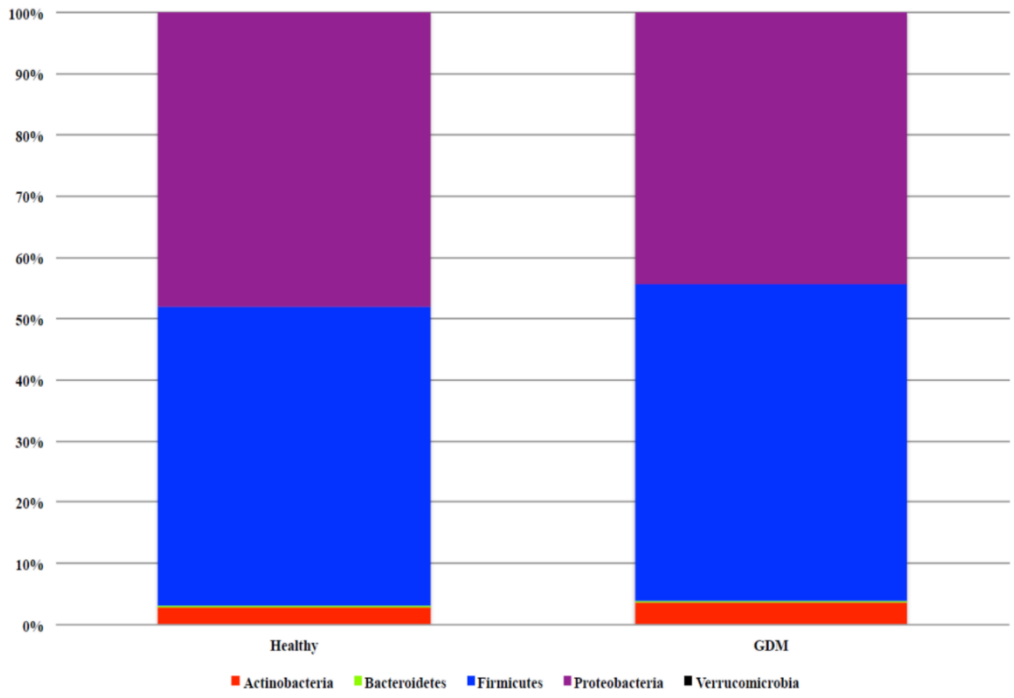
6.2.4 Comparison between gut microbiota from GDM offspring and healthy women offspring

The gut microbiota of the offspring from mothers with GDM was compared with those of 19 breastfed infants from healthy normoglycemic women. A higher α-diversity was evident in the latter (for the Chao-1 diversity index, P = 0.001). However, when comparing to those infants the 10 breastfed infants from GDM women, no significant difference in α-diversity was found. At phylum level, we observed higher abundance of Actinobacteria and Bacteroidetes in the offspring from GDM women (Fig 16 panel A) (P<0.001). At genus level, higher abundance of *Staphylococcus*, *Ralstonia*, *Lactobacillus* and some members of *Enterobacteriaceae* were observed in the offspring from healthy women (Fig16 panel B).

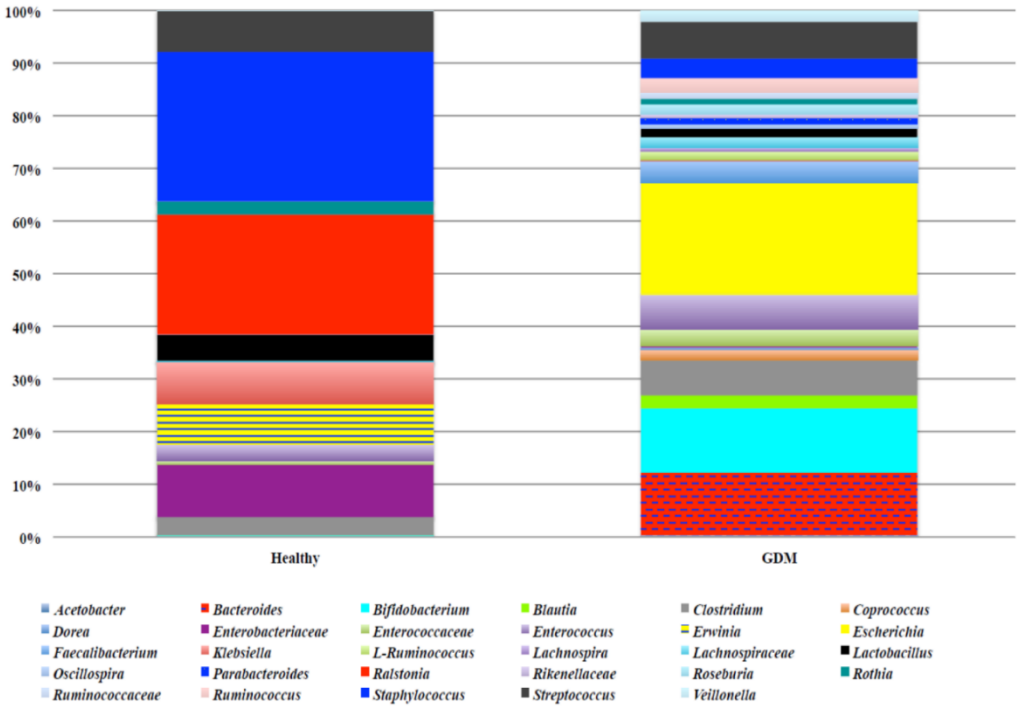
In the comparison between the two groups of breastfed infants, the offspring of GDM women showed a significantly higher relative abundance of *Escherichia* and *Parabacteroides* (P<0.001).

Figure 16. (S5A Fig) Distribution of OTUs between offspring from healthy and GDM women. Plot shows the relative phyla (panel A) and genus (panel B) abundance in the offspring from healthy and GDM women.

Panel A



Panel B



6.3 Discussion

The microbiota of infants from women with GDM showed a low complexity, a high inter-individual variability, and was influenced by early maternal nutrition and breastfeeding. Intriguingly, in comparison with the offspring of healthy normoglycemic women, the infants from GDM mothers showed a higher relative abundance of pro-inflammatory taxa, in particular *Escherichia* and *Parabacteroides*. Furthermore, few mother-to-child oligotype transmissions were found.

The offspring gut microbiota composition substantially differs from the microbiota composition of their GDM mothers, being the former characterized by limited species-level complexity (i.e. a lower α-diversity) and a greater inter-individual variability of genus abundance (i.e. a higher β-diversity), as already reported.(173,215–220) Indeed, the gut microbiota complexity increases progressively with the infant growth, with the gradual development of a microbial community resembling the adult microbiota at the age of 3 years with a lower inter-individual variability.(70,221) We found that Actinobacteria and Proteobacteria dominated the gut microbiota of the infants from GDM women, similarly to the infants of healthy mothers and in accordance with previous reports.(217,218,221,222) The relative abundance of these phyla has been reported to be higher in the meconium of GDM newborns compared to offspring from healthy controls.(223) *Bifidobacterium, Streptococcus, Escherichia, Staphylococcus* and members of the *Enterococcaceae* family presented a higher relative abundance in the gut microbiota of our GDM infants. Accordingly, these bacteria have been detected in the feces or meconium of full-term newborns in several studies.(144,215,216,218,220,224–227)

A distinct microbial composition was found in our breastfed GDM offspring when comparing them with breastfed neonates from healthy controls, consistent with literature.(138,144,228)
In particular, a significantly higher relative abundance of *Escherichia* and *Parabacteroides* was found in the infants' form GDM mothers. These taxa can be considered as pro-inflammatory and have been found to be higher in the meconium of babies from mothers with T2DM.(144) Furthermore, *Parabacteroides* have been reported to be enriched in GDM patients when compared to healthy subjects.(199) In the 1st phase of our study, we have observed an increased, though not statistically significant, relative abundance of *Parabacteroides* across pregnancy from enrolment to study end. (209) Similarly, *Escherichia* is more abundant in pregnant overweight women, particularly in GDM patients.(139,229) Diabetes has been considered as an inflammatory disease and, not surprisingly, the offspring microbiota may be influenced by this pregnancy pathological condition; a direct transmission from mother to child of some pro-inflammatory taxa like *Parabacteroides* or *Escherichia* might be hypothesized, even if the contribution of not analyzed environmental conditions could not be ruled out.
No differences in the Firmicutes-to-Bacteroidetes ratio, indicating an increased risk for developing obesity, was found between breast-fed GDM infants vs breast-fed infants form normoglycemic women.(230)

The microbiota in the first days of life of our GDM offspring might be one of the contributing factors to the 2- to 8-fold increased future risk of dysmetabolic diseases of those offspring when compared to offspring of healthy women.(45)
Various factors have been reported to influence and shape the neonatal microbiota during intrauterine life, among which those that are more frequently implicated are maternal glycemic status, weight gain during pregnancy [64], pre-pregnancy BMI [64–65], and antibiotic use.(144,231–233) We failed to demonstrate any associations between maternal anthropometric indexes with the offspring microbiota composition, indeed the insulin resistance and hyperglycemia of our participants, all suffering from GDM, may have obscured these relationships. Furthermore, other studies did not show relationships between maternal BMI or clinical characteristics and the infant microbiota composition.(144,228)

There is an inverse association between Clostridiales and HbA1c changes across pregnancy. The Clostridiales order includes several genera and in particular *Roseburia* and *Faecalibacterium prausnitzii*, both butyrate-producing bacteria with beneficial functions, which have been found to be reduced in GDM women.(137,173,199)

The microbiota of our GDM offspring did not meaningfully vary according to the way of delivery. Contrasting data are available in the literature regarding this topic, since a different microbial pattern or no differences have been reported between C-section-delivered infants and vaginally-delivered infants.(144,153,217,222,234–236) It could be hypothesized that the exposure to an adverse environment in our GDM offspring could have had a major role on their gut microbiota, partly overshadowing the effects of the exposure during birth. Furthermore, the influence of delivery mode on the infant microbiota rapidly decline after delivery and is quickly overridden by the role of lactation. (70,220,237–239)

A possible vertical mother-to-child transmission of maternal gut bacteria has already been reported, even if, to date, certainty about the way of intrauterine microbial acquisition is lacking. (218,240,241) Besides breastfeeding and vaginal microbiota, also placenta and amniotic fluid have been reported to be a vehicle for this transmission.(173,242) By using the oligotype pipeline, the transmission of different sub-OTUs from our mothers to their infants was observed in line with data of a recent metagenomic study confirming that mother's dominant strains were transmitted to their children.(221) In particular, we have found a few *Bacteroides* and *Blautia* oligotypes significantly shared by the GDM mothers and their offspring, suggesting a maternal microbial imprinting. On the other hand, the infant *Bifidobacterium* oligotypes of the newborns were not present in the feces of the GDM women, and this directs towards a post-natal acquisition. Indeed, the infant *Bifidobacteria* mostly derive from breast-feeding, being either isolated from human milk and vertically transmitted.(242,243) or induced by the milk prebiotic HMO.(244) The *Bifidobacterium* oligotypes were highly different among infants but only one third of them was breastfed and the human milk has a heterogeneous composition and contains variable amounts of glycans and bifidogenic oligosaccharides.(237,245)

Several limitations of the present study should be recognized. The sample size was low, the standard errors were wide, and we could analyze the fecal samples of only 70.7% of the offspring

of the initially enrolled women. Therefore, we might not have detected modest differences in the bacterial composition. Many associations that we found to be not statistically significant, could have become so if we had analyzed a larger sample. However, the post-hoc power was 0.80 with $\alpha = 0.05$; furthermore, we have set-up a lower p-value cut-off value ($p<0.002$) as statistically significant. In doing so, we have considered only the strongest associations; however, we cannot exclude the possibility that a type II error could have occurred.
Infant fecal samples were collected between the 3rd and the 5th days of life. So, we cannot exclude that the difference of a few days could have influenced the results due to the high instability of the infant microbiota.

In conclusion, the gut microbiota changes might be one of the conditions determining the increased metabolic risk of the GDM offspring with both pre and postnatal complications, since in utero exposure to maternal dysmetabolic conditions, and post-natal factors can both modulate the offspring microbial community. This study brings a better understanding of the composition of the gut microbiota in early life in the offspring of women with GDM and the results are worthy to be replicated in large-scale studies due to their potential practical implications on neonatal and infant health.

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