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IMPACT OF PERINATAL FACTORS ON THE MATERNAL – NEONATAL MICROBIOTA AND INFLUENCE ON HEALTH OUTCOMES

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ORIGINAL PUBLICATIONS

Publications used for the literature background section and general discussion:

0- **Selma-Royo M**., Tarrazó M., García-Mantrana I., Gómez-Gallego C., Salminen S., Collado M.C. (2019) Shaping Microbiota During the First 1000 Days of Life. In: Guandalini S., Indrio F. (eds) Probiotics and Child Gastrointestinal Health. Advances in Experimental Medicine and Biology, vol 1125. Springer, Cham.

1- **Selma-Royo, M**.; Calatayud, M.; García-Mantrana, I.; Cortés-Macias, E.; Olivares, L.; Sánchez, G.; Collado, MC. (2020) Gut Microbiota development in infants and children. In: Koletzko, B (ed.) Paediatric Nutrition in Practice (World Review of Nutrition and Dietetics). *Accepted.*

2- García-Mantrana I, Alcántara C, **Selma-Royo M**, Boix-Amorós A, Dzidic M, Gimeno-Alcañiz J , Úbeda-Sansano I, Sorribes-Monrabal I, Escuriet R, Gil-Raga F, Parra-Llorca A , Martínez-Costa C, Collado MC, on behalf of MAMI team. MAMI: A Birth Cohort Focused on Maternal-Infant Microbiota During Early Life BMC Pediatr2019 May 3;19(1):140.

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IV- García-Mantrana, I.*; **Selma-Royo, M***.; González, S.; Parra-Llorca, A.; Martínez-Costa, C.; Collado, M.C. Distinct maternal microbiota clusters are associated

with diet during pregnancy: impact on neonatal microbiota and infant growth during the first 18 months of life. Gut Microbes 2020, 1–17, doi:10.1080/19490976.2020.1730294.
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V- Selma-Royo, M.; Calatayud, M.; García-Mantrana, I.; Parra-Llorca, A.; Escuriet, R.; Martínez-Costa, C.; Collado, M.C. Perinatal environment shapes microbiota colonization and infant growth: impact on host response and intestinal function. Microbiome 2020, Minor *revisions*. doi: 10.21203/rs.3.rs-20279/v1 *(pre-print in Research Square BMC)*

SUMMARY

Background: The importance of the microbiota for host health and disease is now widely appreciated. Microbial communities that inhabit the human body have been proposed as an essential aspect of the hypothesis focused on the developmental origin of health and disease (DOHaD), suggesting that altered microbial exposure during infancy could play a role in the risk of some immune-based diseases, such as obesity, allergy development or T2 diabetes mellitus. Indeed, associational studies have related perinatal factors that could disrupt neonatal microbial colonization, such as antibiotic intake, C-section and formula feeding, to these diseases later in life. Some authors have suggested that the interaction between the initial microbiome and the developing immune system could be crucial for a correct maturation of the latter. However, the exact molecular mechanisms that regulate the host−microbiome interconnection during this period are still unknown. Furthermore, despite the importance of maternal microbiota on initial neonatal microbial seeding, data concerning the effect of these perinatal factors on maternal microbiota are still scarce.

Objectives: The general aim of the thesis was to to ascertain the impact of perinatal factors, such as mode and place of delivery, antibiotic use, maternal diet and gestational age on maternal-neonatal microbiota composition and their implications on health outcomes.

Subjects: Participants in this study were part of a larger longitudinal cohort study conducted in the Mediterranean area, between 2015 and 2019. Mothers were enrolled in the Hospital Universitario y Politécnico La Fe, Hospital Clinico Universitario de Valencia, primary health-care units from Valencia and CEIC-Parc de Salut MAR. In this thesis, faecal samples at birth, 7 and 31 days were used as well as the saliva, amniotic fluid and placenta samples from a subset of participants.

Methods: Next-generation sequencing targeting the V3−V4 region of the 16S rRNA gene was performed to assess the microbiota composition at each time point. Enzyme-linked immunosorbent assay and Luminex assay were used for the determination of the molecular hormonal, metabolic and immune status of the participants and/or cellular models. Quantitative polymerase chain reaction (PCR) and reverse transcription PCR were also performed in a quantitative measurement of microbiota composition and for the assessment of gene expression in placental tissue and cellular models, respectively.

The culture of different cell lines, including intestinal epithelial cells (Caco-2), mucus-producer cells (LSTH-17) and monocyte-like cells (THP-1), were used for the *in vitro* assays.

Results and conclusion: Maternal microbiota at delivery was influenced by several perinatal factors, including immune status of amniotic cavity, diet during gestation and delivery mode. Maternal consumption of fat was positively related to Firmicutes spp. while carbohydrates, fibre and polyunsaturated fatty acids (PUFA) intakes were associated with higher relative abundance of Bacteroidetes and Proteobacteria levels. Some of these relations were also reflected in the neonatal microbiota at delivery. Indeed, children born by C-section and from mothers with higher fat intakes showed higher body mass index (BMI) index z-scores than those born from mothers with higher fibre consumption. Two bacterial profiles were found during the study. The first one was composed of health-related bacteria with short-chain fatty acids (SCFA) production activity, such as *Roseburia*, *Faecalibacterium*, *Blautia*, *Lachnospira* or *Bacteroides* genera. This group was associated with a distinct amniotic cytokine profile characterized by higher concentrations of IL-2, IL-5, IL-17 and TNF-α. Indeed, salivary cortisol concentration also showed positive relations with some of these genera independently of the delivery mode. The other group was characterized by *Finegoldia*, *Peptoniphilus*, *Anaeroaoccus*, *Porphyromonas* and *Campylobacter* genera. Those were associated with another amniotic cytokine profile dominated by IL-4, IL-13, IL-18, and IL-10 cytokines and more highly represented in mothers who had undergone a C-section. Some of these genera were negatively associated with salivary cortisol concentration. Thus, our results suggested that amniotic fluid immune status and cortisol concentration were related to maternal microbiota at delivery with implications also in neonatal microbiota.

Furthermore, we described the effect of mode and place of birth in the microbial colonization evolution during the first month of life, showing that neonates born by C-section had a distinct microbial pattern and higher BMI index z-scores than vaginal-born infants, both at home and hospital. We proposed an *in vitro* molecular mechanism by which the differences observed in C-section-born neonates in terms of microbiota composition would promote a shift in immune system maturation through the lack of immune stimulation observed in these samples compared with those from vaginal-born infants, especially those from the home birth group. Thus, disruptions in the

colonization process during the first month of life could have long-lasting consequences through an alteration of immune system development during this period.

RESUMEN

Introducción: El gran impacto del microbioma humano sobre la salud del huésped es ampliamente conocido. Se cree que las comunidades microbianas que habitan el cuerpo humano serían un aspecto fundamental en la hipótesis promulgada sobre el posible origen de la enfermedad durante el desarrollo (DOHad) sugiriendo que alteraciones en la exposición microbiana durante la infancia podrían estar implicadas en el mayor riesgo de algunas enfermedades de base inmunológica como la obesidad, la alergia o la diabetes mellitus de tipo II. Además, se han encontrado relaciones significativas entre este tipo de enfermedades con algunos factores perinatales que se han descrito como disruptores del proceso de colonización, como la cesárea, el uso de antibióticos o la alimentación mediante leche de formula. Algunos autores sugieren que la interacción entre la microbiota inicial y el sistema inmunológico que está desarrollándose sería crucial para una correcta maduración de este. Sin embargo, los mecanismos exactos que mediarían en esta relación huésped-microbiota durante este periodo todavía se desconocen. Además, a pesar de la importancia de la microbiota materna para el inicio del proceso de colonización, todavía es escasa la información sobre como los diferentes factores perinatales afectan a la microbiota materna.

Objetivos: El objetivo general de esta tesis fue definir el impacto de los diferentes factores perinatales como el tipo de parto, el uso de antibióticos, la dieta materna o la edad gestacional en la composición de la microbiota materno-neonatal y sus posibles implicaciones para la salud.

Participantes: Los participantes del presente análisis son parte de un estudio longitudinal de cohorte desarrollado en el área mediterránea entre 2015 y 2019. Las madres fueron reclutadas en el Hospital Universitario y Politécnico de la Fe, el Hospital Clínico Universitario de Valencia, centros de atención primaria de la ciudad de Valencia y el CEIC-Parc de Salut MAR. En el presente trabajo se utilizaron las muestras fecales recogidas al parto, a los 7 y 31 días, así como las muestras de saliva, líquido amniótico y placenta de un grupo reducido de participantes.

Métodos: Se utilizaron técnicas de secuenciación de nueva generación dirigidas a la región V3-V4 del gen 16S rRNA para la determinación de la composición de la microbiota de cada muestra a los tiempo estudiados. Además, el ensayo por inmunoadsorción ligado a enzimas y la técnica Luminex fueron usadas para la

determinación de moléculas relacionadas con el estado hormonal, metabólico e inmune de las muestras y/o de los ensayos celulares. La PCR cuantitativa y la de transcripción reversa fueron usadas para realizar una medida cuantitativa de la composición microbiana y de la expresión de algunos genes seleccionados en las muestras de placenta y modelos celulares, respectivamente. EL cultivo de diferentes líneas celulares, como líneas epiteliales (Caco-2), productoras de moco (LSTH-17) y líneas similares a macrófagos (THP-1) fueron utilizadas en los ensayos in vitro.

Resultados y conclusión: La microbiota materna al parto estuvo influenciada por diversos factores perinatales, incluyendo el estado inmunológico de la cavidad amniótica, la dieta durante el embarazo y el tipo de parto. El consumo materno de grasa estuvo positivamente relacionado con Fimicutes spp. Mientras que el consumo de carbohidratos, fibra y ácidos grasos poliinsaturados fueron asociados con mayores abundancias relativas de los filos Bacteroidetes y Proteobacteria. Algunas de estas relaciones fueron observadas también en la microbiota neonatal. Además, los niños nacidos por cesárea y de madres con altos consumos de grasa mostraron mayores IMC (índice de masa corporal) z-scores que aquellos que nacieron de madres consumidoras de fibra.

Dos patrones microbianos fueron encontrados a lo largo de todos los análisis. El primero de ellos estaba compuesto por especies asociadas a la salud y productoras de SCFA como los géneros *Roseburia*, *Faecalibacterium*, *Blautia*, *Lachnospira* o *Bacteroides*. Este grupo estuvo asociado a un perfil de citoquinas caracterizado por IL-2, IL-5, IL-17 y TNF-α. Los niveles de cortisol en saliva estuvieron positivamente correlacionados con algunos de estos géneros independientemente del tipo de parto. El otro patrón microbiano estuvo caracterizado por los géneros *Finegoldia*, *Peptoniphilus*, *Anaeroaoccus*, *Porphyromonas* y *Campylobacter*. Estos estuvieron relacionados con un perfil de citoquinas en la cavidad amniótica dominados por la presencia de IL-4, IL-13, IL-18 y IL-10 y con el parto por cesárea. Además, algunos de estos géneros estuvieron negativamente correlacionados con los niveles de cortisol en saliva. Así, nuestros resultados sugieren que el ambiente inmunológico de la cavidad uterina y la concentración de cortisol estuvieron relacionados con la composición de la microbiota materna al parto con implicaciones para la microbiota neonatal.

Además, describimos el efecto del tipo de parto y el lugar en el proceso de colonización durante el primer mes de vida mostrando que los niños nacidos por cesárea tienen un perfil diferencial de microbiota y mayores IMC z-scores que los niños nacidos de forma

vaginal, tanto en el hospital como en casa. En nuestra investigación, hemos propuesto un mecanismo molecular por el cual las diferencias en la composición microbiana de los niños nacidos por cesárea podrían provocar una alteración en la maduración del sistema inmunológico mediante la falta de imnunoestimulación observada estas muestras comparadas con aquellas obtenidas de los niños nacidos por parto vaginal, especialmente de aquellos nacidos en casa. Así, las alteraciones en el proceso de colonización durante el primer mes de vida podrían tener consecuencias a largo plazo en la salud infantil debido a una alteración del desarrollo del sistema inmunológico durante este periodo.

RESUM

Introducció: El gran impacte del microbioma humà sobre la salut humana del hoste és amplament reconegut. Es creu que les comunitats microbianes que habiten el cos humà serien un aspecte fonamental en la hipòtesi promulgada sobre el possible origen de la malaltia durant el desenvolupament (coneguda com DOHad) suggerint que alteracions en l'exposició microbiana al llarg de la infància podrien estar implicades en el augment del risc a patir algunes malalties amb base immunològica com són l'obesitat, l'al·lèrgia o la diabetis mellitus tipus II. Així mateix, s'han trobat relacions significatives entre aquest tipus de malalties amb alguns factors perinatals que s'han descrit com disruptors del procés de colonització, com la cesària, l'ús d'antibiòtics o l'alimentació mitjançant llet de fórmula. Alguns autors suggereixen que la interacció entre la microbiota inicial i el sistema immunològic que estaria desenvolupant-se seria crucial per a una correcta maduració d'aquest últim. No obstant això, els mecanismes exactes que mediten en aquesta relació hoste-microbiota durant aquest període encara es desconeixen. A pesar de la importància de la microbiota materna en l'inici del procés de colonització, encara és escassa la informació sobre els diferents factors perinatals que afecten a la microbiota materna.

Objectius: L'objectiu general d'aquesta tesi fou definir l'impacte dels diferents factors perinatals com el tipus de part, l'ús d'antibiòtics, la dieta materna o l'edat gestacional en la composició de la microbiota materno-neoantal i les possibles implicacions sobre la salut.

Participants: Els participants del present anàlisi són part d'un estudi longitudinal de cohort de l'àrea mediterrània entre el 2015 i 2019. Les mares van ser reclutades en l'Hospital Universitari i Politècnic La Fe, l'Hospital Clínic Universitari de València, centres d'atenció primària de la ciutat de València i el CEIC-Pac de Salut Mar de Catalunya. En el present treball s'utilitzaren les mostres fecals recollides al part, als 7 i als 31 dies, així com les mostres de saliva, líquid amniòtic i placenta.

Mètodes: S'utilitzaren tècniques de seqüenciació de nova generació dirigides a la regió V3-V4 del gen 16S rRNA per a la determinació de la composició de la microbiota de cada mostra als temps estudiats. Així mateix, l'assaig per immune adsorció lligat a enzims i la tècnica Luminex foren utilitzades per a la determinació de molècules relacionades amb l'estat hormonal, metabòlic e immunològic de les mostres i/o els

assajos cel·lulars. La PCR quantitativa i la de transcripció en mode revers foren utilitzades per a realitzar una mesura quantitativa de la composició de la microbiota i de l'expressió d'alguns gens seleccionats en les mostres de placenta i models cel·lulars, respectivament. El cultiu de diferents tipus cel·lulars, com tipus epitelials (Caco-2), productors de moc (LSTH-17) i línies similars a macròfags (THP-1) foren utilitzades en els assajos in vitro.

Resultats i conclusió: La microbiota materna en el moment del part va estar influenciada per diversos factors perinatals, incloent l'estat immunològic de la cavitat amniòtica, la dieta durant l'embaràs i el tipus de part. El consum matern de greix es va vore associat amb Fimicutes spp. Pel contrari, el consum de carbohidrats, fibra i àcids grassos poliinsaturades es van veure relacionats amb majors abundàncies relatives dels phylum Bacteroidetes i Proteobacteria. Algunes d'aquestes relacions foren també observades en la microbiota neonatal. Tan mateix, els nounats nascuts per cesària i de mares consumidores de greixos van mostrar majors índexs de massa corporal (IMC) z-scores que els nonats nascuts de mares consumidores de fibra.

Dos patrons microbians es trobaren al llarg de totes les anàlisis. La primera d'elles va estar composta per espècies associades amb la salut i productes d'àcids grassos de cadena curta (SCFA), com són els gèneres *Rosburia*, *Faecalibacterium*, *Blautia*, *Lachnospira* o *Bacteroides*. Aquest grup va estar associat a un perfil de citocines caracteritzat per interleucines (IL)-2, IL-5, IL-17 i TNF-alpha. Els nivells de cortisol en saliva al part estigueren positivament correlacionats amb alguns d'aquests grups , independentment del tipus de part. L'altre patró microbià estava caracteritzat per els gèneres *Finegoldia*, *Peptoniphilus*, *Anaeroccocus*, *Porphyromonas* i *Campylobacter*. Aquests grups foren relacionats amb el part per cesària i un perfil de citoquines en la cavitat amniòtica dominat per la presència de IL-4, IL-13, IL.18 i IL-10. A més, alguns d'aquests gèneres estigueren negativament relacionats amb els nivells de cortisol en saliva en el moment del part. Així, els nostres resultats suggereixen que l'ambient immunològic de la cavitat uterina i la concentració de cortisol tenien una relació amb la composició de la microbiota materna al part amb implicacions per a la microbiota neonatal.

Tan mateix, descrivim l'efecte del tipus de part i el lloc de naixement (hospital vs. casa) en el procés de colonització durant el primer mes de vida, mostrant que els nonats nascuts per cesària tenien un perfil diferencial de microbiota i majors índexs de massa corporal (IMC) z-scores que aquells nonats nascuts de forma vaginal, tant a l'hospital

com a casa. En la nostra investigació, hem proposat un mecanisme molecular pel qual les diferències en la composició microbiana podrien provocar una alteració en la maduració del sistema immunològic mitjançant una manca de inmuno-estimulació, observada en les mostres obtingudes dels nounats nascuts per cesària, especialment si es compara amb aquells nascuts a casa. Així, les alteracions en el procés de colonització, durant el primer mes de vida podrien tenir conseqüències a llarg termini en la salut infant debut a alteracions en el desenvolupament del sistema immunològic durant el període.

ABBREVIATIONS

AA	Amino acids
ALA	Omega-3 alpha-linolenic acid
ANOVA	Analysis of variance
APC	Antigen presenting cells
ASV	Amplicon sequence variant
BMI	Body mass index
CESNID	Centro de Enseñanza Superior de Nutrición Humana y Dietética
CH	Carbohydrates
CHI	Calinski Harabasz Index
CLA	Omega-6 Conjugated Linoleic Acid
CS	C-section
CSIC	Centro Superior de Investigaciones científicas
DAG	Direct acyclic graph
DAPC	Discriminant analysis of principal components
DGGE	Denaturing gradient gel electrophoresis
DHA	Omega-3 docosahexaenoic acid
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
DPA	Omega-3 Docosapentaenoic acid
ECS	Emergency C-section
ELISA	Enzyme-linked immunosorbent assay
EMEM	Eagle's Minimum Essential Medium
EPA	Omega-3 Eicosapentaenoic acid
FDR	False Discovery rate
FFQ	Food frequency questionnaire
FISH	Fluorescence in situ hybridization
GALT	Gut-associated lymphoid tissue
GDM	Gestational diabetes mellitus
GLM	Generalizes linear model
GPAC	Gram-positive anaerobic cocci

HB	Home birth
HEC	Hospital ethics committees
HFD	High fat diet
HMO	Human milk oligosaccharides
IAP	Intestinal alkaline phosphatase
IBD	Intestinal bowel disease
IBSP-CV	Biobanco para la Investigación Biomédica y en Salud Pública de la Comunidad Valenciana
IG	Immunoglobulin
IQR	Interquartile range
IL	Interleukin
KEEG	Kyoto Encyclopaedia of Genes and Genomes
LEfSE	Linear discriminant analysis effect size
LPS	Lipopolysaccharides
LW	Low wright
LY	Lucifer Yellow
MD	Mediterranean Diet
MUFA	Monounsaturated fatty acids
NCD	Non-communicable disease
NEAA	Non-essential amino acids
NF-kB	Nuclear factors kappa b
NMDS	Non-metric multidimensional scaling
NW	Normal weight
OTU	Operational taxonomical unit
OW	Overweight
PAM	Partitioning around medoids
PAPP	Apparent permeability coefficient
PCA	Principal component analysis
PCoA	Principal coordinates analysis
PMA	Phorbol 12-myristate 13-acetate
PPWR	Post-partum weight retention
PREDIMED	PREvención con DIeta MEDiterránea
PRR	Pattern recognition receptor

PSA	Polysaccharide A
PTB	Preterm birth
PUFA	Polyunsaturated fatty acids
q-PCR	quantitative polymerase chain reaction
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic acid
RT-PCR	Reverse transcription polymerase chain reaction
SCFA	Short-chain fatty acid
SD	Standard deviation
SEAP	Soluble excreted alkaline phosphatase
SFA	Saturated fatty acids
SMRT	Single molecule real time sequencing
TEER	Trans-epithelial electrical resistance
TGGE	Temperature gradient gel electrophoresis
Th	Lymphocytes T helper
TLR	Toll like receptors
TRANS	Trans-unsaturated fatty acids
Treg	Lymphocytes T regulatory
TSS	Total sum scaling
VAG	Vaginal delivery
WFL	Weigh-for-length
WHO	World Health Organization

LITERATURE BACKGROUND

LITERATURE BACKGROUND

GENERAL INTRODUCTION

The human species has been commonly defined as a large-brained bipedal primate with the capacity for language and use of complex tools [1]. However, nowadays this description is inexact, or at least incomplete. Currently, it is known that humans coexist in a sophisticated symbiosis with a community of microbes, including bacteria, archaea fungi and virus [2–4], which is called microbiota. The definition of the human microbiome has been challenging due to the confusion in the terminology. Microbiota and microbiome have been used interchangeably, but the first one refers to the microbial taxa associated with the human body, and the term 'microbiome' defines the collection of the genes from these microbes [5].

The human microbiota comprises a wide variety of microorganisms, including bacteria, virus, archaea and fungi, that collaborate in the homeostasis and the maintenance of human health. Several studies have described the complexity of microbial communities that inhabit different body niches, including the gut [6], vagina [7], oral cavity [8], skin [9], reproductive organs, etc. Human beings have co-evolved with several trillions of microorganisms, which perform essential functions related to several pathways in human physiology [10]. Among the broad range of these functions, they can be classified into metabolic and immunological activities. Although the microbial beneficial activities for the host have been described in more depth for gut microbiota, bacterial populations in other parts of the body also have a crucial role in health preservation. In this regard, vaginal ecosystem microbiota are involved in host defence through pH regulation and reactive oxygen substance productions, among other mechanisms [7]. Similarly, diverse pathways have been associated with skin microbiota, including the production of bactericidal compounds and the influence on cytokine production and regulatory T cell activity in the epidermis [9].

The evidence of the influence on human health and disease has led to the design of some big studies describing the structure and diversity of the microbiome. The most important projects in the field are MetaHit and the Human Microbiome Project (HMP), which provided a global overview of the human microbiota composition [11,12]. Data from these studies have described 2172 different species isolated from human

microbiota and classified them into 12 phyla [13]. Proteobacteria, Firmicutes, Actinobacteria and Bacteroidetes phyla represent approximately 95% of all the species found in these projects. Around 20% of the identified microbes were founded in mucosal regions, including oral cavity and gut, and were strictly anaerobes [13]. However, these data are constantly challenged since emerging studies are increasing the bacterial repertoire in human microbiota, especially in the gut [14].

As we mentioned above, the most important niches for human microbiota are those related to mucosal areas. Thus, the oral cavity is the second largest and most diverse microbial community after the gut, with approximately 392 taxa found [15]. Indeed, in their review of the literature, Diop et al. found a total of 206 genera classified in 96 families [16]. These microbes are involved in several functions of diverse processes, including digestion and maintenance of oral health [17]. Similarly, the vaginal mucosa harbours a community of microbes known as vaginal microbiota, which also participates in health preservation and even in reproduction [18,19].

Besides this, the human colon is the most studied niche in the human body due to the huge microbial biomass present in this habitat and its importance to human health. Gut microbiota is the term used for the several hundred bacterial species that have co-evolved with the host in a perfect symbiotic relationship that is able to interact with the body's systems, including the nervous [20], immune [21] and endocrine systems [22]. Although initially the estimation of the number of microorganisms in the gut was 10^{14} cells, meaning a bacterium: human ratio of 10:1, recent studies have revised this assumption and have proposed that the real ratio is closer to 1:1 [23]. Gut microbiota is considered as a 'superorganism' [24] due to these characteristics and its dramatic influence in host health. Indeed, the microbiota composition undergoes changes along the gastrointestinal tract, from the low numbers of microbes in the oesophagus to the huge diversity in the colon [25]. The bacterial communities are shaped at each gastrointestinal tract site by local environmental conditions such as oxygen content or the pH [26]. Gut microbiota is involved in the extraction and absorption of many nutrients and metabolites and the processing of digestible polysaccharides [27,28], harvesting energy [29] and the production of several vitamins from their precursors [30,31], such as thiamine, riboflavin, niacin and biotin [32]. Indeed, the microbiota plays a crucial role in the prevention of pathogenic infection through several mechanisms, including competition for nutrients, pH regulation or antimicrobial peptide production [33]. Moreover, microbiota also contribute to

maintaining the gut barrier integrity, shaping the intestinal epithelium activity [34] and modulating the host immunity, which is discussed below [35].

Growing evidence suggests that bacterial populations that differ in composition may have a functional redundancy resulting in similar activity and influence in the host [36,37]. This functional redundancy enables the microbiota to respond to the huge number of exogenous challenges to remain stable and not cause alterations in the symbiotic relationship that lead to disease [36], favouring a feature of a health-related microbiota named resilience (discussed below). Thus, studies that go beyond the characterization of microbiota composition and describe the community functionality are required. Metabolomics has also been suggested as a valuable tool in order to determine the bacterial metabolites that could interact with the host [38,39].

In this sense, nutrients that escape digestion in the upper part of the intestinal tract, such as dietary fibre or non-digestible starches, could be fermented by bacteria, producing beneficial metabolites for the host, such as short-chain fatty acids (SCFAs) [40]. These could be used by colonic cells as a source of energy [41] but also develop other functions not yet fully understood. With regard to this, the presence of SCFAs has been related to health benefits in some diseases, including obesity and cardiovascular disease [42], as well as interacting with the immune system, participating in the function of intestinal epithelial cells and leukocytes [43].

IMPORTANCE OF MICROBIOTA FOR HUMAN HEALTH

Microbiota and disease

Many studies have been conducted with the aim of clarifying what would constitute a healthy microbiota. Early research proposed a ‘core’ of bacterial species, which could be universally present in healthy humans [44,45]. However, this hypothesis was no longer supported by the results of the big microbiome sequencing projects, which observed the considerable variation in microbiota composition among healthy individuals, varying in gender, geographical location, age, among other factors [46–48]. In line with this, a health-associated ‘functional core’ has been proposed as a more physiologically realistic hypothesis. Indeed, the acquisition of prokaryotic genes related to starch or bile acid metabolism would be essential for nutrient absorption and in consequence for growth and adult life [49–51].

Considering that a description of a healthy microbiota is impossible, the features of a health-related bacterial community have been proposed and include a balance between beneficial and 'pathogenic' bacteria, compositional and functional richness and diversity, and resilience [52], which is defined as the characteristic of any ecosystem that is capable of maintaining the stability of returning to its baseline functional profile after internal or external perturbations [53,54]. However, sometimes these features are difficult to define and the term 'health-associated' microbiota is preferred to the term 'healthy microbiota' [55], since microbiota alone cannot describe a state of health or disease.

Despite these discrepancies it has become clear through the recent studies with large cohorts that there exist some alterations in microbiota composition or functionality associated with the risk of some diseases, especially those related to immune system disorders, including obesity [56], inflammatory bowel diseases [57,58], allergy [59] and even neurological disorders through the gut–brain axis [60]. In this scenario, the hypothesis of the single pathogen causing diseases has expanded to include the possibility of the disruption of the structure or function of the gut microbiota playing a key role in the development or perpetuation of some diseases. The term 'dysbiosis' has been coined for this physiological state characterized by the loss of the homeostasis in the relationship between the host and the microbiota [61]. Although dysbiosis is defined as an alteration of the microbiota, the exact groups of bacteria that constitute a disruption are impossible to describe as specific to any one individual.

One of the most studied disorders with a possible relation to a 'dysbiotic' state is metabolic syndrome due to its association with the mentioned diseases. Metabolic syndrome is the term used to define a condition characterized by the loss of glycaemic control, hypertension and the increase in adiposity, which could lead to obesity or diabetes mellitus type 2 [62]. This condition has been related to diminished compositional and functional diversity [63]. Indeed, some transplantation studies in germ-free mice have shown that microbiota transference from obese humans could reproduce the obese phenotype, such as insulin resistance and the increasing of body fat [64,65], supporting the idea that microbiota play a role in the obese phenotype.

Another hypothesis highlights the link between immune and metabolic systems. Alterations in gut microbiota could increase the lipopolysaccharide content, which could alter the intestinal permeability and induce the activation of a chronic low-grade inflammation, which is observed in obesity [66].

Early microbial colonization: impact on infant health programming

In the first year of life, microbiota and several host systems, including immune, endocrine and metabolic pathways, are highly interdependent and co-evolve together in order to establish a beneficial mutualistic relationship. In this context, it has been proposed that the first 1000 days of life, which would comprise the period from conception to the age of two years of life, as a window of time in which microbiota is developing and highly susceptible to exogenous alterations [67]. It has been hypothesized that priority events occurring during very early stages of infancy could have long-lasting effects on a microbial community [68] and therefore on infant development. Thus, these alterations in neonatal microbial exposure could induce permanent changes in foetal physiology and influence the risk of disease later in life ('Foetal programming hypothesis'; Barker's Hypothesis) [69]. We are beginning to understand that exposure to microbes during gestation and in the neonatal period has profound effects on health. Indeed, assuming the close relation between microbiota and the other systems in the host during this time, it has been suggested that disruptions in microbiota maturation could have important effects later in life. Thus, alterations in the colonization process have been proposed as determinant in the susceptibility for some diseases in the adult period [68,70,71]. A detailed list of the associational studies between these microbial alterations and immune-based disease in early life is reviewed in Stiemsma et al. [72] (**Table 1**).

Table 1. Association studies between microbiota dysbiosis in childhood and risk of disease development later in life

Research objective	***Study design***	***Health outcomes***	***Findings***	***Refs***
Allergy and asthma				
Variations in gut microbiome 35d postpartum associated to asthma	Prospective cohort (n=168)	Asthma 4y of age	Variations in bacterial and fungal taxa at 35d post birth associated to higher risk of asthma.	[73]
Variations in the gut microbiome in 3m old infants	Nested case-control (n=319)	Asthma by 4y of age	Decreased *Lachnospira*/*Clostridum neoantale* ratio at 3m, associated with asthma at 4y	[74]
Variations in the gut microbiome at 3-6m of age associated with resolution of cow's milk allergy	Nested case-control (n=226)	Milk allergy resolution by 8y of age	Higher Firmicutes and clostridia in children whose milk allergy resolved.	[75]
Association between gut microbiota at 3m of age with food sensitivity	Nested case-control (n=166)	Food sensitivity at 1y of life	Decreased α-diversity and higher Enterobacteriaceae/Bacteroideaceae ratio associated with food sensitivity	[76]
Variations in the nasopharyngeal	Prospective human cohort (n=234)	Chronic wheezing at 5-10y of life	*Streptococcus* colonization associated with chronic wheezing and atopy	[77]

microbiome at 7-9m post birth associated with respiratory disease				
Variations in skin microbiota at 2m associated with atopic dermatitis	Nested case-control design (n=20)	Atopic dermatitis at 1y of life	*Staphylococcus* colonization associated with lower incidence of atopic dermatitis	[78]
Variations in Bifidobacterium spp. at 1w and 3m of age associated with eczema or atopy	Nested case-control (n=117)	Atopic dermatitis at 1y of life	Variations in Bifidobacterium spp. associated with atopic dermatitis.	[79]
Associations between proportions of IgA coating at 1w and 1y and allergy development	Nested case-control (n=48)	Asthma, allergy, rhino conjunctivitis, allergic urticaria and eczema at 7y of life	Decreased proportion of IgA bound to faecal bacteria associated with allergic disease at 1y of life. Differences also in the microbial patterns bound to IgA	[80]
Associations between microbial diversity at 1m of age and allergy or asthma later in life	Nested case-control (n=47)	Asthma at 7y of age	Lower overall microbial diversity in children with asthma displayed compared with non-allergic children	[81]
Variations in the gut microbiota at 7,14,28,80 and 110d post-partum associated with maternal stress and infant health	Nested case-control (n=56)	GI symptoms and allergic response by 3m of age	More GI symptoms and allergic reactions in infants exposed to prenatal stress, which was also associated with microbiome variations, including less lactic acid bacteria and *Akkermansia*.spp.	[82]
Obesity and metabolic disorders				
Variations in gut microbiome at 3m of age associated with childhood BMI and antibiotic treatment	Nested case-control from 2 cohorts (n=87, n=75)	BMI at 5-6y	Streptococci was positively and *Bifidobacterium* negatively associated with BMI at 5-6y of age. In antibiotic treated children, BMI was associated with Firmicutes spp.	[83]
Variations in placental microbiome associated with birth weight	Prospective human cohort (n=24)	Birth weight	Lower birth weight displayed lower gut microbiome richness and variations in microbiome composition, including Lactobacillus spp.	[84]
Variations in infant microbiome and function associated with maternal pre-pregnancy BMI	Nested case-control (n=39)	Infant metabolism at 18m of age	Fimicutes spp. enriched in children born by mothers at normal weight. Bacteroidetes spp. enriched in children born by obese mothers.	[85]
Variations in microbiome composition at 6-12m of life associated with weight development in infants	Nested case-control (n=49)	BMI measured at 7y of life	Higher abundance of *Staphylococcus aureus* in children who were obese in infancy. *Bifidobacterium* spp. associated with normal weight.	[86]
Association between perinatal factors and childhood adiposity mediated by variations in the microbiome before 6m of age associated with	Prospective human cohort (n=75)	Adiposity at 18m of age	*Bifidobacterium* and *Collinsella* displayed lower adiposity at 18m of age. Acquisition of these taxa influenced by length of gestation and delivery mode.	[71]
Variations in the microbiome composition within 1y of life associated with BMI in childhood	Prospective human cohort (n=138)	BMI SD score between 1y and 3y of age	*Bacteroides fragilis* at 3-26w of life positive associated with BMI SD score. Contrary observation for *Staphylococcus* spp.	[87]
Associations between cognitive ability and infant gut microbiota profiles	Prospective human cohort (n=89)	Cognitive outcomes at 1-2y of life	3 groups of subjects according to their microbiome characterized by *Faecalibacterium*, *Bacteroides* and Ruminococcacee. Differences in Mullen scales between groups	[88]
Variations in gut microbiome before diagnosis with T1D	Prospective human cohort (n=33)	T1D diagnosis at 3y of age	TD1 disease distinguishable by the gut microbome composition and diversity	[89]

Modified from Stiemsma et al. [72]. Only studies in humans were included.

Therefore, factors considered as disrupters of microbial colonization in early life have been related to both alterations in colonization and immune-based disorders later in life. Thus, studies have associated these perinatal factors with increased prevalence of some of these diseases. The most studied among them is the relationship of the associations between C-section with obesity [90–92] and allergy [93,94]. However, other authors have not found a significant relation between this practice and increased body mass index (BMI) later in life [95,96]. The relationship between antibiotics consumption in early life and these disorders has also been widely studied. It has been reported that a greater risk of asthma and a higher body mass index was also observed in children who received more than two courses of antibiotics [97,98]. Other studies have linked antibiotic intake to the risk of some diseases, including obesity [70,83,99,100], asthma and other atopic diseases [97,101,102], and diabetes [103–105]. Furthermore, formula [106–112] feeding, known as a disrupter of infant microbiota, has also been related to these same diseases.

The relationship between allergic disease and asthma and the microbiota development is among the most studied in the field. Bunyavanich et al. observed in their study in a cohort of children with milk allergy that microbiota from 3 to 6 months could be related to allergy symptoms at 8 years, with the enrichment of species from the Clostridia class and Firmicutes phylum being especially relevant [75]. Indeed, a higher incidence has been found of asthma and allergy in infants with a specific gut alteration in the first 100 days of life [113]. It has been proposed that alterations in the maturation of the intestinal microbiome in 1-year-old infants could increase the risk of asthma at 5 years in the asthmatic mother's offspring [114]. In this study, non-asthmatic children showed a more mature microbiota characterized by a higher presence of *Faecalibacterium, Roseburia*, *Lachnospiraceae incerae sedis* and *Ruminococcus* genera compared to asthmatic children. It has been proposed that early exposure to microbes could activate the T helper 1 (Th1) cells and suppress the Th2 activity, which is commonly observed in allergic disease (discussed further below) [115,116].

All these observations have included the microbiota as a key factor in the Developmental Origins of Health and Disease hypothesis [72], which suggests that infant health could be programmed by environmental exposure during the first years of life. With regard to this, the 'hygiene hypothesis' has been proposed as a mechanism by which microbiota could participate in the early programming of infant health. The term was coined for the first time in 90 when scientists began to suspect that improvements

in hygiene and the increase in the rates of Crohn's disease, allergies and type 1 diabetes were interconnected [117]. However, epidemiological and experimental evidence has led to the suspicion that this idea may not be entirely correct, and a new hypothesis has risen, the so-called 'old friends hypothesis', proposed by Graham Rook and colleagues in 2003 [118]. This idea proposed that an early exposure to a diverse range of 'friendly' microorganisms, not infections agents, could be essential for the immune system to react appropriately later in life [119]. Thus, the new hypothesis defends the idea that it is just as important to recognize what to attack as what to tolerate, highlighting the role of all disrupter factors in the infant-microbes more than in just 'hygienic conditions'.

However, most of the human studies have described only correlative associations in cohort observational studies [72] and mechanistic studies that reveal the possible causality of the observations are still scarce. In addition, studies in germ-free mice have provided some evidence of this causality and have related this long-lasting effect to the important role that intestinal colonization could have in the maturation of the immune system shaping the host response [120], which is discussed below in this literature review.

Furthermore, antibiotics administration in early life increased adiposity and other metabolic hormones [121] in mice. Moreover, in the case of allergy, the relation between oral tolerance to foreign antigens and microbiota has been described in animal studies, since germ-free mice did not establish oral tolerance [122], although this result is still controversial [123]. Arrieta el al. showed that inoculation of a specific bacterial community that was diminished in children with high risk for asthma, reduced the airway inflammation associated with allergic disease [113].

Growing evidence has been found in studies focused on rural areas. Riedler et al. described in their cross-sectional survey that children exposed to stables and farm milk intake before the age of one showed lower incidence of asthma and atopy [124]. The authors suggest that the exposure to microbial compounds were in part responsible for this observation. Several authors have found similar relations between farms and rural environments, microbiota and the prevalence of allergic disease [125–127].

Although we have a lot of hints, mechanistic studies in humans that go beyond associative relationships are required in order to be capable of designing strategies that intervene in the early programming through microbiota modification. In this regard, longitudinal studies would be useful, since in this type of analysis each individual could

serve as its own control. However, improved statistical and bioinformatics methods are needed to analyse the data produced.

MICROBIOTA ACQUISITION PROCESS

In utero environment

Women's microbiota during gestation and lactation

During gestation and the breastfeeding period there exists a very close relationship between mother and neonate. The vertical transmission of maternal microbes to the infant has been widely shown, the bacterial populations from the mothers being the first seed for neonatal microbiota [128–130]. It is considered that the colonization process mainly begins during delivery when the neonate encounters a great variety of new bacterial antigens from the maternal vagina and environment. Thus, the maternal microbiota greatly affects the beginning of the colonization process and it is essential to determine which factors could alter not only the infant's microbiota, but also the bacterial communities in the mother.

Indeed, some authors have described that maternal microbiota, in both vaginal and gut niches, undergoes several changes that could play a role in the gestational process [131,132] . It has been found that maternal gut microbiota changes dramatically during pregnancy (**Fig. 1**), with the enrichment of species from Proteobacteria and Actinobacteria phylum, as well as the reduction in microbial richness, being especially relevant [132]. The increment of Proteobacteria has been traditionally associated with inflammation and diseases, including those observed in obesity and metabolic syndrome [133,134]. It is commonly accepted that, during pregnancy, symptoms occur in a woman's body that resemble metabolic symptoms, including body fat augmentation, reduced insulin sensitivity and pro-inflammatory state [135]. However, during pregnancy these could report benefits for foetal growth and energy accumulation [136,137]. Koren and colleagues speculated that this dysbiosis could promote the metabolic inflammation observed in pregnancy and could be one of the mechanisms required for the metabolic changes essential for gestation. Nevertheless, other studies have reported no significant microbial changes in the gut during pregnancy [138], which indicates that further research is needed to clarify the impact of pregnancy on the gut microbiota.

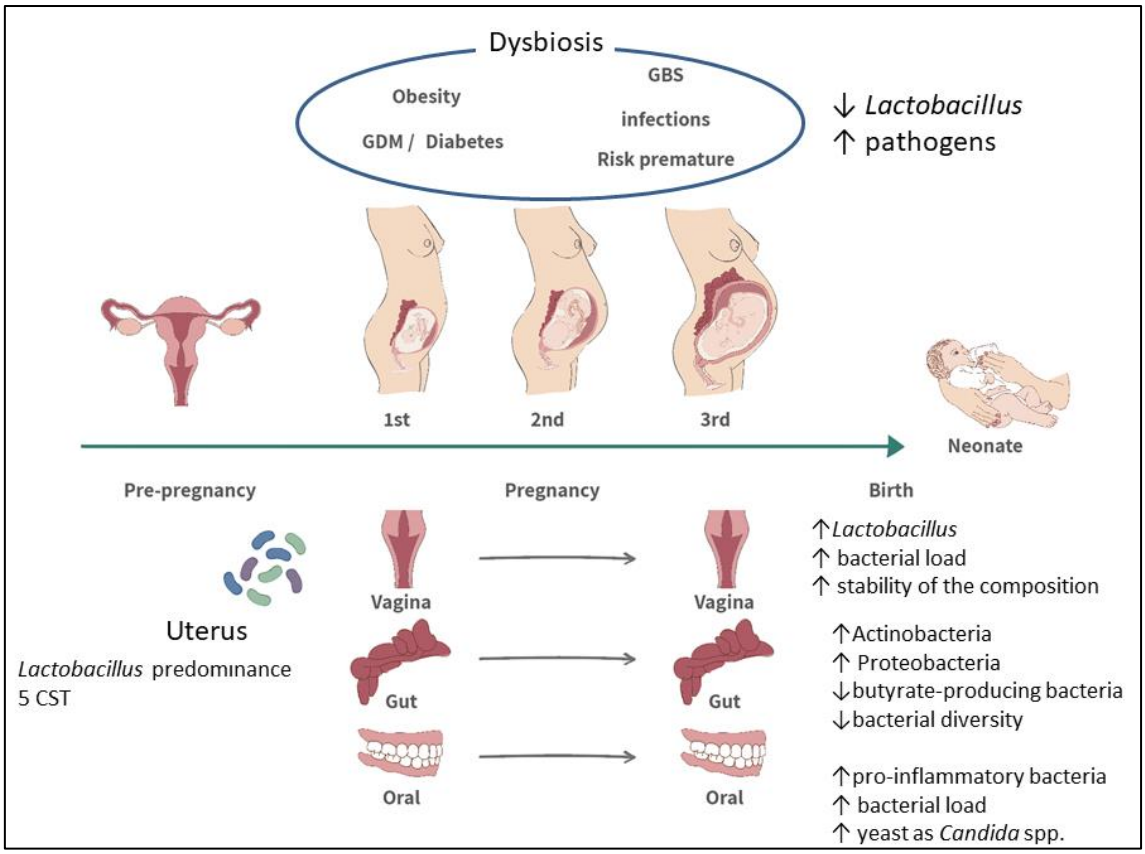


Figure 1. Changes in maternal microbiota over the gestation (Copyright Collado et al. [139], with permission).

Furthermore, recent studies have shown that other microbial niches in the maternal body could have a role not only in the gestation and neonatal period, but also in conception [7]. Vaginal microbiota is the first bacterial community that meets the neonate during the vaginal delivery. Furthermore, it is one of the preferred transmission routes for bacteria invading the gestational cavity and stimulates the inflammatory cascade that could lead to preterm birth [140–142]. Changes have been observed that occur in the vaginal microbiota in pregnant compared to non-pregnant women [143,144]. During pregnancy, vaginal microbiota increases its stability and shows less diversity, with *Lactobacillus* species being the most relevant in this community. These shifts have been hypothesized to also have a role in the healthy pregnancy and delivery [145,146]. Changes in maternal microbiota during gestation have been related to several pregnancy complications during this period, including preterm birth [147,148], preeclampsia [149,150], gestational diabetes [151] and obesity [152], although in most cases it is still not clear whether these shifts are a cause or a consequence of these

conditions. Yet, despite the growing knowledge base, the different prenatal factors that can affect the maternal microbiota during pregnancy remain unclear.

The crucial role of maternal microbes for neonatal colonization has recently been described [153]. The authors reported that maternal skin and vaginal strains colonize the infant microbiota only transiently, but the mother continues contributing to neonatal microbial colonization by other routes. Interestingly, they found that maternal gut microbiota preferentially persists in the infant gut compared to those strains from other sources, highlighting the importance of the maternal intestinal microbiome in the colonization. Although this fact is well known, most studies are focused on infant microbiota and little evidence is available of the effect of the perinatal factors on maternal microbiome.

However, recent studies have suggested that maternal microbiota could influence the infant health even during pregnancy. Thus, it is possible that the placenta harbours metabolites or pathogen-associated patterns that still affect foetal development, as we discussed above. It has been found in a mouse model that maternal high-fibre diet increased the SCFA content in the offspring and affects the immune system in the pumps [154,155]. Gray et al. reviewed the evidence of the possible relationship between the maternal diet and bacterial metabolites during pregnancy and the prevalence of asthma in the offspring [156].

In utero microbial contact

As we mentioned, traditionally, microbial colonization has been considered to start during delivery through the birth canal. However, this statement of the *in-utero* sterility has been recently questioned. Improvements in sequencing technologies and computational analysis have enabled the study of microbial niches in the human body that had been considered sterile, including amniotic fluid [157], foetal membranes [158,159], placenta [160,161] and meconium [37,162,163]. Moreover, some studies have identified bacterial species in those niches using culture methodologies [164,165]. However, results in this field are still not consistent, since several recent studies have suggested that a placental or amniotic fluid microbiome does not exist other than in pathological situations [166–168]. In this regard, there are important technical issues that make these projects difficult, including the low amount of deoxyribonucleic acid (DNA) and thereby the interference of contaminant DNA, its detection and filtering. Furthermore, due to these problems it is essential to design accurate sample collection

protocols, something that has not been well defined in all studies conducted in the field [169].

Despite these technical issues, evidence generates several questions concerning the possible origin of this bacteria and whether they have any function in foetal development in terms of immunological or metabolic maturation. In this regard, researchers have discussed two principle routes whereby microorganisms could reach the gestational cavity. The most commonly accepted hypothesis is based on the haematogenous route, which would include the gastrointestinal–foetal route, the vaginal–placental and oral–foetal placental [170]. Animal studies have shed light on the possible foetal–maternal microbial transmission during gestation [171]. Results from Jimenez et al. have provided evidence of how bacteria administrated orally could reach gestational tissues in mice [172]. Indeed, other animal studies have also shown the increased bacterial translocation in the maternal gut during gestation and lactation [173,174], which could be facilitated by dendritic cells in the gut [175].

Although some authors have described the composition of placental microbiome [160], growing evidence supports the hypothesis that bacterial populations observed in these studies were the result of contamination and were not distinguishable from controls [166,167]. Similarly, microbial populations have been described in amniotic fluid, which is one of the best sources of information about foetus health. This liquid is continually swallowed by the foetus and it is in contact with the foetal gut throughout the gestation. Similarly to what happens in the placental microbiome, the existence of amniotic fluid microbiome in healthy pregnancies is still not confirmed. Although some studies observed different bacterial populations in amniotic fluid in preterm birth (PTB) children [164] and other complicated pregnancies, studies in healthy gestations are still scarce and the results are controversial [167,176]. Other authors have not found bacterial DNA in amniotic fluid obtained from pregnancies without rupture of membranes [167]. However, few studies have described bacterial DNA in the amniotic cavity in pregnant healthy women without any anatomopathological symptoms [160,165,177].

Recently, it has been proposed that bacteria-derived products, including structural products [178], macrovesicles [179] or metabolites [156] could reach the gestational cavity. Thus, the presence of viable bacteria in this *in utero* environment might not be necessary for a microbiota effect on the foetal development. In adults, it has been described how bacterial metabolites, such as SCFAs [180] or neuro-active molecules

[60] could be disseminated and have an impact at systemic level. Thus, some authors have suggested that bacterial products could cross placental membranes and affect foetal tissues [155,181]. However, this hypothesis is still poorly studied, and little is known about the metabolites that could have this capacity and how they would have an effect.

In contrast, most studies in the field have been performed in meconium microbiota. Meconium is defined as the earliest stool of a mammalian infant. Thus, it is considered as an illustration of the intrauterine environment. For this reason, together with the easy and non-invasive collection, meconium has become the preferentially selected sample for researchers to elucidate the role of bacteria for a healthy pregnancy and neonatal period. This hypothesis is supported by studies that have consistently found a bacterial population in meconium by culture-dependent and independent methods [172,182,183].

Similar to what was found in placental and amniotic fluid microbiome [184], meconium microbiota harbours a bacterial population characterized by the presence of species from the Proteobacteria and Firmicutes phyla, including the Enterobacteriaceae and Enterococcaceae families [164,183]. Some authors have hypothesized that this composition could reflect the maternal origin of this bacterial population since almost all these genera would be typically present in greater abundance in adulthood microbiota and are not commonly considered as environmental contamination.

In recent years, several perinatal factors have been proven to modulate meconium bacterial populations, which could have an effect on the initial pioneers of infant development, since the immune system and microbiota maturation run parallel during this important period. Thus, relations between meconium or first-pass faeces microbiota and several maternal clinical conditions, such as prenatal antibiotic use [162], maternal BMI [152], diabetes [185], gestational age [186], have been described.

All these studies concerning bacterial communities present in the gestational cavity and tissues have suggested the possible role of microbes in a healthy pregnancy. Despite the effort that has been made in the field, almost all the studies are focused on the meconium, placental or amniotic fluid microbiota composition, and the possible effect on the neonatal metabolic or immunological routes has been not addressed. Thus, the molecular mechanisms that mediated the implication of the microbial shifts observed in meconium microbiota in pathological or unhealthy conditions in the host homeostasis remain undescribed. It is necessary that research in the field turns to a more mechanistic

analysis in order to respond to the questions about the real consequences of these bacterial populations for infant and adult health.

Maternal diet and clinical factors

Other maternal factors that directly or indirectly alter the mother's microbiota may affect the neonatal microbiota. In this regard, some studies have been performed concerning the effect of maternal BMI and weight gain during pregnancy. Although the importance of these factors in microbiota's constitution and its relation to the host has been seen in the general population, studies in pregnant women are still scarce. Specific groups, including *Bacteroides* and *Bifidobacterium* genera, have been found to be influenced by the body weight in pregnant women. It has also been suggested a relation to metabolic biomarkers showing a connection between gut microbiota and women's metabolism [85,187]. Early neonatal microbiota composition has been shown to be dependent on pregestational weight status but only in vaginally born infants [152]. However, other researchers suggest that obesity is commonly related to other factors, such as weight gain [188], higher prevalence of C-section [189] and other gestational diseases [190,191], which may confound the observed effects in infant microbiota composition. Moreover, maternal viral infections during delivery could modify intestinal microbiota and alter foetal development [192].

Some authors have provided evidence of the effect of diet during gestation on maternal–neonatal microbiota. A high fat diet (HFD) in non-human primates has been found to result in the reduction of *Campylobacter* and Bacteroidetes spp. in the microbiota of offspring compared to control-fed mothers [193], while *Ruminococcus* and *Dialister* genera were enriched. This group partially confirmed their results in humans, in which they found lower relative abundance of *Bacteroides* genus in the offspring's microbiota from mothers who followed a HFD [194]. Interestingly, it has been suggested that the effect of the maternal diet on infant microbiome could be influenced by delivery mode [195]. Other authors have reported the influence of the intake of several vitamins by the mother on the neonatal microbiome [196,197]. Recently, Maher et al. reviewed the studies conducted focused on the relationship between maternal diet and the gut microbiota of mother and infant [198]. However, the effect of these relations and whether they are the cause or consequence of the microbiota alterations observed remain unclear.

From birth to first year of life: towards a complex microbiota in postnatal period

The acquisition of microbiota is a complex process that is influenced by numerous environmental factors as well as those intrinsic to the host (**Fig. 2**). Despite the mentioned recent studies on the *in-utero* colonization, the pass through the birth canal is considered the first bacterial challenge to the newborn. During vaginal delivery, the neonate encounters the maternal faeces and vaginal microbiota and initiates the colonization process. Thus, maternal microbiota determines the first bacterial population that comes into contact with the newborn.

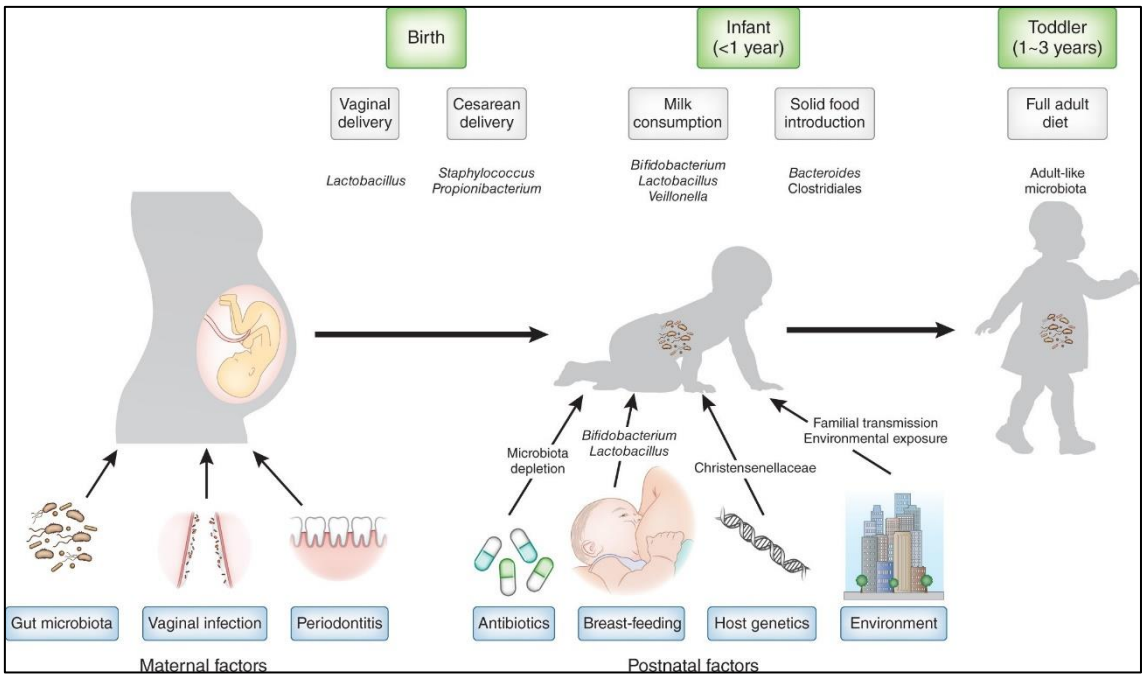


Figure 2. Factors that could influence the infant colonization process in early life (Copyright Tamburini et al. [199], with permission).

At the beginning of the colonization process during the first days, infant microbiota is characterized by the presence of facultative anaerobes, including species from *Escherichia*, *Shigella* and *Enterobacteria* genera. These species trigger the consumption of the available oxygen and favour the colonization of obligate anaerobes, including *Bacteroides* and *Bifidobacterium* genera [130]. The presence of *E. coli* could contribute to reducing the oxygen content, altering the pH and lowering the redox potential, increasing the carbon dioxide to facilitate the colonization of these other genera [200]. Microbial profiles from meconium microbiota determined by 16S

ribosomal ribonucleic acid (rRNA) gene sequencing differ from those observed in faecal samples from early life. Researchers describe that while meconium has a dominance of Firmicutes phylum, faecal samples from neonates are characterized by Proteobacteria spp [164,182].

During the first week of life, newborn gut microbiota shows lower diversity and higher inter-individual variability, but the bacterial community is increasing in diversity and stability during the developmental process. It has been commonly accepted that by the age of two years, infant microbiota reaches a stable state resembling those observed in adulthood [6]. However, some authors suggest that this process could take longer – up to five years. During this time, neonatal microbiota is thought to go through essentially two adaption periods during early life [201]. The first one is a consequence of lactation and causes the predominance of *Bifidobacterium* genus in postnatal microbiota. The other is triggered by the introduction of solid food and establishes the adult microbiota characterized by the increment of the Firmicutes and Bacteroidetes species [202,203].

From delivery to this moment of stabilization, the colonization process is influenced by several factors that possibly interact with each other and modify or alter the acquisition of the gut microbiota. The most relevant among these factors are discussed in the following sections.

Factors influencing neonatal microbiota development

The gut microbial community is very dynamic in infancy and, therefore, susceptible to host-related and environmental factors that could modulate the microbiota composition and functionality [6,204]. Thus, as we mentioned above, a large number of studies have been conducted concerning the effects of several perinatal factors on neonatal microbiota in order to find whether there exists an ‘adequate’ developmental microbial pattern for a ‘healthy’ infant maturation or not.

Gestational age: preterm neonates

Several studies have been found differences in terms of microbiota composition between preterm (<37 weeks of gestation) and term infants. Tirone et al. reviewed the alterations of gut and lung microbiota in these infants [205]. Pre-term (PTB) new-born colonization is determined by the immaturity of the organs and the co-occurrent factors

such as antibiotic use, hospital stay and enteral feeding [206]. The microbiota of preterm infants has been observed to be characterized by an enrichment of Enterobacteriaceae spp., *Enterococcus* or *Staphylococcus* genera and a delayed *Bifidobacterium* colonization [205,207,208]. Compared to term infants, preterm neonates shown a decreased relative levels of anaerobic bacteria while abundance of potential pathogenic and facultative anaerobic microbes would be increased [209]. Furthermore, gestational age has been also observed to influence the milk composition, altering the metabolites that affect the colonization, such as oligosaccharides (HMO) [210].

Delivery mode

In the recent decades, the incidence of C-section (CS) has increased worldwide with an annual increment of 3.7% from 2000 to 2015 [211,212]. The world health organization (WHO) estimates that the optimal rates for a C-section that provides a medical benefit would be 10–15% [213]. However, Europe shows an average of CS prevalence of 28% [214]. In this regard, one of the most studied factors among those that influence the composition of the neonatal microbiota is the delivery mode. It has been widely observed that in those children born by vaginal birth, the skin, gut and oral microbiota are initially similar to those bacterial populations found in the maternal vagina [37], principally lactobacillus strains. However, in C-section-delivered infants who have not be in contact with the canal birth, their microbiota is characterized by the presence of skin and environmental microbes, including species from *Streptococcus, Staphylococcus, Corynebacterium or Propionibacterium* genera or *Proteobacteria* genus [203,215]. Indeed, researchers have found differences between elective and emergency C-section, showing the first one lower in diversity and richness [216]. The authors speculated that the infant could be exposed to maternal microbes before the initiation of the surgery.

With regard to this, several authors have described the shifts in the microbiota in children born by C-section. Rutayisire et al. reviewed, a few years ago, the studies focused on the effect of delivery mode on meconium microbiota [217]. They highlighted the lower relative abundance of *Bifidobacterium* species in CS neonates compared to those born vaginally [71,218–220], at 1 week and beyond [218,221,222]. The other consistently observed result is the delay in colonization of some bacterial groups, specifically from Bacteroidetes phylum, in CS-born children's intestinal

microbiota. A delayed Bacteroidetes colonization at the age of 2 [223] was found in children born by C-section. Other authors confirmed this observation [218,219,224]. Shifts in other groups have also been described with less consistency. *Clostridium* and *Klebsiella* genera species have been related to C-section [71,219,220]. In addition, in almost all these studies C-section-born infants have a microbiota characterized by lower diversity. Recently, these described results have been confirmed in a large cohort of almost 600 children, including the disrupted transmission of *Bacteroides* strains, and the enrichment of opportunistic pathogens of *Enterococcus*, *Enterobacter* and *Klebsiella* genera in the C-section-born infants compared to those born by vaginal delivery [225].

Moreover, the length of the effects of delivery on the microbiota has not been firmly established. While some studies have detected differences due to C-section at 7 years of age [226], other authors observed the effects in less extensive time [37]. In the case of *Bifidobacterium*, despite the evidence not being coincident in all studies, some authors report differences up to the age of 3 [219]. For *Bacteroides* spp., differences have been observed at 3 months of age [224]. Despite the short period in which the authors could find these differences, it is necessary to notice the importance of this period for immune system education, as we have discussed in this literature review.

Although a cause–consequence relationship could not be established between the delivery mode and these shifts in neonatal microbiota, it seems clear that these variations related to the delivery mode may have a consequence for infant development. Indeed, it has been proposed that C-section is one among other practices that could also be responsible, partially, for the effects observed in those children, including antibiotic administration, instrumentalization, delayed initiation of breastfeeding … In this regard, it has been described that mothers who had undergone a C-section had difficulties in starting their milk [227]. It has been suggested that these other factors, including diet, antibiotic exposure and gestational age could be responsible in part for the observed differences in C-section-born infants [228]. A study from this group found that although there were minor shifts in these infants in terms of microbiota composition, no differences were observed in bacterial community function [37].

As we mentioned above, C-section has been associated in observational studies with increased risk of some non-communicable diseases; however, the causal relation between the microbiota alterations and these disorders has not been demonstrated and mechanistic studies are still lacking. In this regard, Domíngez-Bello et al. conducted a study with the aim of restoring the microbiota of the C-section-born infants, exposing

them to maternal vaginal fluids at birth [215]. Although they succeeded in partially reproducing the vaginal-delivered infants' microbial pattern in those born by C-section, it was a pilot study with very few participants and the possible benefits in the health outcomes of those children are still unclear.

In any case, this body of evidence has generated a growing interest in deliveries with less intervention. In this regard, practices such as home birth are increasing worldwide, being considered as a more 'natural' environment in which to give birth [229,230]. However, the effects of this practice on infant microbiota and neonatal health are still not fully understood since very few studies have been conducted [231,232].

Mode of delivery have been also associated with relevant health outcomes on mothers during post-partum period which could also affected the neonate during this time. Very little studies have described the relation between mode of delivery and post-partum weight retention (PPWR) [233–236]. In two of them, the authors did not find any differences in weight change after delivery according to delivery mode [234,235]; however, both were retrospective in design. In one of them, the authors described that mothers who undergone a C-section showed higher PPWR at 1-year after delivery even after controlling for relevant covariates including maternal age, education, exercise levels, breastfeeding duration, pre-gestational BMI and gestational weight, among others [236]. Any of these studies were focused on the possible role of microbiota shifts associated with delivery mode in these observations, and the it remains totally unexplored, despite of the extensive literature that showed the relation between body weight and gut microbiota [237]

Antibiotic exposure and bacterial infections

Although other medications have been found to significantly affect microbiota composition, including laxatives, anti-inflammatory drugs, hormones, antihistamines and antidepressants or proton pump inhibitors [254,255], antibiotics are one of the most studied factors affecting microbiota. Indeed, they has been a widespread increase in their use in recent decades [238] and they are the treatment most prescribed to infants.

Some authors highlight that antibiotics could affect neonatal health, not only in the postnatal period but also prenatal antibiotic exposure could disturb the foetal development through the alteration of maternal microbiota [239]. Thus, some authors have observed that antimicrobial treatment during pregnancy could affect the neonatal

gut microbiome [240] with possible consequences for infant health [241–243]. Maternal antibiotic treatment has been described to impact the development of neonatal gut microbiota and antiviral immunity [244]. Vaginal microbiota has also been observed to be affected by antibiotic treatment prior to birth [245]. Furthermore, an increase in antibiotic-resistant genes has been described in infants exposed to antibiotics early in life, mostly in intrapartum antibiotherapy [246,247]. Besides the gut microbiota, intrapartum antibiotics have been found to alter the initial oral microbiome in neonates, causing the enrichment of infant microbiota in species form Proteobacteria phylum in detriment of Streptococcaceae family members [248], including the promotion of increased abundance of Enterobacteriaceae [206].

Although it is known that antibiotic intake could modify microbiota in terms of composition, structure and resilience, the effects of this in neonates is not fully understood [199]. It has been found that macrolide intake in 2–7-year-old infants showed differences in gut microbial composition and function, such as a decreased Actinobacteria relative abundance, while Bacteroidetes and Proteobacteria phyla increased. Indeed, in children who received more than two courses of these antibiotics, a greater risk of asthma and increased BMI was also observed [97,98]. Similar trends in Actinobacteria and Proteobacteria phyla were observed by other authors [249–252]

As mentioned above, most studies in children have been focused on the use of antibiotics in early life and the susceptibility to some immune-related diseases. Several studies have focused on the effects of antibiotic intake on the risk of some diseases, including obesity [70,83,99,100], asthma and other atopic diseases [97,101,102], diabetes [103–105] and even neurocognitive outcomes [253]. However, the impact of long-lasting antibiotic consumption in infants is poorly understood, partly due to the unusualness of those long-term treatments in children. It has been suggested that high-dose antibiotics can cause important reductions in the microbiota population, which may be related to the weight loss observed in some studies [254]. However, lower antibiotic doses would cause microbiota composition shifts, more than population size variation, and trigger the weight gain shown in the above-mentioned studies.

Animal studies have allowed us to deepen the knowledge of the effects that these microbial alterations could have on the host physiology, including the reduced intestinal host defence [255], their effect on toll-like receptor (TLR) expression in mice colon [256] and reductions of *Lactobacillus* genera and segmented filamentous bacteria after antibiotic administration, resulting in the activation of the T helper 17 cells in the colon

[257,258]. Further studies focused on humans are needed in order to improve our knowledge of the effects of antibiotics on human microbiota and health.

Furthermore, the presence of early exposure to bacteria and subsequent infections have been related to some beneficial effects for immune system development in the neonate [259]. While the early-onset sepsis have been associated with an increment in the mortality rates [260,261], birth cohorts studies have explored the effect of bacterial endotoxins exposure during infancy on the risk of later atopy and asthma [262]. Results from a murine model revealed that the chronic exposure to low d-dose endotoxin could protect mice form asthma development in airway epithelial cells [263]. As we discussed below, it has been suggested that the immune-maturing infections could contribute to "correct" the Th-2 biased neonatal immune system to a Th1 response improving the immune response in neonates and infants [264]. However, this hypothesis is being also revised, since the increase of Th1-mediated autoimmune disease have been also related to an excessive hygiene [265]. Thus, could be due to the most experimental data showing Th1-Th2 response are based on observations made in adult animals with mature immune systems [264] which differ form that in adults. Further studies with more accurate models that mimic the conditions of the neonatal immune system are needed to uncover the remaining questions.

Infant diet: breastfeeding practices and introduction of solid foods

Approximately 25–30% of the infant gut microbiota derives from breast milk [266]. Moreover, exclusive breastfeeding could partially re-establish the shifts on infant gut microbiome induced by C-section delivery, reducing the differences with vaginally delivered infants [267]. The most isolated breast milk genera include *Staphylococcus*, *Streptococcus*, *Lactococcus*, *Propionibacterium*, *Lactobacillus*, *Bifidobacterium*, and Proteobacteria phylum [268,269]. Indeed, breastmilk possesses other molecules that could affect infant microbiota and immune system, such as antimicrobial compounds (lactoferrin and lysozyme) and antibodies or cytokines [270]. A growing body of evidence has described the diverse microbiota that is present in breastmilk, which could even be influenced by several maternal and infant factors, including maternal weight, delivery mode or genetical variants [271,272]. Some authors have proposed that secretor status influences the composition of milk microbiota, especially the HMO content [273,274]. Indeed, it has been found that this status is transferred to the infant

since microbiota from children with secretor mothers has been observed enriched in *Bifidobacterium* genus compared to those born from non-secretor mothers [273,275].

Breastfeeding significantly alters the infant microbiota. Breastfed children have higher relative abundance of *Bifidobacterium* and *Lactobacillus* genera in their microbiota compared to formula-fed children [276]. Both groups are commonly recognized as a source of probiotics due to their immunomodulatory capacities and their functional activities, including the reduction in pH and the production of SCFAs, in the case of *Bifidobacterium* species. Indeed, it has been speculated that milk microbiota could be among the sources of infant gut microbiota, at least for *Bifidobacterium* species. A high percentage of sharing in the *Bifidobacterium* species between milk and faecal samples collected from the same mother–neonate dyad has been observed [277]. Furthermore, some *Bifidobacterium* spp. had the ability to ferment HMOs, a group of unconjugated glycans, from milk to produce SFCAs [278], which suggests a prebiotic effect of the HMOs on breastfed children favouring a selective growth of bifidobacteria. Interestingly, fermentation of HMOs increased the secretory immunoglobulin A (IgA) and improved the defence immune function [279]. In contrast with breastfeeding, formula-fed infants showed a microbiota with higher diversity and an enrichment of *Clostridium coccoides*, *Staphylococcus* spp. and those from the Enterobacteriaceae family [216,280,281].

The introduction of solid food is the second transition period in the microbial colonization. During this process, microbiota is rapidly modified, increasing the functional diversity that will participate in the metabolism of the compounds that will be supplied by the adult-like diet [201]. It is characterized by the acquisition of bacteria with the ability to digest glycans, mucin and complex carbohydrates and produce bioactive molecules such as SCFAs [51], including species from *Ruminoccocus*, *Blautia*, *Lachnospira*, *Faecalibacterium* genera, among others. In a study conducted in a Bangladesh cohort, the authors found that *Faecalibacterium praunitzii*, and species from *Ruminococcus* and *Dorea* genera were those that discriminate more between the age-related maturation of the infant microbiota [282].

Some studies have been performed exploring the effect of the early introduction of infant complementary feeding (consumption of solids or non-water liquids before 3–4 months). Differding et el. found a higher relative abundance of *Roseburia* genus and butyric acids in children with early complementary feeding (vs later introduction) at 12 months of life [283]. However, these authors explained later that these observations

were influenced by the duration of breastfeeding, describing that among breastfed infants (>4 months) and with early solid food introductions, higher BMI-z scores were seen at 5 years of life [284].

Genetic background

Very little information is available regarding the possible effect of different genetic background on microbial colonization, partially due to the difficulty in studying this field without the confounding factors bias. Ethnicity has been identified as one of the main drivers of neonatal microbial colonization, along with delivery mode and breastfeeding, even in children from the same geographical location [285]. Authors have shown that the effects of ethnicity in infant microbiota was apparent at 3 months post-birth, even before the introduction of complementary foods.

Some studies on twins have found that there are some bacterial taxa in the gut microbiota that are heritable [286]. The most studied among them are the family Christensenellaceae and methanogenic archaea [287]. Indeed, these families have also been related to lower BMI [288] in several studies that highlight the interconnection between these heritable taxa and genes related to diet, metabolism and immune systems.

The *fucosyltransferase 2* (FUT2) is one of the most studied genes related to human microbiota composition. Secretor status refers to the expression of the ABH and Lewis histo-blood group antigens in the intestinal mucosa [289]. Differences in microbiota composition according to secretor status have been described, including lower species richness [289] and reduced bifidobacterial diversity, richness and abundance in non-secretor individuals [290]. Indeed, it has been observed that *Bifidobacterium* spp. were established earlier and more often in children fed by secretor mothers (vs non-secretor) [273], likely due to the effect of secretor status in breastmilk HMOs, as we mentioned above [274]. However, recently no association has been found between the FUT2 genotype and faecal microbial composition in the adult population [291]. Thus, further research is needed to ascertain the possible influence of the FUT2 gene genotype in microbiota composition.

Geographical location and environment

Along with the maternal source, the environment is the natural habitat of the bacteria that contribute to the microbial colonization. Increasing evidence suggests that exposure to a wide array of antigens, such as **living in rural areas**, or having **siblings**

or **pets,** may influence the early colonization and development of the microbiome and consequently the immune system response and tolerance induction. It has been observed that family members have a more similar microbiota than to other unrelated humans [292,293]. Indeed, it has been shown that this fact was not related to genetic proximity, since cohabiting parents and children genetically non-related also shared more similarities between them than to other children [294]. In this regard, studies have shown that the colonization by *Bifidobacterium*, *Bacteroides* and *Lactobacillus* increases with the number of siblings, whereas first-born infants presented higher levels of enterobacteria and clostridia [295,296]. Other results showed that having older siblings was associated with differences in gut microbial composition at 9 and 18 months [297]. It has been found that children with families with a large number of members resulted in an increment in *Bifidobacterium* species and the reduction of *Clostridium difficile* relative abundance during the first 2 months of life, which has been related to allergic disease [298].

However, early exposure to pets and animals has been hypothesized to also be related to a protective effect through the promotion of microbial tolerance. On the other hand, microbiota richness and diversity tended to be increased in infants living with pets, whereas an under-representation of *Bifidobacteriaceae* and over-representation of *Clostridium, Veillonella, Peptostreptococcaceace* and *Coprococcus* were exhibited [100]. Early exposure to pets has been associated with a reduction in allergy and asthma incidence [299]. Moreover, in a recent study with 746 infants, the exposure to pets influenced the gut microbiota of infants at the age of 3–4 months, independently of delivery mode, with higher levels of *Ruminococcus* and *Oscillospira* in the stool samples of infants in contact with pets. Previous studies have inversely associated these bacterial groups with atopic disease in children and obesity [300], even though the molecular mechanisms behind this effect and their relationship with the microbiota are not clear. In this regard, it has been hypothesized that rural areas could have an impact on the microbiome composition and its response to an immunological challenge compared to more urban areas [301]. Indeed, it has been suggested that children living on farms have higher bacterial diversity than those children who never visit farms, and these farm-living infants showed a decreased risk of developing allergies [301,302]. Furthermore, postnatal exposition to these rural environments could also reduce the allergy prevalence in the offspring [303].

GUT MICROBIOTA AND IMMUNE SYSTEM

Physiology of the immune system in the gut

Gut epithelium is the first barrier that meets the large number of bacterial antigens present in intestinal microbiota. It consists of an epithelial monolayer, which is thin and permeable due to its functions related to the uptake of nutrients, water and electrolytes [304]. Furthermore, in order to facilitate these processes, the epithelium has numerous projections, called villi, to increase the surface of action [305]. Integrity of gut epithelium is regulated by the transmembrane multiprotein complexes, called tight functions, between cells that form the monolayer [306]. The maintenance of these unions is extremely important since damage to them could facilitate the invasion of commensal bacteria, promoting inflammation and having effects at systemic level [307,308]. Enterocytes are responsible of the nutrient transport and generate a membrane-bound mucin that forms a dense protective cover [309].

Along with the epithelial cells, the goblet cells release a group of glycoproteins, known as mucins, that constitute a barrier that protects the gut epithelium. Actually, these proteins assemble two protective mucus layers. The outer one is the environment of the most commensal microbes, and the inner layer, which has a high density and strength, is impermeable to bacteria and prevents contact between bacteria and the epithelium. Thus, alterations in the permeability or the composition in the mucus layer could lead to inflammation through excess bacterial stimulus on the epithelium. In this regard, microbiota could also modulate the mucus production by the TLR pathways [310].

The third main epithelial cells are the Paneth cells, which participate in the gut innate immunity [311] and are characterized by the secretory granules that control the mucosal tissue colonization via several antimicrobial molecules [312]. Besides these, enteroendocrine cells respond to specific stimuli to secrete numerous hormones to control the GI tract functions [313].

The immune system in the GI has been termed the gut-associated lymphoid tissue (GALT) (**Fig. 3**) and its activity is driven mainly by four mechanisms: the mucus layer and gut barrier modulation; the antimicrobial peptides; the Paneth cells activity and the secretion of IgA [314]. Below the gut epithelium, the lamina propria, which is a layer of connective tissue, is the reservoir to several lymphoid tissues and immune cell populations [315]. Peyer's patches are present, mainly, along the small intestine and are

the main site for the gut adaptative induction [316]. The epithelium that covers the Peyer's patches is called follicle-associated epithelium and about 10% are unique microfold (M) cells [317]. These areas are relatively free of mucin to promote the interaction with microbes and other materials in the gut lumen, which facilitates the transcytosis and the presentation of the antigens to antigen-presenting cells (APCs), such as macrophages and lymphocytes [318]. Enterocytes and these immune cells express the pattern recognition receptors (PRRs) that recognize the microbe-associated molecular patterns, such as lipopolysaccharides (LPS) of Gram-negative bacteria, and their signal triggers the downstream immune response, such as the reinforcement of the gut barrier or the secretion of mucus [21].

Then, in the adaptative immune system, B cells activated by these antigens and with the contribution of the helper T cells (Th) initiate a process that concludes in the release of IgA, which is secreted as a dimer compound called secretory IgA [319] and is an essential molecule in the maintenance of the gut-microbiota homeostasis. IgA is considered as one of the most important mechanisms in the defence of the intestinal mucosa blocking the microbial adhesion to epithelial cells [1]. The action of the intestinal secretory (s)IgA could be affected by the gut microbiota through the control of its production or the modulation of the sIgA repertoire [2,3]. Commensal bacteria or their antigens are taken up by M cells and in turn captured by DCs in Peyer's patches. This caused the activation and maturation of B cells in lymph nodes leading to IgA production which leads to the prevention of microbiota penetration [4]. Thus, microbiota could also affect the B cells driving of isotype switching [5,6]. Furthermore, IgA may control the composition of gut microbiota with the ability to select species with less inflammatory activity on the host tissue [7]. Indeed, IgA have been described to be polyreactive and to have a great board but defined subset of microbiota which suggest that it have innate-like recognition characteristics [8]. The GALT system responds to exogenous antigens in a different way from the systemic immune system since it exists in a constant balance between the defence of the organism and the induction of tolerance to maintain the homeostasis with the gut microbiota in order to not trigger an excessive inflammatory response [320].

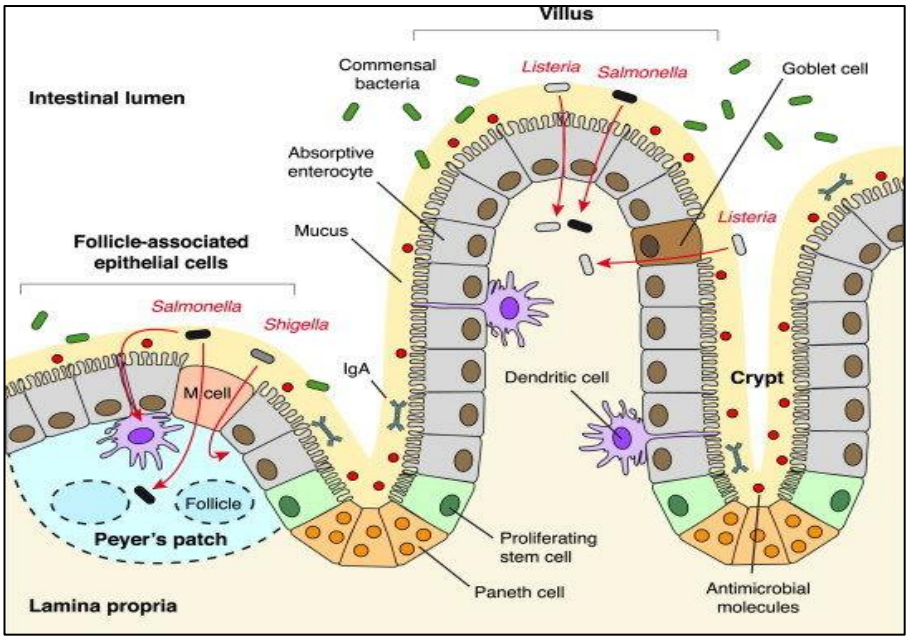


Figure 3. Scheme of the organization of the gut associated lymphoid tissue (Copyright Ribet et al. [321], with permission).

Other essential subsets of cells in the gut-microbiota homeostasis are the lymphocytes T (T-cells). Naïve T cells from the thymus migrate to secondary lymphoid tissues to encounter antigens and the become activated and differentiated into effector T-cells [322]. In this process, Th cells help B cells make antibodies to interact and neutralize the possible pathogens. Effector Th cells can be classified into Th1, Th2, Th17 and regulatory T (Treg) cells subpopulations based on their unique cytokine properties [323]. Among the various immune cells, these T-helper cells have been specially related to microbiota [257,324,325]. Several researchers have been shown the alterations in T-cells maturations and response in germ free mice which has been no exposed to microorganisms [325,326]. Especially regulatory T-cells have been deeply study in their role of maintaining the intestinal homeostasis [327,328]. Several commensal microorganisms as well as bacterial metabolites may induced the Treg cells in the gut [329,330] being that one of the mechanisms of action of health-associated bacteria in their capacity to induce benefit to the host [331]. However, as we mentioned above, most of these results have been described in adult mice whose immune system shows important functional differences compared to neonatal immune response. Thus, the exact mechanisms by which the initial period of the bacterial colonization could contribute to the maturation of these immune cells needs further evaluation.

Microbiota and immune system development

As we mentioned above, it has been shown that the shifts triggered by the perinatal factors in neonatal microbiota could not be maintained beyond early childhood. However, during this period the development of the immune system is taking place, and alterations in this process, in which microbiota may play an essential role, could have important consequences later in life.

There is a mutualistic relation between microbiota and the immune system's maturation. The accumulation of Paneth cells in the intestine would modulate the microbiota composition and may facilitate initiation of the host tolerance [332]. At the same time, microbiota is essential for the complete maturation of the immune system [333]. In this regard, animal studies have made relevant contributions to this field. Several authors have described immunological dysfunctions in germ-free mice, including the increased susceptibility to immune-related diseases [120]. The gut of these animals is composed of fewer and smaller Peyer's patches [334], a weaker mucus layer [335,336], and poor maturation of lymphoid tissues [337]. Indeed, functional alterations have also been observed, including dysfunctions in cytokine [338,339] and immune cell [340] production and in antigen sensing by TLRs [341]. Furthermore, animal studies have suggested that despite Peyer's patches appearing during foetal development, their complete maturation with the ability of IgA secretion is dependent on the gut colonization [342,343]. Indeed, microbiota could also affect the balance between the different T cell subtypes [325,344], including the equilibrium between Tregs and Th1/Th2.

On the other hand, metabolites resulted from bacterial metabolism have also been highlighted as a mechanism of action in the bacteria–host relationship, with SCFAs being especially relevant. Regarding this, butyrate, which is used by the colonocytes as an energy source [41], can modulate the proliferation of several immune cells [43], such as Treg cells, and could enhance the mucus secretion promoting the expression of muc2 genes [345]. Additionally, SCFAs could facilitate the differentiation of naive T cells to Treg cells promoting tolerance in the gut environment [346,347].

Gut microbiota and TLR are closely related since they are the most important sensors for bacterial antigens. Indeed, microbiota could also alter the host system through the TLRs' activity. Indeed, polysaccharide A (PSA) from *Bacteroides Fragilis* could induce the production of anti-inflammatory cytokines and the induction of Treg

cells by TLR2 sensing [325,348,349]. This mechanism has been proposed, among others, as the means by which bacteria could promote tolerance to a complex gut microbiota. Similarly, it has been described that total LPS from the human gut microbiota would silence the TLR signalling supporting the immune tolerance of the microbiota [350]. Besides this, some bacterial indoles could be sensed by routes involving TLR4 and affecting the integrity of the mucus layer [351].

Furthermore, microbiota have the ability to regulate the TLR response, and hence the energy homeostasis since it is closely related [304]. Microbiota could also modulate the permeability and the systemic energy balance. In this regard, germ-free mice also exhibited metabolic alterations [352,353]. Reduced weight and length prior to weaning have been found in germ-free mice compared to conventionally raised animals [354].

Although it is not possible to establish a causality between microbiota dysbiosis in early life and immune-based diseases, some authors have hypothesized some mechanisms by which this relationship could affect infant development. Initially, neonates are born with the immune system biased towards Th2-cells-cytokine pattern resulting from the foetal period, which also contributes to avoiding the excessive pro-inflammatory signals due to the new antigens that the neonate receives [355,356]. During ;infant growth, the child encounters multiple pathogenic challenges that are caused by the balance between Th1 and Th2 polarization towards Th1, since a larger Th2 bias is associated with allergy development [116,357]. Thus, a constant equilibrium between Th1/Th2 profiles is essential for the development of a correct immune system, and microbiota could play a role in this period. Delayed Bacteroidetes colonization that has been associated with C-section could be related to the reduced Th1 activity observed in these children [223]. As we mentioned, the PSA from *B. fragilis* could restore the Th1/Th2 balance in germ-free mice [325]. Thus, not only the altered microbiota composition but also the timing of the steps in the colonization could be important for the correct immune system maturation. It has been shown that a delay of 1 week in the colonization was sufficient to provoke a disruption in the mononuclear cell population and higher levels of regulatory cytokines [35].

Variations in this orchestrated process could favour the emergence of some diseases during childhood or adult stage. With regard to this, neonatal dysbiosis could lead to a strong Th1 response, which would trigger a continuous pro-inflammatory state with long-lasting consequences [199] or it might not induce the Th1 activity necessary to equilibrate the Th1/Th2 balance. In this regard, a few studies have been performed

concerning the role of the LPS from species transferred during delivery to the neonate. It has been suggested that specific strains transferred from the mother to the infant in the vaginal delivery trigger the activation of the immune system by a higher function related to LPS biosynthesis, which induces the increased levels of TNF-a and IL-18 in those infants compared to C-section-born infants [358]. Macrophages from a foetal murine model were found to be non-responsive to LPS, but after birth the acquisition of LPS resistance and the activation of intestinal epithelial cells occur and that is mediated by TLR4 [359]. Thus, this indicates that the activation of the immune system is essential for correct infant development. Monocytes derived from neonates born to obese mothers showed a lower responsiveness to LPS, leading to an altered cytokine promoter methylation [360]. Although evidence in animal studies are available, mechanistic studies in humans are still scarce.

In their study, Vatanen et al. analysed more than 200 children from Russia, Finland and Estonia. Children's microbiota from the last two areas were dominated by the *Bacteroides* genus, which has a structurally different LPS. This difference causes *Bacteroides* LPS to not trigger the activation of TLRs and therefore the tolerance by accommodation processes [361]. The authors hypothesized that this could be the cause of the higher incidence of autoimmune diseases in these countries compared to Russian children.

Briefly, some mechanisms have been suggested by which dysbiosis could affect immune system development. However, very few studies in humans concerning this question have been performed, especially including a mechanistic analysis. Several questions remain unanswered, including how perinatal factors could influence this process and exactly which mechanisms are involved in this effect.

METHODOLOGIES TO STUDY MICROBIOTA

Before the microbiota era, the characterization of bacteria was focused on the detection of pathogenic organisms. However, the description of the importance of the microbiome–host relationship in human health has caused the development of new tools for microbe investigation. **Table 2** summarizes the most used techniques and their advantages and limitations for the microbiome study.

Table 2. Summary of the most used techniques in microbiota analysis.

Technique	Advantage	Limitations	Applications
Culture-microbiology	▪ Traditionally used ▪ Allow exact mechanistic studies	▪Existence taxa not cultivable ▪Difficult to reproduce the required conditions	[164]
Molecular fingerprinting	▪Permit the following of the variations of a community ▪Easy and rapid	▪Only comparisons between patterns ▪Difficult to performed	[362,363]
Sanger	▪The cheapest sequencing technology ▪Easy and rapid	▪Low throughput ▪Relatively expensive	
Targeted amplification (used in the studies of this thesis)	▪Offer taxonomical information ▪Cheaper than metagenomics	▪Limited taxonomic level identification (fragment size limitation) ▪Biased by PCR amplification ▪Low abundant taxa underrepresented	[138,364]
Metagenomics	▪Permit functional studies ▪No biased by PCR amplification	▪Required more bioinformatical analysis ▪Functional analysis does not reveal active genes/activities ▪Higher cost than targeted	[37]
Transcriptomics	▪Provided information of gene expression and viability	▪Elevated cost ▪Experimental issues (instability of RNA) ▪Poor database quality	[365]
Metabolomics Proteomics	▪Great amount of data generated. ▪Functional information	▪Still expensive techniques ▪Complex analysis ▪Poor database quality	[366–368]
Culturomics	▪Discovery of novel strains ▪Facilitate future molecular studies	▪Still difficult to imply	

Examples of studies focused on maternal-infant relation in the microbiome field are shown in the "Applications" column. All of the studies described in this dissertation have been performed using the targeted amplification approach by Illumina protocol for 16S rRNA gene sequencing.

Culture-based techniques

Methods based on cultures have been traditionally used in microbiology [369]. However, most of the environments studied including human niches, harbor species that are not culturable or require a complex conjunction of culture conditions that are

difficult to reproduce [370]. Furthermore, some species in the gut share a symbiotic relation among them, and the culture and isolation of the specific strains are difficult to perform [371]. In the large studies of human microbiota, the limitations of the culture methodologies prompted interest in culture-independent technologies. However, in order for the objectives of some studies to be achieved, culture methodologies are still essential, such as in some mechanistic analyses that aim to associate a specific strain with an observable effect on the host cells.

Culturomics

Culturomics has been recently developed in order to avoid the possible bias of the other techniques and to provide new approaches that could be useful in combination with other technologies, especially in terms of studying new bacterial species [372]. It consists of the high-throughput cultivation with different media types and conditions to cover all the possible requirements of the target species [373]. Although initially only 20% of intestinal bacteria were thought to be cultivated, recent studies have estimated that approximately half of the total gut bacteria found by sequencing techniques have a cultured representative [374,375].

Molecular approaches

One of the first technologies developed was that based on the banding patterns of DNA, called molecular fingerprinting [376]. This approach consists of generating bands from DNA extracted from the sample based on their physical or chemical properties (e.g. GC content), but with the limitation that it gives a comparison and the exact identification of the microbial composition [376]. Among the molecular technologies used the most known are restriction fragment length polymorphism (RFLP), and denaturing (DGGE) and temperature (TGGE) gradient gel electrophoresis [377,378]. Although they can be useful for some preliminary analysis, such as the testing of variations in a microbial community by a treatment, they have been supplemented by other techniques.

Besides the sequencing technologies, quantitative polymerase chain reaction (qPCR) is the most used technique in the study of microbiota and offers quantification of the targeted DNA, which is amplified with specific primers [379]. It confers an important advantage compared to sequencing because it provides a relative abundance

of each detected taxa. However, the design of the specific primers that target the analysis of each taxa is necessary and the information that it is possible to obtain in each reaction is limited. Thus, it is especially useful in combination with sequencing approaches.

Other techniques are also used in microbial analysis, including microarrays, such as the human intestinal tract chip (HITChip) [380] and fluorescence *in situ* hybridization (FISH) [381], which are designed with the aim of detecting known specific taxa among a bacterial population in a sample. Thus, this limit their application in the big microbiome studies.

Sequencing-based methods

The development and improvement of the sequencing technology were the most determinant factors that contributed to the advance in microbiome analysis. Among these methods, Sanger sequencing was the first used, but it has a higher cost and is more time-consuming compared to the new next-generation sequencing technologies.

Targeted amplicon sequencing represents the current standard methodology for profiling the composition of microbial communities. By far the most regions used for this approach are the hypervariable regions of the 16S rRNA gene [382], which has been widely used in the microbiome studies. These regions are flanked by DNA sequences that are highly conserved, which facilitates the primers' design [383]. Several platforms from the different biotechnology companies are available for DNA sequencing, including the Roche 454 sequencing approach, Ion Torrent from LifeTechnologies, Illumina systems such as HiSeq and MiSeq and the recently developed PacBio and Heliscope [384]. The first mentioned systems require a pre-clonal local amplification of the initial template into polonies [385] in order to increase the signal-to-noise ratio, while PacBio and Heliscope have been classified as single molecule real-time sequencing (SMRT) and do not require any amplification step due to their capacity for single molecule detection [386]. Although the most used approach is the Illumina system, SMRT technology users are growing in number since some of its characteristics, such as the long-read data or the absence of GC bias, among others, make them suitable for some applications that the other sequence platforms cannot address [384].

Moreover, bioinformatical analysis by pipelines such as QIIME [387], mothur [388] or DADA2 [389] are used for extracting the compositional information from the raw sequence. Currently, the most common analyses consist of operational taxonomic units (OTUs) construction based on the similarity between the sequences (usually threshold of 97% of similarity), which reduces the possible impact of technical variation and the computational requirements [390]. However, recently, new denoising methods, such as DADA2, have appeared, which are based on the amplicon sequence variants (ASVs). These permit the improvement in the reproducibility and comparability of microbiome studies [391]. Further analyses are performed to characterize the bacterial community and provide information about the composition, alpha and beta diversity, among others. **Table 3** describes the main characteristics of a microbial population that are described in most microbiome studies. Although some authors have recently suggested that a correct analysis of 16S rRNA gene amplicon would permit taxonomic identification at strain level [392], it is still commonly accepted that this approach offers reliable assignation up to genus level.

Table 3. Terms commonly used in microbiome studies

Term	Brief description
Microbiota	All the microorganisms (bacteria, viruses, archaea, etc.) that habit a specific site or habitat.
Microbiome	The combined genetic material of the microorganisms in the specific environment.
Alpha-diversity	Indices used to characterize a community (e.g. richness, evenness). Expressed by Chao, Shannon, Simpson index, etc.
Richness	The number of the different species of a community.
Diversity	A mathematical index that consider the number of different species and their proportion within a microbial community.
Beta-diversity	Indices used to compare communities based on the membership and the structure of them. The most used representation is the principal coordinate analysis (PCoA) and non-metric multidimensional scaling (NMDS).

Table modified from Costa et al. [376].

The selection of the bioinformatical approach have been described to impact on the microbiome studies results [393,394]. The main algorithms and pipelines used in microbiome studies are listed in Table 4. While QIIME1 has been one of the most used platforms for microbiome studies [395], it has been partially replaced by other algorithms such as DADA. It has showed the best sensitivity and resolution in the comparison of the main bioinformatic approaches for microbiome data [393] based on 16S rRNA amplicon sequencing and it was the algorithm that was used in the studies described in the present thesis. However, some authors have highlighted that while DADA algorithm has been shown to drastically improve the analysis results of Illumina data, it lacks proper evaluation and parameters optimization in other sequencing techniques such as Ion Torrent [394].

Table 4. Comparison among 16S rRNA amplicon analysis tools.

	QIIME (used in this thesis)	**UPARSE**	**MOTHUR**	**DADA** (used in this thesis)	**MED**
Core algorithm		UPARSE-OUT UPARSE-REF	DOTUR SONS TreeClimber LIBSUFF UniFrac	DADA Divisive Amplicon Denoising algorithm Meedleman-Wunsch algorithm	MED
Short description	Pipeline for microbiome analysis from raw sequencing data through graphics and statistical analysis (demultiplexing, quality filtering, OUT picking taxonomical assignment, phylogenetics, visualization, etc.	Approach for producing clusters (OTUs) from sequencing reads of markers genes.	Algorithms from previous tools and additional features such as ecological parameters, visualization, and screening sequence collections.	DADA 2 (open source R package) that implements amplicon sequence variant (ASV) workflow, including filtering, dereplication, sample inference, chimera identification, merging of paired ends, etc.	An algorithm extending the principals of oligotyping to entire marker gene data sets into ecologically meaningful and phylogenetically homogenous units.
Availability	Open source	Tutorials	Open source	Open source and tutorials	Basic command lines and tutorials

Modified from Niu et al. [396]. QIIME1 and DADA2 pipeline (DADA algorithm) were used in the studies described in this dissertation.

Due to the problems of targeted amplicon sequencing, metagenomic analysis or 'shotgun sequencing' is used to sequence the DNA directly from the sample without the requirement of the previous PCR amplification, which eliminates the bias that it could introduce [397].

The most important limitation of these techniques based on DNA sequencing lies in the fact that the presence of a specific sequence does not imply the viability of these microbes, which would be important in some analyses (e.g. placental and amniotic fluid microbiome studies), and that they provide information about the relative abundance of the detected taxa in a sample, not absolute numbers of them. Furthermore, several authors have suggested that there remain methodological issues to resolve in the field of sequencing-based technologies, mainly due to the effect that the different DNA extraction kits and next-generation sequencing tools could have in microbiome studies [398]. To avoid the problem of the viability of the bacteria found in the samples, the sequencing of the messenger ribonucleic acid (RNA), known as transcriptomics, has also been developed [399]. However, strategies to adapt the DNA-based technologies have also been developed to make them suitable to discriminate the total and the viable microbes in a sample, such as the use of propidium monoazide (PMA), which intercalates into double-stranded DNA, preventing it from being amplified by PCR to remove free DNA from dead microbes [400].

Recently, single-cell genomics has been proposed as a new tool for microbiome characterization [401]. However, this approach is still poorly developed, since several technical issues need to be addressed, including the improvement of microfluidic technologies and the systems of controls to avoid the contamination in results [402].

Other methodologies

Some studies have shown that not only microbial composition define the properties and host–microbiome interaction, but also the functionality of the community may play a role in this relation. For this reason, some studies have been performed focused on the analysis of the bacterial-derived products using metabolomics, which allows the identification and even the quantification of these metabolites using mass spectrometry or nuclear magnetic resonance [403,404]. These techniques have also been used for the identification of the bacterial proteins in the samples [405,406].

Future perspectives

Although microbiological technologies have been developed in recent years, some aspects need to be improved. These include the establishment of bioinformatical tools that permit easy and standardized pipelines to analyse the large amount of data resulting from the omics technologies. In this regard, it is essential to develop specific pipelines for the identification and diminishment of contamination, which is a common problem in microbiome studies, especially those focused on the low-biomass environments, such as placenta or amniotic fluid [166,167]. For this reason, a significant amount of effort has been expended in identifying new techniques that can reduce the amount of false positive results with regard to microbiota analysis, and also facilitate contaminant detection in samples with a low microbial biomass [407,408]. Other factors that are receiving attention in microbiome research include the reduction of the effect of host DNA in the sequencing depth and the improvement of experimental techniques, which increase the integrity of microbes during sample collection and storage, among others [390]. The design of computational tools that facilitate the interconnection of the results obtained from different approaches would be especially relevant for advances in the knowledge of the role of microbiota in human health.

Moreover, the development of new tools for the study of molecular mechanisms that mediate the host–microbiome relationship was also required. In this regard, especially valuable has been the analysis in germ-free mice and the faecal transplantation experiments, which have provided most of the knowledge in the field [120]. However, animal studies have been reported to have some inherent disadvantages, including the lack of colonization of the total human microbiota in mice [27] and the genetic, behavioural, physiological and anatomical differences between host species [409].

For these reasons, great efforts have been made to develop other models that allow the mimicking of the gut environment and the relations between microbes and the different cellular types. Thus, the co-cultures of different cell types in special systems such as Transwell® are expanding their use in the exposure of studied analytes or microbes [410,411]. Indeed, Transwell® systems with apical and basolateral polarization enable studies of bacterial adhesion to the epithelium as well as bacterial transport [412]. Among the cell lines in *in vitro* studies, the most used are Caco-2, HT-29 and T84, all of them being derived from human colon cancer. Although transformed

cell lines have been considered the most cost-effective approach, primary cell lines are often considered to be more biologically and physiologically similar to *in vivo* models, but more difficult to maintain and to obtain reproducible results. The main disadvantage of the cell line culture is that the intestinal epithelium is composed of several cell types and the conclusions of these studies need to be confirmed by other technologies [413].

Recently, the organoids technology has been developed as an improvement on these co-culture systems. Organoids are primary human intestinal epithelium derived from inducible pluripotent stem cells (iPSCs). Despite the great advantage of this system compared to previous approaches, the 3D culture in microbiological studies is still difficult to develop, mainly due to the position of the lumen inside the spherical structure [414]. However, in the last few years, improvements in the technology have been achieved, including the 2D organization [415], the microinjection in the sphere [416] and the co-culturing with immune cells or neurons [417]. In addition to this, the most recent system is the one known as organ-on-a-chip devices, which mimic complex multilayer systems found *in vivo* using microfluidics [418]. Pearce et al. reviewed the advantage and disadvantage of these systems [413]. In general, *ex vivo* models are more complex and more physiologically relevant, but they are still difficult to work with and less cost-effective than the *in vitro* models, such as the cell line culture. Thus, new advances in the biotechnology of cell models are required to improve the current systems in order to better mimic the host environment and reduce the complexity and the cost of these new methodologies. Indeed, these new techniques would allow individualized models to be designed that provide input more clinically relevant, which will lead to personalized medicine in the future.

STUDY DESING AND OBJECTIVES

RATIONALE OF THE THESIS

Recent years have seen important advances in the field of nutrition and personalized medicine, partly due to the inclusion of other practices such as microbial ecology and nutritional genomics. Furthermore, the development of several 'omics' technologies, including metabolomics or transcriptomics, has provided new approaches in their study. The recent sequencing technologies and the developed bioinformatical tools have facilitated the analysis of the human microbiome and revealed its essential role in the host development, especially during early life. Several studies have highlighted the possible relation between shifts in the microbial colonization and immune-based diseases, such as obesity, diabetes, allergy or inflammatory bowel disease later in life [72,419].

However, most of these evidences have been found in associational studies and the possible mechanisms behind these observations are still unknown, despite the results from animal studies that suggest a possible relation between alterations in the colonization process and the immune system maturation [334–336,338–340]. Based on this hypothesis, practices that disrupt the contact between microbes and the neonate during the first years of life could impede the correct establishment of the symbiotic relationship between the microbial community and the host.

In this regard, it has been suggested that perinatal factors could modify this process of microbiota acquisition and, therefore, the immune system development, with long-lasting consequences. However, data about the impact of perinatal factors, such as maternal diet, antibiotic exposure, gestational age, and lifestyle, among others, on maternal and neonatal microbiota and infant development, are still limited. Since maternal microbiota is the first inoculum for the infant colonization, it is a key variable that contributes to these observations and we consider it essential to elucidate the perinatal factors that could affect the maternal microbiota composition and their transference to the neonate.

STUDY DESING AND PARTICIPANTS

The results of all the projects included in this thesis are based on the observational prospective longitudinal MAMI study (The power of maternal microbes on infant health). MAMI is a mother-infant birth cohort from the Spanish-Mediterranean area who were enrolled at the end of pregnancy and follow-up during the first two years of life [420]. The overall objective of the study is to analyse the early microbial exposition, focusing on the maternal microbiota, shaping the neonatal oral and intestinal microbiota development and the immune system maturation to determine the impact of perinatal and environmental factors on maternal-neonatal microbiota [420].

Participants

The hospitals that participate in the MAMI project (registration number 2015/0024) are Hospital Universitario y Politécnico La Fe, Hospital Clinico Universitario de Valencia, Atención Primaria Comunidad Valencia, and CEIC-Parc de Salut MAR and approvement by Hospital ethics committees from all those centres were obtained. The study is registered on the Clinical- Trial.gov platform (NCT03552939).

A total of 250 mothers-infant dyads were recruited in the hospitals via gynaecologist and midwives and, in the primary health care units. The recruitment of the participants started at 2015 and the collection of all follow-ups finished in early 2019. All the mothers received oral and written information about the study and verbal and written consent were provided. In order to limit the number of drops-off during the following period, three manners of participation. Mothers that decided to participate only at delivery without following collection points, mothers recruited at delivery and who decided to continue in the study, and mothers who were recruited after delivery during the first week post-partum (no samples at delivery available).

Data and sample collection

Perinatal information as well as data from gestation were obtained from clinical records collected by clinicians at hospitals and primary health centres. Delivery data was extracted from the intrapartum medical records by the medical staff and

gynaecology team. Additionally, infant health outcomes and growth, including weight, height and diseases were provided by the paediatricians. Dietary intake during pregnancy and follow-up period were assessed by a comprehensive 140-item food frequency questionnaire (FFQ) [421] validated in adult population [422]. Furthermore, a 14-item validated test (PREDIMED: PREvención con DIeta MEDiterránea) to determine the adherence of participants to the Mediterranean diet [423]. The mothers were asked to fill the questionnaire within 24h post birth with the dietary intake during pregnancy, and then, during the follow-up period (12 and 24 months).

The biological samples were collected at different time points (**Fig. 4**). Samples at delivery, including faecal swabs, saliva, amniotic fluid, placenta and vermix were collected at delivery room by the clinical staff. At the following time points faecal sample, urine, breastmilk and saliva were collected by collaborators of primary health care units or by participants mothers who received verbal and written instructions.

All samples collection was centralized at the biobank "Biobanco para la Investigación Biomédica y en Salud Pública de la Comunidad Valenciana (IBSP-CV)" and the samples stored in sterile cryovials under their standardized protocols. Then, the specific sample were shipped at IATA-CSIC for the analysis where they were stored at -80ºC.

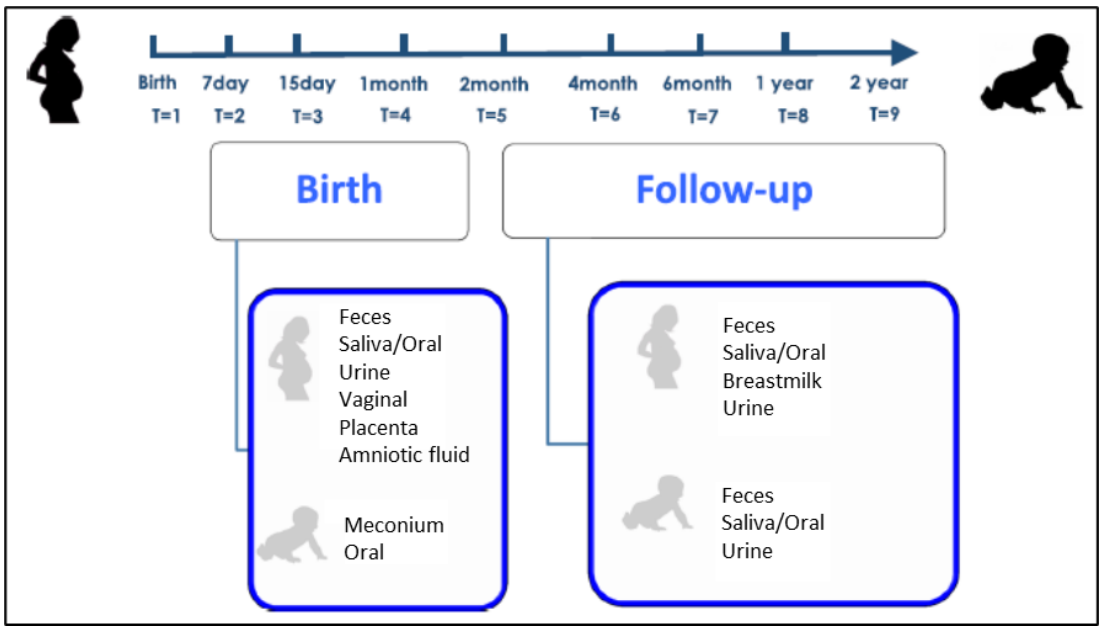


Figure 4. Flow chart of sampling procedure (modified from García-Mantrana et al. [420], with permission).

OBJECTIVES OF THE THESIS

The overarching aim of this project was to determine the impacts of various perinatal factors, including the mode and place of delivery, antibiotic use, maternal diet and gestational age, on the composition of the maternal–neonatal microbiota as well as to examine their influence on mothers' and neonates' health outcomes (**Fig. 5**).

The specific aims of the individual chapters were as follows.

I – To determine the association between the maternal–neonatal microbiota at the time of birth and the biochemical markers in the blood, the immunological profile of the amniotic fluid and the placental metabolic status.

II – To study the influence of various perinatal factors, including the mode of delivery, antibiotic administration, ruptures of membranes, gestational age and instrumentalisation, on the maternal microbiota as well as their association with maternal weight retention during the post-partum period.

III –To evaluate the possible associations between maternal dietary intake during pregnancy and neonatal microbiota at birth, and to ascertain the relation with maternal intestinal markers.

IV – To assess whether the maternal gut microbiota is shaped by both the mother's diet and specific nutritional components during pregnancy and to evaluate the potential impact of it on infant development during the first 18 months of life.

V – To investigate the impacts of various birth-related factors, including the place and mode of delivery, on early gut colonisation during the first month of life as well as on infant growth during the first 18 months. Additionally, to determine the potential biological mechanisms involved using *in vitro* gut models to study the impacts of distinct microbiota patterns on both intestinal function and innate immune system maturation.

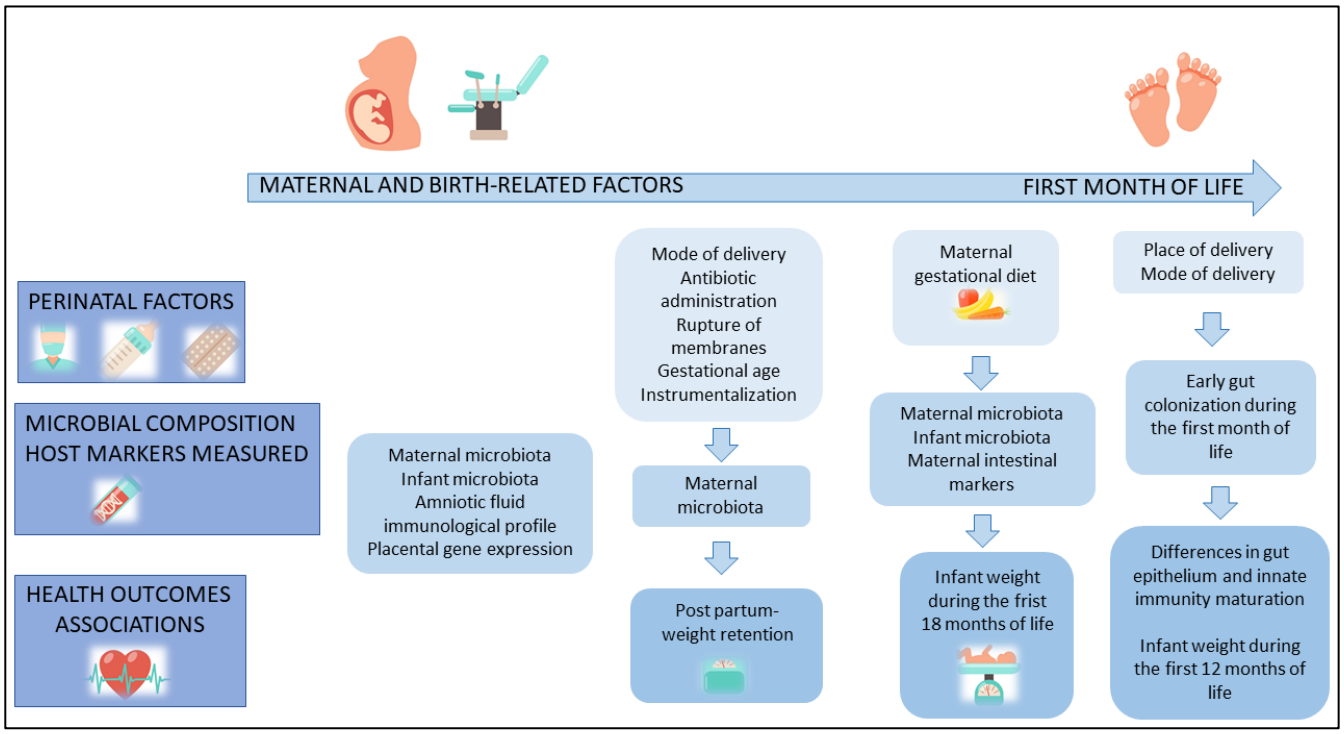


Figure 5. Summary of the main research objectives of the present thesis (own drawing).

CHAPTER I

MATERNAL MICROBIOTA IS RELATED TO AMNIOTIC FLUID CYTOKINE PROFILE AND PLACENTA GENE EXPRESSION

CHAPTER I – MATERNAL MICROBIOTA IS RELATED TO AMNIOTIC FLUID CYTOKINE PROFILE AND PLACENTA GENE EXPRESSION

Manuscript in preparation.

ABSTRACT

The immune system starts to develop early in foetal life. The microbial changes in the gut as well as the immune and metabolic adaptations that occur during pregnancy may influence neonatal immune training and maturation. However, little is currently known about the mechanisms that affect the interplay between the maternal microbiota and foetal immune development.

To determine the associations between the maternal–neonatal microbiota at the time of birth and various blood biochemical markers, the amniotic fluid immunological profile and the placental metabolic status.

In a cross-sectional study, the profiles of maternal–neonatal faecal microbiota at the time of birth (n=15, C-caesarean deliveries) were assessed by means of 16S rRNA gene sequencing. The concentrations of several cytokines in sterile amniotic fluid collected prior to delivery were determined, as were some placenta metabolic and antioxidant status-related gene expression levels. Furthermore, maternal–neonatal clinical and anthropometric data, in addition to blood biochemical makers such as the total cholesterol, triglyceride and glucose levels, were collected.

The maternal microbiota was associated with specific metabolic parameters. Higher serum cholesterol and triglyceride levels were related to Firmicutes such as *Roseburia* and *Bacteroides* genera, and several Ruminococcaceae groups. Additionally, the maternal microbial profile was also related to the amniotic fluid immune pattern. *Faecalibacterium*, *Roseburia* and *Lachnospira*, among others, were positively associated with IL-2, TNF-alpha, IL-1beta, IL-5 and IL-17A. Some of these associations, especially the association with IL-2, were reflected in the composition of the neonatal microbiota. These pro-inflammatory cytokines were negatively associated with the maternal taxa, including *Peptoniphilus*, *Prevotella*, *Finegoldia*, *Porphyromonas* or *Corynebacterium* genera. The other cytokine pattern, which was composed of IL-4, IL-13, IL-18 and IL-10, was associated with several Ruminococcaceae groups as well as with the *Ezakiella* and *Murdochiella* genera. This latter cytokine profile was also positively correlated with the placental mRNA expression of the glucose transporter *GLUT3* and the glutathione peroxidase (*GPX*)-

Our results suggested that the maternal microbiota was associated with both the immune status of the amniotic cavity and placental metabolic gene expression, thereby having possible consequences for neonatal microbial colonisation at the time of delivery and, therefore, for foetal and neonatal immune system development.

Keywords: amniotic fluid, microbiota, pregnancy, cytokines, neonate.

INTRODUCTION

A growing body of evidence has demonstrated the crucial role played by the early microbiota in relation to health outcomes later in life [72,424]; however, little is currently known about the mechanisms behind the association between the microbiota and health. The developmental origin of adult disease has been proposed in terms of an alteration in immune system maturation related to shifts in the composition and diversity of the microbiota [124,201,333]. From this perspective, several perinatal factors, including the mode of birth, maternal antibiotic intake, maternal diet and maternal body mass index (BMI), could shape the neonatal microbiota [206,217,281,425], which has implications for immune system development with regard to an imbalance between the Th1/Th2 and Treg responses [116,223,426]. In turn, this could lead to a higher risk of non-communicable diseases (NCDs) such as diabetes, allergy, and obesity [115,427–431].

Furthermore, it is commonly accepted that the maternal microbiota plays a crucial role in the initial neonatal microbial colonisation. However, only a few studies have focused on the maternal microbiota during pregnancy [131,132], which have been found to undergo changes in terms of their composition and diversity [132] as well as their immune, hormonal and metabolic processes [432–434]. Such changes could participate in the maintenance of pregnancy and/or appropriate foetal development, especially toward the end of gestation. Yet, the possible relation between these changes and the immune systems of pregnant women remains unknown.

Beyond the possible effects of the microbiota that may colonise the intrauterine cavity, bacteria-derived products, such as short-chain fatty acids (SCFA), have been shown to impact both gut homeostasis [345,435,436] and systemic health, including the immune [43] and neurological systems [437]. Some studies have proposed that these products could influence susceptibility to certain diseases through epigenetic markers that could modify the maturation of the immune system during the foetal stage [155,156].

Thus, the main aim of the present study was to determine the associations between the maternal–neonatal microbiota at the time of birth and various blood biochemical markers, the amniotic fluid immunological profile and the placental metabolic status. Furthermore, the potential associations with the neonatal microbiota at the time of birth were also analysed.

MATERIALS AND METHODS

Description of the study

A subset of 15 mother-neonates from the prospective and observational MAMI cohort enrolled during 2015-2017 [420], were selected for the study. Written consent was obtained from all participants in the MAMI All the participants in the MAMI project. The study is registered on the ClinicalTrial.gov platform, with the registration number NCT03552939.

Maternal and neonatal data were obtained at hospital, including clinical and anthropometrical data such as pregestational maternal body mass index (BMI), weight gain during pregnancy, antibiotic intake, as well as other variables. Neonatal characteristics were also collected. Mothers were classified according their weight gain during pregnancy following the Institute of Medicine for each BMI categories [438]. Only elective C-section deliveries were included in the study in order to avoid the delivery mode effect and also, due the "sterile collection" of the amniotic fluid.

Sample collection

Serum samples were obtained by trained medical staff during the last clinical check-up of the third trimester. Analytical assessment of total cholesterol, triglycerides, glucose, urea, haematocrit, haemoglobin (Hb) and creatinine were measured using the routine analysis at hospital facilities (University and Polytechnic hospital La Fe, Valencia, Spain) at 35-38 weeks of gestation. The oral glucose tolerance test, known as O'Sullivan test, was performed in the second trimester of the pregnancy by the medical staff (week 24-28 of gestation).

Amniotic fluid samples (approximately 10 mL) were collected by amniocentesis with sterile syringe before C-section surgery and stored at 80ºC immediately. Placental tissue was immediately sampled after delivery from the central area surrounding umbilical cord and they were immediately stored at -80ºC.

Maternal fecal samples at delivery were collected using a sterile cotton-tipped by trained clinical staff before birth at the entry of delivery room following protocol described previously [420] Neonatal fecal swabs were similarly collected immediately after neonate extraction.

All samples collection was centralized at the biobank "Biobanco para la Investigación Biomédica y en Salud Pública de la Comunidad Valenciana (IBSP-CV)" and the samples stored in sterile cryovials under their standardized protocols.

Faecal DNA extraction and sequencing

Master-Pure DNA Extraction Kit (Epicentre, Madison, WI, United States) was used for total DNA isolation from faecal swabs. An initial step of cell lysis was added to the manufacturer's instructions in order to improve the efficiency. Briefly, a chemical treatment with the lysis buffer provided in the kit, supplemented with lysozyme (20 mg/ml) and mutanloysin (5U/ml) was performed during 60 min at 37ºC, followed by a mechanically disruption step using 3-µm diameter glass beads 1 min at 6 m/s in a FastPrep 24-5g Homogenizer (MP Biomedicals). Resulted DNA was purified by DNA Purification Kit (Macherey-Nagel, Duren, Germany) and quantified by Qubit 2.0 Fluorometer (Life Technology, Carlsbad, CA, United States).

Total DNA was used to study the microbiota by sequencing the specific V3-V4 variable region of the 16S rRNA gene by Illumina protocol on a MiSeq-Illumina platform (FISABIO sequencing service, Valencia, Spain) as described in Selma-Royo et al. [439]. DADA2 pipeline [389] was followed for the quality-trimmed and filtering of the obtained sequences. Briefly, default quality filtering options were used, and the reads were trimmed at the 270^{th} and 210^{th} nucleotide in forward and reverse, respectively, after quality examination. Then, the reads were merged, and chimera removed following the mentioned protocol. Silva v132 database [440] was used for the taxonomic assignment with the specie level classification. Additional quality filters were assessed including the filtering of taxa occurring <5 reads in at least 20% of the samples and those that were present in less than 0.01% of the total read across all the samples. Besides this, sequences from contaminants and those classified as Cyanobacteria and Chloroplast, considered ingested plant material were also filtered. Samples with less than 1000 reads were also removed from the final analysis and bacterial counts were transformed to relative abundance.

Amniotic fluid analysis

Immunological profile

Amniotic fluid samples were thawed immediately before use, vortexed and centrifuged 5 min at 4,000 rpm. Supernatant was diluted 1:50 for cytokine assay in

sample dilution buffer. The following cytokines were measured in amniotic fluid using a premixed Luminex Human Magnetic Assay (13-Plex) LXSAHM-13 (R&D systems, Inc., MN, USA) according to the manufacturer's instructions: interleukin (IL)-2, IL-8, IL-1 betta, IL-13, IL-18, IL-5, TNF-alpha, IFN-gamma, IL-10, IL-17A, IL-4, IL-6, IL-1 alpha. The plate was read on the Bio-plex 200 array reader (BioRad, Hercules, CA, USA). Fi

Microbial SCFA profile

SCFA extraction from amniotic fluid were performed by a modification of a protocol for mammalian feces previously published [441]. Briefly, the modifications were as follows: amniotic fluid aliquots (500 µl) were transferred to 2 ml tubes in presence of 1 ml of isobutanol/H_2O followed by 2 vertexing pulses of 30s. Then, the tubes were centrifuged 21,000g for 5 min and 675 ul of the supernatant were transferred to a new tube when the protocol was continued with the experimental procedures described in Takeshi et al. [441]. The samples were processed the same day of the injection in the gas chromatography mass spectrograph (GC-MS). A blank control and standards of the main SCFA (formic, acetic, butyratic, isobutyratic, propionic, isopropionic, valeraric and isovaleric acid) at 10, 25, 50, 100 ppm were also prepared in the same way. Separation of SCFAs was performed on an Agilent 7890B GC/5977 MSD (Agilent, Santa Clara, CA, USA) using a Agilent HP-5 ms column (30 m × 0.25 mm × 0.25 µm). The injector temperature was set at 260°C in split mode (10:1) and an injection volume of 1 µL. The column temperature was initially 40°C for 5 min and then increased to 120°C at 10°C/min and then ramped to 310°C at 40°C/min and held for 2 min. The MS transfer line was maintained at 280°C and the ion source at 230°C.

Placenta tissue specific gene-expression

A placental cotyledon was collected to a region near of the umbilical cord. For the maternal tissue RNA extraction, basal plate and chorionic surface were removed and villus tissue was kept for further analysis. Villus tissue was then manual mechanically disrupted with pestle and mortar in liquid nitrogen. RNA was isolated from the resulted homogenised placental tissue (100-200 mg) following a protocol modified from Chomczynski et. al [442]. Briefly, tissue powder was further homogenized in presence of 1ml of Trizol reagent (Invitrogen, Carlsbad, CA, USA) as well as stainless grinding balls of 4 mm (Restsch, Haan, Germany) by vertexing 30s at 6 m/s in a FastPrep 24-5g Homogenizer (MP Biomedicals). Then, 200 µl of chloroform (PanReac AppliChem,

Darmstadt, Germany) were added and the mixture was centrifuged at 11,000g for 15 min at 4ºC. The RNA was precipitated from the aqueous phase by mixing with isopropyl alcohol (Sigma-Aldrich, San Luis, MI, USA) and incubate the resulted mixing at room temperature for 10 min. The RNA was collected by centrifugation at 11,000g for 10 min at 4ºC and two times washed with 75% ethanol, followed by a centrifugation at 7,000g for 5 min at 4ºC. The ethanol was completely air-dried before further analysis and the resulted RNA was resuspended in RNAse/DNAse free water. Finally, RNA was quantified using a NanoDrop (ThemoFisher Scientific, Waltham, MA, USA).

Total RNA was diluted with RNAse/DNse free water to 500 ng/µl and converted to cDNA by Transcriptor First Strand cDNA Synthesis Kit (Roche, Basilea Switzerland) using both random primers and OligoDT, following manufacturer instructions. RT-qPCR reaction was performed using 1 µl of resulted cDNA reaction and 0.25 µM of the specific primers using Lightcycler 480 SYBR Green I master mix (Roche, Basilea, Switzerland). Plates were read in the LightCycler 480 (Roche, Basilea, Switzerland) at annealing temperature of 58ºC. Sequences of the primers used in the study are listed in **Supplementary file I-1**. Due to their importance for placenta nutrients transport and antioxidant metabolism the following genes were selected to study: Actin beta (*ACTB*) expression was used to the assessment of the relative expression.

Statistical analysis

Calypso online platform (v. 8.72) [443], SPSS software [444] and Rstudio environment [445] were used for the statistical analysis. Vegan package was used for alpha diversity determination including Shannon index for diversity measure and Chao1 as richness index. Heatmaps showing the Spearman rank correlation between taxonomic groups and amniotic fluid cytokines and clinical parameters were performed through "ggplot" package [446]. Other spearman correlations were assessed by the SPSS software v.25. Calypso platform were used for the network analysis of the relations between maternal microbiota, clinical parameters, and some amniotic fluid cytokines. For the gene expression data analysis, expression value for each gene was normalized by the ACTB expression.

RESULTS

Description of participants

A total of 15 mothers was recruited in the last week of gestation. Anthropometrical and clinical characteristics of study participants are showed in **Table I-1**. Most of women (75%, n=11) were classified as normal weight (BMI<25 Kg/cm^2) and 50 % (n=7) gained excessive weight during pregnancy according to pregestational BMI and the recommendations by the Institute of medicine [438]. Biochemical markers in blood including serum cholesterol, glucose or triglycerides among others as well as the oral glucose tolerance test (O'Sullivan index) were below pathological thresholds and were considered normal for pregnant women [447].

Table I-1. Participants characteristics (n=15)

Maternal data	
Maternal weight (Kg)	63.97 ± 20.11
Pre-gestational BMI (Kg/m^2)	24.67 ± 7.89
NW	75 %
OW	18.75 %
Maternal age (years)	32.87 ± 9.92
Gestational weight gain (Kg)	13.92 ± 6.51
Recommended	50.0 %
Excess	31.25 %
Lower	12.5 %
Smoking habits	
Yes	18.75%
No	81.25%
Maternal biochemical markers	
O'Sullivan index	123.70 ± 57.21
Cholesterol (mg/dl)	243.33 ± 1165.47
Triglycerides (mg/dl)	231.42 ± 113.42
Haematocrit (% whole blood)	38.47 ± 13.37
Hb (g/dl)	12.63 ± 4.40
Glucose (mg/dl)	70.07 ± 29.38
Urea	20.77 ± 9.75
Creatinine (mg/dl)	0.57 ± 0.22
Neonatal data	
Gestational age (weeks)	38 ± 15
Neonatal weight (g)	3120 ± 500
Neonatal sex	

Female	50%
Male	43.75%
Amniotic fluid cytokines (pg/ml)	
IL-8	740.44 ± 422.6 (1.32 - 1059.99)
IL-1 betta	13.93 ± 1.57 (4.99 - 3911.24)
IL-13	522.67 ± 76.85 (121.45 - 90153.43)
IL-18	30.73 ± 8.24 (16.74 – 13498.56)
IL-2	91.19 ± 12.97 (8.61 – 7620.16)
IL-5	2.74 ± 0.59 (2.10 – 1581.98)
TNF-alpha	12.46 ± 1.76 (2.82 – 2159.72)
IFN-gamma	66.2 ± 17.38 (13.00 – 12099.13)
IL-10	2.89 ± 0.84 (0.91 – 880.07)
IL-17A	6.01 ± 2.45 (11.98 – 3064.09)
IL-4	81.34 ± 9.52 (14.30 – 3828.87)
IL-6	340.47 ± 340.67 (1.42 – 1095.97)
IL-1 alpha	93.57 ± 65.29

Mothers were classified according body mass index (BMI) and gestational weight gain following the categories reported in [438]. Clinical data were extracted by the clinicians by the clinical records of the last trimester. Maternal clinical data was extracted by clinical records. Maternal biochemical makers were measured following hospital protocols at 35-38 weeks of gestation. Limits of quantifications are expressed in brackets for each measured cytokine.

We found some clinical characteristics which were related between them (**Supplementary file I-2**). Pre-gestational BMI was positively correlated with O'Sullivan index (rho= 0.62, *p=<0.001*) and with maternal age (rho= 0.28, *p=0.032*) and maternal weight gain during pregnancy was negatively correlated to pre-gestational BMI (rho= -0.71, p<0.001) and O'Sullivan index (rho= -0.49, *p=0.001*). O'Sullivan index was found a good marker of metabolic status in pregnancy since it was correlated to glucose (rho= 0.41, *p=0.062*) but also showed a negative association with cholesterol (rho= -0.39, *p=0.035*), triglycerides (rho= -0.63, *p=0.001*) and creatinine (rho= -0.41, *p=0.007*).

Maternal microbiota was related with some biochemical markers in blood

Maternal microbiota at delivery was characterized by the dominance of the four main phylum of human gut microbiota, Firmicutes (61.2 %, 53.91-64.49; median, IQR),

Bacteroidetes (27.75 %, 18.64-32.23; median, IQR), Proteobacteria (5.31 %, 2.86-10, median, IQR) and Actinobacteria (4.07 %, 3.16-5.63, median, IQR) (**Fig. I-1, A**).

We found significant associations between biochemical markers and maternal microbiota composition at delivery (**Fig. I-1, B**). Cholesterol was positively correlated to some Firmicutes genera including *Roseburia* (rho= 0.613, *p=0.034*), *Bacteroides* (rho= 0.55 *p=0.062*) and *Parabacteroides* (R=, p=) as well as several Lachnospiraceae family groups, including *Lachnospiraceae_UCG_004* (rho= 0.62, *p=0.033*) and *Lachnospiraceae_ND3007_group* (rho= 0.58, *p=0.049*). Similarly, triglyceride levels were positively associated with *Lachnospir*a genus (rho= 0.55, *p=0.062*) and negatively related to *Prevotella_9* (rho= -0.68, *p=0.014*) and *Corynebacterium* (rho= -0.61, *p=0.037*) genera. No significantly associations were found between microbiota diversity and any of the measured clinical parameters. Furthermore, higher neonatal weight was positive related to *Ezakiella* (rho= 0.52, *p=0.05*), *Murdochiella* (rho= 0.54, *p=0.036*) and *Dialister* (rho= 0.72, *p=0.002*) genera.

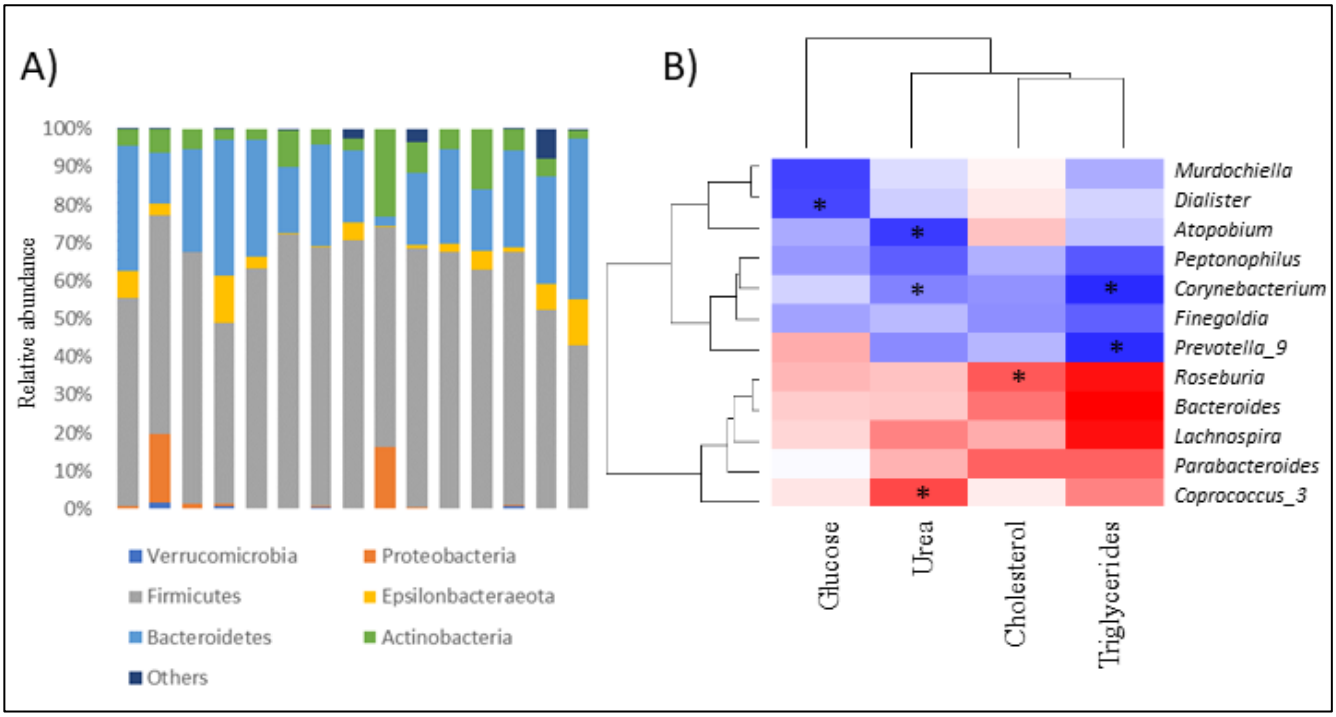


Figure I-1. Maternal microbiota at delivery time. A) Maternal microbiota composition at phylum level at delivery mode of each participant of the study. **B)** Heatmap of the Spearman correlations between clinical parameters measured in maternal blood in last trimester and maternal microbiota genera at delivery. Only relevant relations were shown. Red color represents the positive correlations whereas blue color show negative relations. Bacterial genera and clinical variables were grouped at the square edges. Symbols marked the significance of the association. * p<0.05

Maternal microbiota composition was related to amniotic fluid cytokines at delivery

Amniotic fluid cytokine profiling is detailed in **Table I-1**. We found a cluster of microbiota genera significantly associated to a group of cytokines in amniotic fluid, composed by interleukin (IL)-2, IL-1b, IL-5, TNF-alpha and IL-17A (**Fig I-2**). Amplicon sequence variant (ASV) from *Akkermansia, Butyricicoccus, Roseburia, Faecalibacterium, Lachnospira* or *Bacteroides* and Parabacteroides were positively correlated to this cluster of amniotic fluid (AF) cytokines. Among these, IL-2 and IL17A were the cytokines most significant associated with maternal intestinal microbiota. Thus, IL-2 was positively correlated *Lachnoclostridum* genus (rho= 0.61, *p=0.016*), *Faecalibacterium* (rho= 0.62, *p=0.055*), *Roseburia* (rho= 0.68, *p=0.005*) or *Lachnospira* (rho= 0.53, *p=0.044*) and negatively correlated to *Peptoniphilus* (rho= -0.62, *p=0.016*), *Finegoldia* (rho= -0.55, *p=0.032*), *Anaerococcus* (rho= -0.48, *p=0.069*). Indeed, this cluster of cytokines was observed to be negatively associated to another microbial group dominated by *Peptoniphilus*, *Corynebacterium*, *Porphyromonas*, *Dialister*, *Prevotella* or *Finegoldia* genera. Another group of cytokines composed by IL-4, IL-13 IFN-gamma was related to *Porphyromonas*, *Corynebacterium*, *Ezakiella* and several Ruminococcaceae family genera such as Ruminococcaceae_UCG-002 (rho= 0.63, *p=0.011*), Ruminococcaceae_UCG-005 (rho= 0.60, *p=0.019*) and Ruminococcaceae_UCG-014 (rho= 0.59, *p=0.021*) genera. This group follows a similar pattern of associations with IL-10 and IL-18 in amniotic fluid. A positive relation was found between IL-10 levels and *Murdochiella* (rho= 0.65, *p=0.009*), *Negativicoccus* (rho= 0.61, *p=0.015*), *Ezakiella* (rho= 0.56, *p=0.029*) genera and the mentioned groups of Ruminococcaceae family.

Indeed, we found a positive correlation between serum cholesterol and amniotic IL-2 (rho= 0.52, *p=0.012*) and IL-5 (rho= 0.48, *p=0.021*). On the contrary, serum cholesterol and triglyceride levels had a negative association with IL-6 levels (rho= -0.42, *p=0.046* and rho= -0.56, *p=0.005*, respectively) (**Supplementary file I-3**).

In terms of alpha-diversity, positive associations were found between microbiota richness measured by Chao1 index and the groups of cytokines IL-10 (rho= 0.52, *p=0.046*), IL-13 (rho= 0.55, *p=0.034*), and IFN-gamma (rho= 0.63, *p=0.012*). IL-4 showed the same trend than those but with no significant association (rho= 0.49, *p=0.062*) (**Supplementary file I-3**).

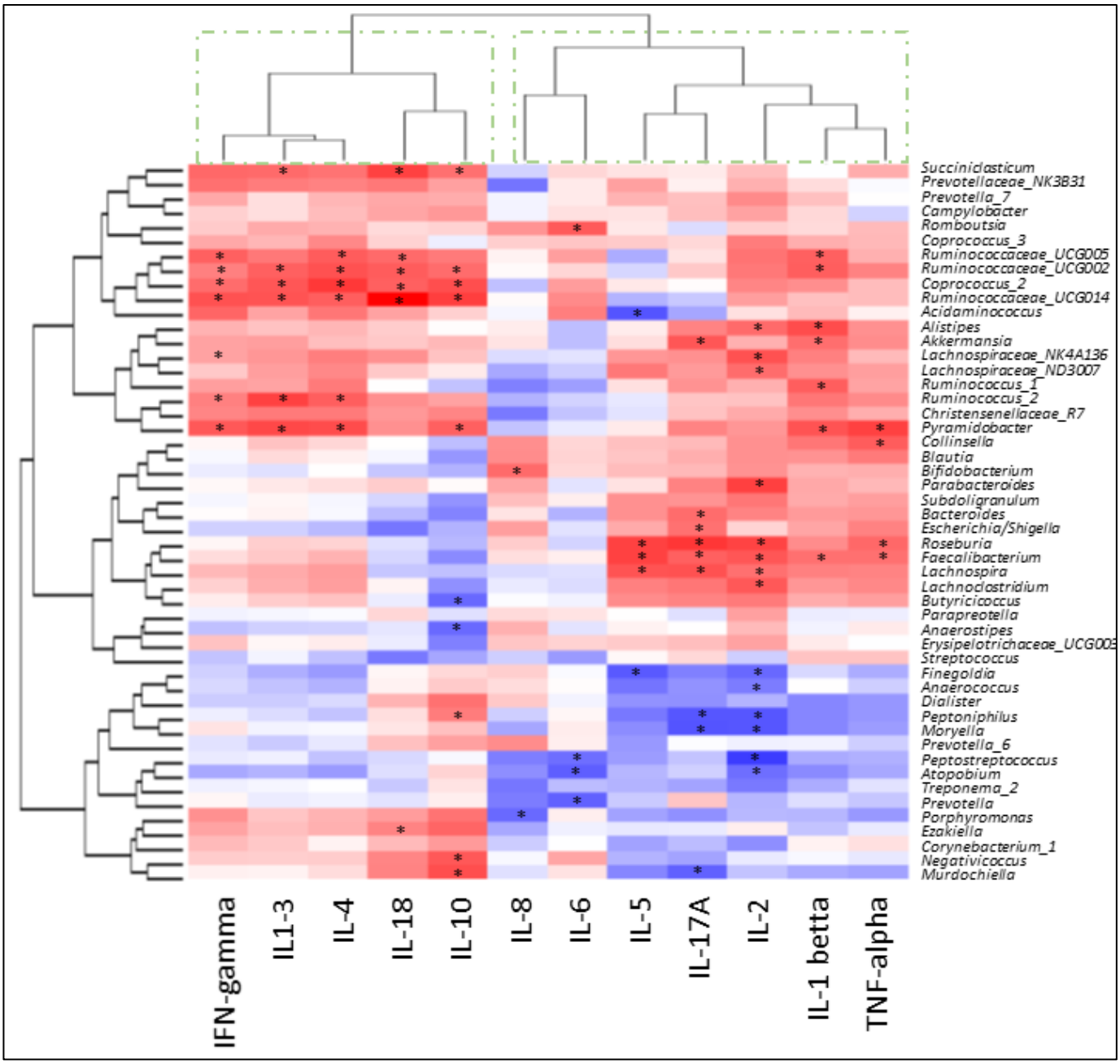


Figure I-2. Maternal microbiota was associated with cytokines concentration in amniotic fluid at delivery. Heatmap of spearman correlations between amniotic fluid cytokines at delivery and maternal microbiota at genus level at birth. Significant correlations ($p<0.05$) were marked by an asterisk (*). Red color represents the positive correlations whereas blue color shown negative relations. The top 50 genera in relative abundance were shown in the graph. Bacterial genera and amniotic fluid cytokines were grouped at the square edges.

Maternal biochemical markers and microbiota related to gene expression on placenta

A positive association was found between placental mRNA expression of glucose transporter *(GLUT) 3* and glutathione peroxidase (*GPX*) and the cluster of

cytokines composed by IL-4 (rho= 0.64, *p=0.013* and rho= 0.65, *p=0.011*), IL-13 (rho= 0.62, *p=0.019* and rho= 0.69, *p=0.007*), IFN-gamma (rho= 0.62, *p=0.018* and rho= 0.56, *p=0.037*), IL-10 (rho= 0.69, *p=0.007* and rho= 0.74, *p=0.003*), IL-18 (rho= 0.76, *p=0.002* and rho= 0.77, *p=0.001*), respectively (**Table I-2**).

The groups observed to be correlated to these cytokines were also associated to GLUT3 including several genera from Ruminococcaceae family such as *Ruminococcaceae_014* (rho= 0.71, *p=0.005*), *Ruminococcaceae_002* (rho= 0.56, *p=0.025*) and *Ruminococcaceae_005* (rho= 0.55, *p=0.039*), as well as *Coproccocus* (rho =0.66, *p=0.010*) and *Negativicoccus* (rho= 0.61, *p=0.020*). The same groups were also associated to the expression of the antioxidant enzyme GPX (**Table I-2**). No associations were found between maternal intestinal microbiota alpha diversity and the expression of the assessed genes.

In general, we found a relation between the triglycerides and cholesterol serum concentration, the amniotic fluid cytokines profile and the expression of these metabolism-related genes, both GLUT3 and GPX. This relationship is also associated with changes in maternal intestinal microbiota at delivery (**Fig. I-3**). Indeed, maternal blood cholesterol followed a negative relation to catalase gene (CAT) expression (rho= -0.59, *p=0.042*). On the other hand, the expression of hypoxia induced factor (HIF-1a) gene expression showed a positive correlation to pre-gestational BMI (rho= 0.829, *p=0.042*) and a tendency towards O'Sullivan Index (rho= 0.60, *p=0.053*).

Table I-2. Associations between placenta gene expression and clinical parameters measured, amniotic fluid cytokines and maternal microbiota at delivery

Gene	Clinical parameters	Amniotic fluid cytokines	Maternal microbiota
GLUT3		▪ IL13 (R=0.62, *p=0.019*) ▪ IL18 (R=0.76, *p=0.002*) ▪ IFN (R=0.62, *p=0.018*) ▪ IL10 (R=0.69, *p=0.007*) ▪ IL4 (R=0.64, *p=0.013*)	▪ Ruminococcaceae_UCG014 (R=0.71, *p=0.005*) ▪ Ruminococcaceae_UCG005 (R=0.56, *p=0.039*) ▪ *Negativicoccus* (R=0.61, *p=0.020*) ▪ *Coprococcus_2* (R=0.66, *p=0.010*) ▪ Ruminococcaceae_UCG002 (R=0.60, *p=0.025*) ▪ *Ruminiclostridium_5* (R=0.53, *p=0.05*) ▪ Lachnospiraceae_FCS020 (R=0.64, *p=0.014*) ▪ Lachnospiraceae_NK4A136 (R=0.57, *p=0.034*)
GPX		▪ IL13 (R=0.68, *p=0.007*) ▪ IL18 (R=0.77, *p=0.001*) ▪ IFN (R=0.56, *p=0.037*) ▪ IL10 (R=0.74, *p=0.003*) ▪ IL4 (R=0.65, *p=0.011*)	▪ *Ruminococcus_2* (R=0.57, *p=0.032*) ▪ Ruminococcaceae_UCG014 (R=0.71, *p=0.005*) ▪ *Negativicoccus* (R=0.55, *p=0.043*) ▪ *Coprococcus_2* (R=0.59, *p=0.027*) ▪ Ruminococcaceae_UCG002 (R=0.56, *p=0.036*)
CAT	▪ Cholesterol (R=-0.59, *p=0.042*)		▪ *Mobiluncus* (R=-0.56, *p=0.037*) ▪ *Romboutsia* (R=0.68, *p=0.008*) ▪ *Negativicoccus* (R=0.55, *p=0.041*)
HIF	▪ Pregestational BMI (R=0.57, *p=0.034*) ▪ Weight gain (R=-0.63, *p=0.016*) ▪ O'Sullivan index (R=0.60, *p=0.053*)		▪ *Prevotella_6* (R=0.55, *p=0.040*) ▪ *Akkermansia* (R=0.58, *p=0.030*)

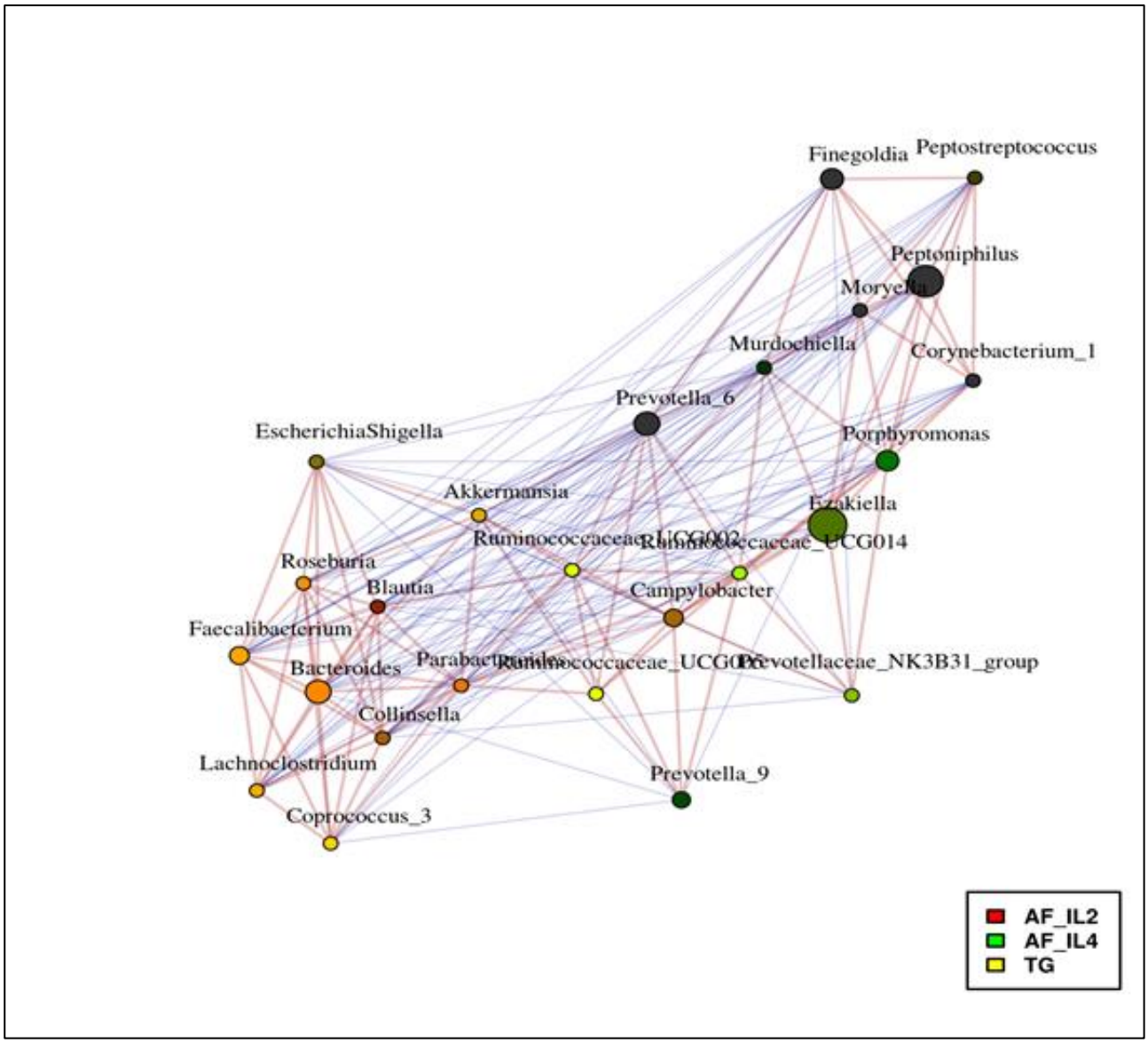


Figure I-3. Network of associations between maternal biochemical markers, amniotic fluid content and maternal microbiota at delivery. IL2 and IL4 were selected as representative of the two cytokines groups showed in the hierarchical clustering of the Fig.3 (edge at the top) with most of the significant relations with bacterial genera in the maternal microbiota. Furthermore, triglycerides (TG) concentration in maternal serum at third trimester was also included in the network. The colour of the nodes represents the association with some of the studied variables, IL2 (red), IL4 (green) and TG (yellow). Red colored lines link genera with positive correlations whereas those blue colored showed negative relations.

SCFA determination in amniotic fluid

No detectable levels of the measured SCFA were found in any of the studied amniotic fluid samples. Despite of we observed very low levels of acetic and iso-propionic acid in all the samples, they were below than those observed in blank injections.

Neonatal microbiota was related to amniotic fluid cytokines concentration

The cytokines in amniotic fluid with higher number of significant relations with neonatal microbiota were IL-17A and IL-18 (**Fig. I-4, A**). A group of taxa in neonatal microbiota composed by *Prevotella_9* (rho= -0.71, *p=0.004*) and Christensenellaceae_R7 (rho= -0.55, *p=0.043*) was negatively associated with amniotic fluid IL-17A. Similarly, IL-2 showed a negative relation with *Eggerthella* (rho= -0.61, *p=0.02*) and *Clostridium sensu stricto* (rho= -0.67, p= *0.008*) and a positive association with Bacteroides (rho= 0.54, *p=0.044*).

The pattern of associations between neonatal microbiota and cytokines are similar for IL-5, IL-2 and IL-17A (**Fig. I-4, B**). The other group of cytokines was composed by the IL-18. IL-10 and IL-4. Thus, IL-10 was negatively associated with Lactobacillus (rho= -0.55, *p=0.039*) in neonatal microbiota and IL-18 showed a positive relation to some groups from Ruminococcaceae family including Ruminococcaceae_UCG014 (rho= 0.58, *p=0.03*), Ruminoclostridium_5 (R=0.62, p=0.017) and Ruminiccocaceae_UCG002 (R=0.69, *p=0.006*).

Regarding placenta gene expression, only one significant association was found. *Bifidobacterium* relative abundance in neonatal microbiota was positively associated with the mRNA expression of *GPX* (rho= 0.65, *p=0.017*) and *CAT* (rho= 0.58, *p=0.039*).

In terms of alpha diversity of neonatal microbiota, no significant relations were found between any of the studied diversity index and measured cytokines in AF, maternal biochemical markers, or placenta gene expression.

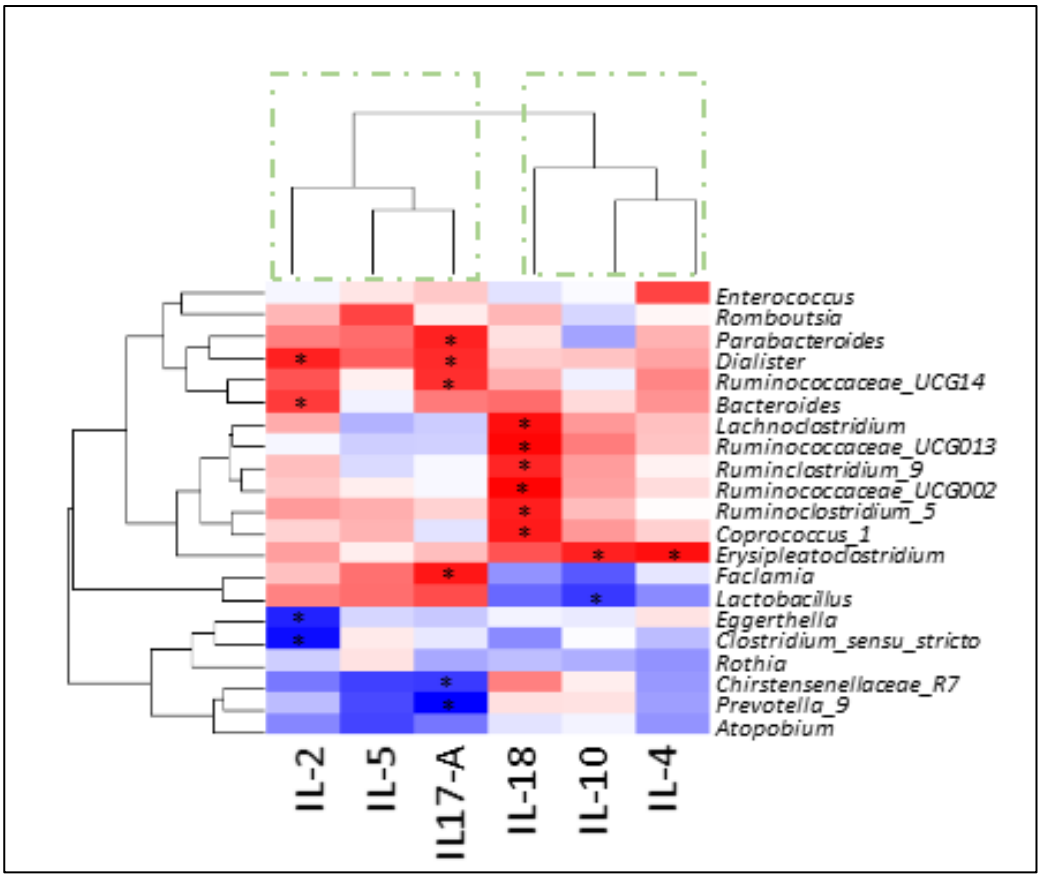


Figure I-4. Amniotic fluid was associated with neonatal microbiota at delivery. Heatmaps of the spearman associations between selected amniotic fluid cytokines and genera from neonatal microbiota at delivery. Red color represents the positive correlations whereas blue color shown negative relations.

DISCUSSION

Gestation is a process that requires the adaption of all the systems of the body, including hormonal [432], metabolic [433] and immune system [434,448] adaptations, as well as changes in the maternal microbiota [131] in order to provide an adequate environment for foetal development. Similarly, as gestation progresses from conception to labour, both women's bodies and foetal systems modify their responses so as to maintain the homeostasis of the dyad and to trigger the initiation of labour [449,450]. Our study showed that the maternal and neonatal microbiota were associated with distinct cytokine profiles in the amniotic fluid as well as with the gene expression levels of GLUT3 and GPX in the placental tissue.

During pregnancy, the gut microbiota is involved in metabolic, immune and hormonal control [42,43,451,452]. Among our cohort of mothers, we observed a slight

increase in the relative abundance of Proteobacteria phylum as well as a reduction in the relative abundance of Firmicutes when compared with the non-pregnant adult population [453,454]. It has been shown that the maternal microbiota decreases in terms of its diversity from the first to the third trimester of gestation, while the relative abundance of Proteobacteria phylum species increases during the same period [132]. These changes may be involved in the metabolic inflammation observed during pregnancy, including body fat augmentation, reduced insulin sensitivity and a pro-inflammatory state [135], which could be essential for foetal growth and energy accumulation [136,137]. In this sense, both the Proteobacteria phylum and the diminished microbial diversity are related to inflammation and metabolic syndrome [133,134].

Despite the influence of the immune status of the host and the composition of the gut microbiota having been widely reported in animal [35,333,455] and human studies [43,223], the interaction between the gut microbiota and the host immune system during gestation has been only poorly described to date. In fact, to the best of our knowledge, this is the first study to explore the immune status of the amniotic cavity as well as the maternal and neonatal microbiota at the time of delivery. One group of maternal taxa composed of the *Faecalibacterium*, *Roseburia*, *Lachnospira* and *Bacteroides* genera, among others, was related to a cytokine profile dominated by IL-1beta, TNF-alpha, IL-2, IL-17A and IL-5. These cytokines were negatively associated with the other pattern of bacteria, which was characterised by the *Peptoniphilus*, *Anaerococcus*, *Prevotella*, *Finegoldia*, *Porphyromonas* and *Murdochiella* genera, among others. The other cytokine profile, which was composed of IL-13, IL-4 and IFN-gamma, was positively associated with several groups from the Ruminococcaceae family as well as with the mRNA expression of both *GLULT3* and *GPX* in the placental tissue.

Placental GLUT3 was observed to be up-regulated in human pregnancies with late-onset intrauterine growth accompanied by an increased HIF-1alpha concentration, which suggests that hypoxia would be involved in the up-regulation [456], while reductions in the expression and activity of placental GPX have been linked to oxidative damage and preeclampsia [457]. We observed a positive and significant association between the mRNA expression of both *GPX* and *CAT* and *Bifidobacterium* genus in the neonatal microbiota. It remains unclear whether the foetal gut is colonised by microorganisms, although some recent studies have shown that viable bacteria are

limited, albeit still present, in the foetal intestine during mid-gestation, with some of the detected strains exhibiting an immunomodulatory capacity. The relationship between *Bifidobacterium* spp. and the antioxidant enzymes found in the placental tissue may indicate the active role of placental behaviour in relation to the further colonisation of the neonatal gut; however, this hypothesis requires further investigation.

TNF-alpha and IL-1beta have been found to be involved in the healthy triggering of labour [458]. Indeed, the serum IL-1beta, IL-6 and TNF-alpha levels were observed to be elevated in the neonates at one and five days post-birth when compared with the levels found in the umbilical cord and maternal serum [458], which suggested their involvement in the normal parturition process and also reflected the neonate's immune response to environmental changes.

It has previously been reported that, during labour, the IL-2 and IL-4 cytokine concentrations in the umbilical cord are increased when compared with the concentrations in adult and maternal serum, while the IL-2 levels decreased significantly at five days post-partum [459]. The IL-1beta, IL-6, TNF-alpha, sIL-2R and SIL4-R concentrations were found to be dependent on the mode of delivery, being elevated in cases of vaginal delivery when compared with elective caesarean section [458] [460]. Thus, the present study concluded that these cytokines and their receptors participate in the development of the neonatal immune system as well as its regulation during labour [459]. Although all our amniotic fluid samples were collected a few minutes prior to delivery via caesarean section, it is feasible that some of the physiological processes that conclude the triggering of labour, including immune system signals, had been initiated. Interestingly, in a previous study involving the same cohort [461], the bacteria negatively associated with the cytokine profile composed of TNF-alpha, IL-2 and IL1-betta, including *Finegoldia*, *Peptoniphilus* and *Anaerococcus* genera, were observed to be related to mothers who had undergone a caesarean section without labour having been initiated when compared with those who underwent a vaginal delivery. In that study, these bacteria were also negatively associated with the salivary cortisol concentration at the time of delivery, suggesting the relation between these bacterial taxa and the absence of the labour process [461]. IL-2 has traditionally been considered a potent inductor of T-cell growth and expansion, and IL-2 inhibitors have been used to supress the rejection of transplanted organs [462]. However, several studies have highlighted its role in tolerance development through its impact on the function of CD4+CD25+ Tregs [463,464]. Thus, the maternal microbiota could be

associated with the adaptations seen in the immunological environment of the amniotic cavity prior to delivery. This is especially relevant given that some studies have reported that the maternal microbiota could play a role in immune system maturation even during the gestational period.

Thorburn et al. reported that a high-fibre diet during pregnancy could contribute to the production of SCFA by the maternal microbiota, which was related to the suppression of certain genes linked to asthma and also to the generation of Foxp3 Tregs through epigenetic modifications in a mouse model [155]. Moreover, in the case of T-cell development, Li et al. showed that memory CD4+ T-cells could be generated in the human foetal intestine, thereby providing evidence of memory formation during the foetal stage [465]. Fotiou et al. reported that the metabolites found in amniotic fluid are influenced by habitual maternal dietary patterns, although it is not yet known whether the gut microbiota is involved in the observed differences between dietary clusters [466]. Further, the question of whether or not the relation between the maternal microbiota and the amniotic fluid immune environment contributes to foetal development remains unanswered. Immune signals could alter the maternal microbiome, thereby affecting the maternal–neonatal microbial transfer during delivery [155]. Indeed, despite less significant associations found between the amniotic fluid cytokines and the neonatal microbiota when compared with the maternal microbiota, we found that the IL-2 concentration in the amniotic fluid was also related to the neonatal microbiota showing positive association with the relative abundance of the *Bacteroides* genera and the negative relation with the *Clostridium sensu stricto* and *Eggerthella* genera. Thus, the relations between the maternal microbiota and the immune signals in amniotic fluid could be related to the neonatal microbiota at the time of delivery.

Some of these genera showed positive relations with the anti-inflammatory cytokine IL-10 and negative associations with the IL-6 levels. The *Peptoniphilus*, *Murdochiella* and *Negativicoccus* genera, showed positive relations with the IL-10 concentration. However, fewer significant relations were found between the maternal microbiota and the placental expression than between the maternal microbiota and the amniotic fluid immune system. The crosstalk that occurs between the mother–foetus–microbiota during pregnancy through placental and amniotic fluid metabolite exchange requires further research.

Similarly, considerable knowledge is available concerning the special behaviour exhibited by the immune system during gestation and labour [434] in order to maintain

foetal maturation and to maintain the process of labour, respectively. The immune system in the foetal cavity represents one of the main interactions that occur between the developing neonate and the host (or mother). For this reason, during gestation, there exists a well-controlled balance between a "tolerant" state that avoids the rejection or triggering of labour during the early gestational period and a sufficient capacity to respond to an intrauterine infection [434]. Thus, it is commonly accepted that the maternal and foetal immune systems are biased toward a Th2 profile during pregnancy so as to sustain this tolerogenic environment [467]. Aside from birth, pro-inflammatory signals have been suggested to be crucial not only for the triggering of labour, but also for preparing the neonate for the shift from the "tolerogenic" state of the sterile intrauterine cavity to the microbial challenge posed by the canal birth and the surrounding environment [468]. TNF-alpha and IL-1beta have both been observed to be increased in the amnion, amniotic fluid, and decidua at term [458,469], and they can induce prostaglandin production in the amniocytes *in vitro* [470,471]. Hence, some authors have suggested that the activation of the pro-inflammatory Th1 profile is involved in the initiation of labour, including in the case of preterm births [467,472].

In addition to the immunological changes that occur during pregnancy, the metabolic adaptation of the mother is required to maintain both a healthy pregnancy and adequate foetal development. Our results indicated the positive relation between the blood cholesterol and triglyceride levels and a group of taxa composed of the *Roseburia, Lachnospira* and *Bacteroides* genera. Contrarily, these biochemical markers were found to be negatively associated with the *Peptoniphilus*, *Finegoldia* and *Corynebacterium* genera. The cholesterol and triglyceride levels both increase during gestation, and they reach their maximal blood concentrations just before labour through a physiological process that may provide the foetus with the energy necessary for it to grow [473–475]. Thus, the metabolic traits related to metabolic syndrome in an adult population may be beneficial during pregnancy and may also be involved in the labour process. Recently, Marchioro et al. described how mothers who underwent a planned caesarean section showed lower levels of non-esterified fatty acids (NEFA) and acylcarnitines when compared with mothers who underwent a vaginal delivery with labour [476]. We hypothesised that the SCFA found in the amniotic cavity could be responsible for the observed association between the maternal microbiota at the time of delivery and the immune status of the amniotic fluid cavity. However, we were unable to identify detectable levels of any of the SCFA extracted via the utilised protocol.

Other authors have described the capacity of the meconium to produce low concentrations of SCFA equivalent to approximately 10% of adult levels [477]. Indeed, acetic and propionic acid have also been reported in first-pass meconium samples [478]. Although these studies were conducted in the meconium, which had probably been in contact with bacteria prior to the measurement being taken, rather than in amniotic fluid, the extraction protocol and the chromatography column used differed from our experimental procedure, which could explain these discrepancies [361,479].

A more in-depth understanding of the physiological variations that occur during delivery as well as of the relations between the factors involved in non-pathological pregnancies may help with the design of strategies to detect possible alterations that could lead to foetal and/or neonatal disorders.

The limitations of this study include the low number of participants, which could limit the significance of the conclusions drawn from the results. Furthermore, our analysis of the microbiota was based on the taxonomic profiles obtained via 16S rRNA gene sequencing, which offers lower resolution than complete shotgun metagenome sequencing. Among the strengths of the study is the information provided about a significant range of cytokine concentrations in the amniotic fluid at the time of birth as well as their possible relationships with the maternal and neonatal microbiota. Moreover, while most prior studies focusing on the relations between the maternal microbiota and the amniotic fluid cytokines/metabolites have been conducted in animals, our study provided valuable insights into the possible relations in humans.

Our study is among the first to describe the significant relations between the cytokine profiles in the amniotic fluid and the composition of the maternal microbiota at the time of delivery, which also have possible consequences in relation to the expression of the metabolic genes in the placenta. These relations could be involved in the triggering of labour and/or in the development of the foetal and neonatal immune systems. Further studies are required to explore the possible implications of the maternal microbiota in terms of the normal progress of pregnancy as well as the consequence of alterations in the maternal microbiota–immune system relationship for maternal and infant health.

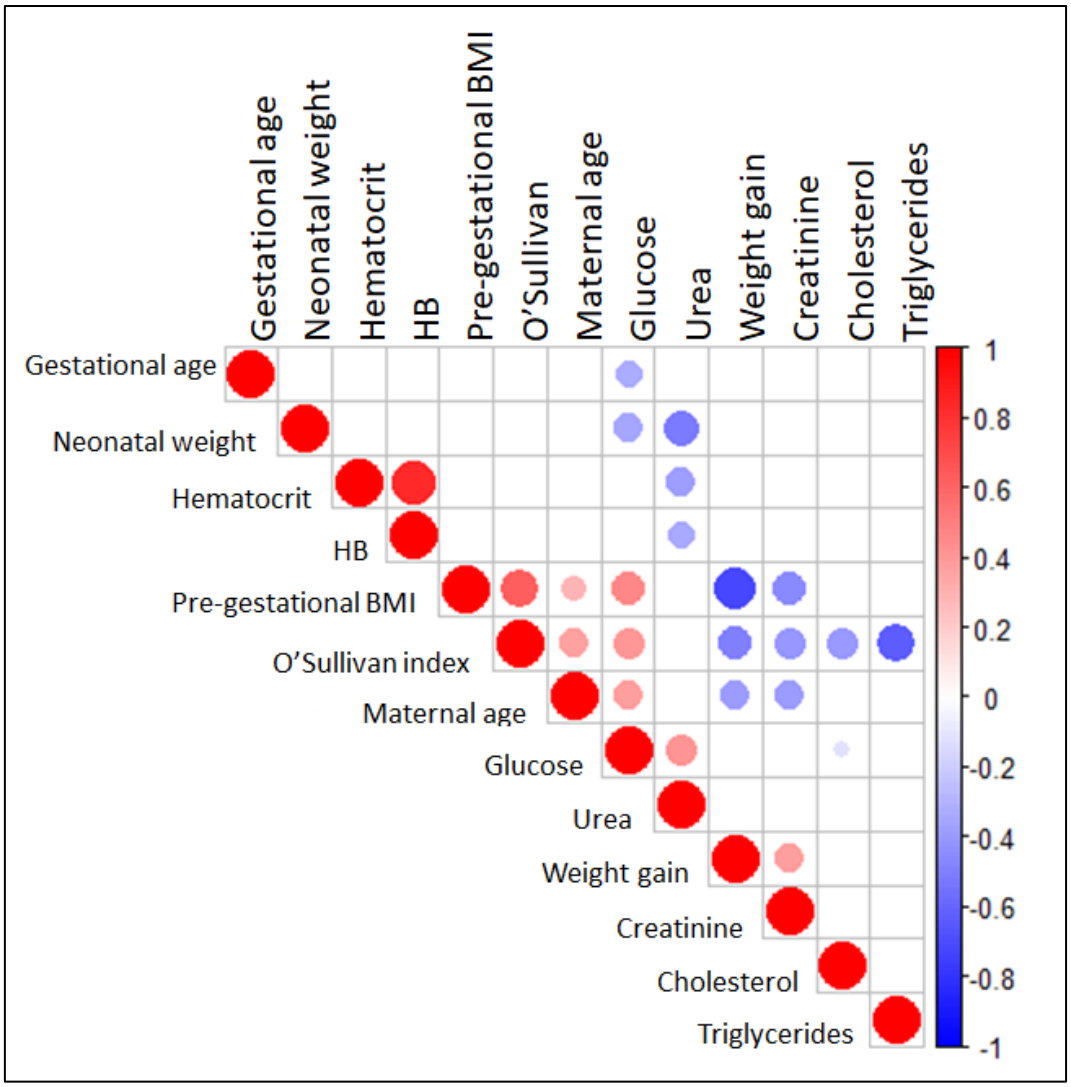
CONCLUSION

Our study is one of the first one that describe significant relations between cytokine profiles in amniotic fluid with maternal microbiota composition at delivery with possible consequences also in the expression of metabolic genes in placenta. These relations could be involved in the labour triggering and/or the foetal and neonatal immune system development. Further studies are needed in order to explore the possible implications of maternal microbiota in the normal progress of pregnancy and the consequence of alterations in this maternal microbiota-immune system relationship in the maternal and infant health.

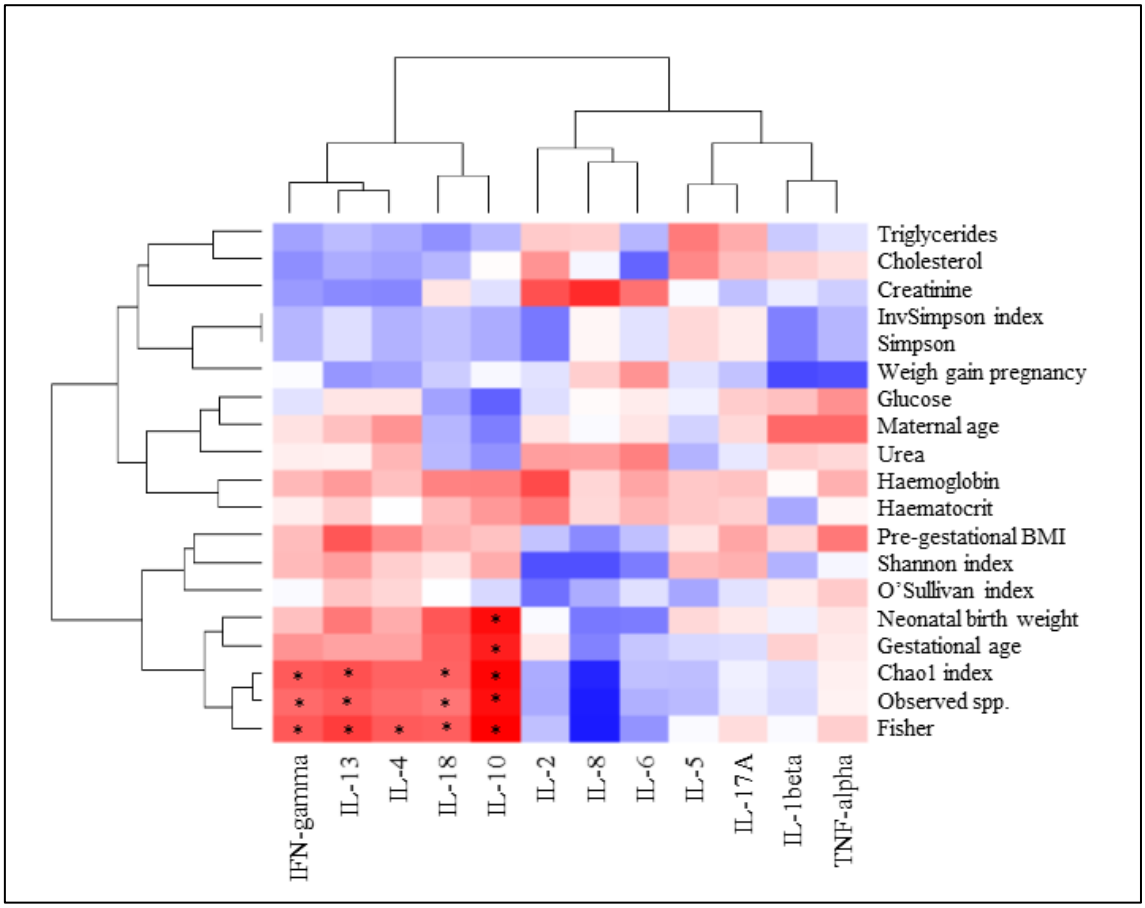
CHAPTER I - SUPPLEMENTARY DATA

Supplementary file I-1. Primers used in the study

Primer	Seq. (5'-3')	Ref
SOD1	AATGGACCAGTGAAGGTGTGGGG CACATTGCCCAAGTCTCCAACATGC	[480]
CAT	GAACTGTCCCTACCGTGCTCGA CCAGAATATTGGATGCTGTGCTCCAGG	[480]
GPX	CGGCCCAGTCGGTGTATGC CGTGGTGCCTCAGAGGGAC	[480]
GLUT3	CGTTGTTGGAATTCTGGTGGC CTTAGCATTCTCCTCTTCTTTT	[481]
VEGF	TGTCTTGGGTGCATTGGAG CCTTGC GTTGTGCTGTAGGAAGCTCATCTCTC	[482]
HIF1-alpha	TTCACCTGAGCCTAATAGTCC CAAGTCTAAATCTGTGTCCTG	[483]



Supplementary file I-2. Relation of the anthropometrical and clinical variables in the study. Spearman rank correlation was performed between them. Blue and red colours represent positive and negative correlations, respectively. Only correlations with a p <0.07 were showed in colours. The size of the circle marking the correlations represent the strength of the correlation (rho). Red colour represents the positive correlations whereas blue colour shown negative relations.



Supplementary file I-3. Heatmap of spearman correlations between amniotic fluid cytokines at delivery, biochemical markers measured at the last trimester of pregnancy and index of alpha diversity of maternal microbiota. Significant correlations ($p<0.05$) were marked by an asterisk (*). Red color represents the positive correlations whereas blue color shown negative relations. Amniotic fluid cytokines and clinical parameters were grouped at the square edges

CHAPTER II

MATERNAL MICROBIOTA, CORTISOL CONCENTRATION AND POST-PARTUM WEIGHT RECOVERY ARE DEPENDENT ON MODE OF DELIVERY

CHAPTER II - MATERNAL MICROBIOTA, CORTISOL CONCENTRATION AND POST-PARTUM WEIGHT RECOVERY ARE DEPENDENT ON MODE OF DELIVERY

Selma-Royo M, García-Mantrana I, Calatayud M, Parra-Llorca A, Martínez-Costa C, Collado MC. Maternal Microbiota, Cortisol Concentration, and Post-Partum Weight Recovery are Dependent on Mode of Delivery. Nutrients. 2020;12(6):E1779. doi:10.3390/nu12061779

Impact factor: 4.17

ABSTRACT

Accumulating data is highlighting the impact of maternal microbiota on the infant health programming; however, little is currently known about perinatal factors that could affect the maternal microbiota, as most authors have focused on the infant microbiome. The aim of this study was to evaluate the effects of various delivery-related factors on the composition of the maternal microbiome at the time of birth as well as on maternal post-partum weight retention. Anthropometric data were collected from mothers enrolled in the MAMI cohort (n=167) during the first four months post-partum. A subset of 100 mothers were selected for the determination of the salivary cortisol concentration and microbiome composition at birth by means of 16S rRNA gene sequencing.

We found that maternal microbiota was classified into two distinct clusters based on their composition and diversity at the time of delivery. Cluster I was characterized by higher diversity ($p<0.001$) and greater richness ($p=0.001$), by the presence of health-related genera, including *Faecalibacterium, Blautia*, and *Roseburia* genera, and by the lower prevalence of births via cesarean section (CS) (31.7%). Cluster II was dominated by *Finegoldia, Peptoniphilus*, *Campylobacter*, and *Porphyromonas* genera, and it was characterized by lower diversity, lower richness, and the higher prevalence among births via CS (70.5 %). Indeed, the maternal microbiota was significantly influenced by the mode of delivery ($p=0.003$), with the differences between the different modes (vaginal [VAG] delivery vs. CS) being more pronounced in Cluster II. Furthermore, the salivary cortisol concentration was significantly higher in those mothers who had a VAG delivery ($p=0.003$), and it was associated with genera from Christensenellaceae (rho=0.32, $p=0.014$) and some Lachnospiraceae groups (rho=0.40, $p=0.001$), which were also related to mothers from vaginal delivery group. Furthermore, the VAG delivery mothers exhibited lower post-partum weight retention than the CS mothers at four months post-partum ($p<0.001$). These results support the hypothesis that the mode of delivery as well as the codominant hormonal changes could influence the maternal microbiota at delivery and possibly impact maternal weight recovery during the post-partum period. Therefore, they could affect the health status of women in the long term.

Keywords: microbiome, delivery-mode, cortisol, post-partum weight retention.

INTRODUCTION

Gestation is a well-orchestrated process that affects all the systems of the body, including the human microbiome [131,484]. The maintenance of pregnancy requires adaptations on the part of hormonal [432], immunological [434,448], and metabolic systems [433] in order to sustain fetal development. Several authors have compared these changes to those that occur in the case of metabolic syndrome, including insulin sensitivity [135] and an incremental increase in adiposity [485], which lead to sub-clinical inflammation [486] characterized by the increased production of pro-inflammatory cytokines. As the gut microbiota is interconnected with all these processes, its bacterial composition and diversity also undergo variations throughout pregnancy [131]. The variations seen in the microbiota during pregnancy tend toward an incremental increase in the presence of the Proteobacteria and Actinobacteria phyla as well as a reduction in diversity and the presence of some health-related genera [132]. In fact, the fecal transplantation of the gut microbiota from pregnant women during the third trimester of gestation to germ-free mice reproduced the symptoms of metabolic syndrome [132].

The maternal microbiota is considered to be the main driver of the initial bacterial seeding of the newborn [153]; however, the perinatal factors that affect the maternal microbiota are only little understood, as prior studies have tended to focus on the factors shaping the infant microbiome [487,488]. Only a few studies have investigated the maternal microbiota and the elements that could modify its composition, including the mother's diet during gestation [196,198,489–491], pre-gestational body mass index (BMI) [187,492], or weight gained during gestation [493]. In these studies, were highlighted the importance of this factors, especially maternal diet (e.g. fiber and fat-related nutrients), on intestinal microbiota at delivery. Thus, little is currently known about the effects of the mode of delivery and associated factors on the composition of the maternal microbiome, while even less is known about their possible effects on the mother's post-partum health status, which can reasonably be expected to affect neonatal development through mother–infant contact and breastmilk.

The aim of this study is, therefore, to clarify the possible influence of factors related to the mode of delivery on the maternal microbiota and its association with maternal weight retention during the post-partum period.

MATERIALS AND METHODS

Participants and sampling information

Data from mother participants in the "Impact of Maternal microbes on Infant health programming" (MAMI) cohort [420] were collected during the first four months post-partum for the present analysis (n=167). Briefly, MAMI cohort includes mothers and their offspring, who gave birth during the 2015-2018 period in the Valencia metropolitan area hospitals, including Hospital Universitario y Politécnico La Fe and Hospital Clínico Universitario. Exclusion criteria, objectives and description of the cohort is described in García-Mantrana et al. [420]. For the microbiota analysis, we selected a subset of 100 mothers from the MAMI cohort according to availability of the sample at delivery.

Anthropometric and clinical data from those mothers were collected including delivery mode, maternal age, pre-pregnancy BMI antibiotic intake during pregnancy, or weight gain during gestation, among others. Delivery-related data such as rupture of membranes mode, instrumentalization and antibiotic administration were also recorded. Dietary intake of mothers were evaluated by a 140-item validated food questionnaire (FFQ) [421] that mothers fill within first week after delivery. FFQ information was analysed using the Nutrients Food Composition Tables developed by the Centro de Enseñanza Superior de Nutrición Humana y Dietética (CESNID), Spain [494].

All mothers received oral and written information about the study and all participants provided written informed consent before enrolment. Ethical approval for the study was obtained from the Ethics/Bioethics Committee for Clinical Research of Hospital Universitario y Politécnico La Fe, Hospital Clinico Universitario and CSIC (Consejo Superior de Investigaciones Científicas) [ClinicalTrial.gov NCT03552939].

Biological samples

Fecal samples at delivery were collected using a sterile cotton-tipped by trained clinical staff before birth at delivery room following protocol described previously [420]. Similarly, saliva samples were also collected in special sponge dispositive (Salivette, Sarstedt AG & Co. KG, Nümbrecht, Germany) at delivery room. All the C-sections mothers, both ECS and CS, received intravenous intrapartum antibiotics during the intervention. Samples were collected before the surgery was initiated. In the case of emergency C-section, the labor process was begun but it was not completed due to complications, including abnormal neonatal position, neonatal size, problems in heart

rate, blood pressure etc... In the case of mothers who undergone a vaginal delivery, all of them had the labor initiated and the samples were collected during the dilatation time.

All samples were sent to biobank, and then, biological samples were managed and stored at -80C in sterile cryovials under specific standardized protocols at "Biobanco para la Investigación Biomédica y en Salud Pública de la Comunidad Valenciana (IBSP-CV)". Once all cohort samples were collected and placed in biobank, aliquots were shipped and centralized at IATA-CSIC for the analysis.

Cortisol concentration quantification

Saliva was collected from the Salivette device by centrifugation of the sponge contained in the tube. Resulted liquid was transferred to a new tube and stored at -80ºC until analysis. Saliva cortisol concentration was assessed by the immunoassay kit "Salivary Cortisol ELISA KIT" (Salimentrics Assay #1-3002, Salimetrics, Carlsbad, CA) following manufacturer instructions.

DNA extraction

DNA was extracted from the fecal material using the Master-Pure DNA extraction kit (Epicentre, Madison, WI, US) following the manufacturer instructions. Some additional steps were added to the basic protocol including a treatment for 60 min at 37ºC with lysozyme (20 mg/ml) and mutanloysin (5U/ml) followed by two cycles of cell disruption with 3-µm diameter glass beads performed by a bead beater FastPrep 24-5G Homogenizer (MP Biomedicals) during 30 s at 6 m/s. Then, purification of the DNA was assessed by DNA Purification Kit (Macherey-Nagel, Duren, Germany) according to manufacturer's instructions and the resulted DNA concentration was measured using Qubit® 2.0 Fluorometer (Life Technology, Carlsbad, CA, US).

Amplicon sequencing and bioinformatics

The amplification of the V3-V4 variable region of the 16S rRNA gene was conducted following the 16SrDNA gene Metagenomic Sequencing Lybrary Preparation Illumina protocol (Cod. 15044223 Rev. A) with the primers selected from [495]. NextERA XT Index Kit (FC-131-2001) (Illumina, San Diego, CA, United States) was used for the multiplexing step and DNA quality of the resulted library PCR product was assessed by a Bioanalyzer DNA 1000 chip (Agilent Technologies, Santa Clara, CA, United States). Then, libraries were sequenced using a 2 x 300 bp paired-end run (MiSeq

Reagent kit v3) on a MiSeq-Illumina platform (FISABIO sequencing service, Valencia, Spain) according to manufacturer instructions. Obtained reads were searched for residual adaptors using the program Trimmomatic [496].

DADA2 pipeline [389] was followed for the quality-trimmed and filtering of the obtained sequences. Reads were trimmed at the 270th and 210th nucleotide in forward and reverse position, respectively, after quality examination. Additionally, adapters were also removed in the filtering process and a maximum of 2 expected errors was considered. Then, the sequences were merged, and chimera removed following the mentioned pipeline with the default options. Silva v132 database [440] was used for the taxonomic assignment with the specie level classification.

Additional filters were also performed as follows: taxa occurring <3 reads in at least 20% of the samples and those that represent less than 0.01% of the total reads across all the samples were also removed. Furthermore, the decontam package [408] in R environment [445,497] was used to determine the presence of potential contaminants-related sequence. Samples with less than 1000 reads were also removed from the final analysis and bacterial counts were transformed to relative abundance.

Statistical analysis

Differences in population anthropometric and clinical data was tested by T-test and Mann-Whitney analysis according to data normality assessed by Shapiro-Wilk test in Graphpad software v. 5.04 (GraphPad Software, La Jolla California USA, www.graphpad.com). Chi-Squared test (2x2) was performed to assess the significance of the differences in the characteristics according to categorical variables. Mothers were classified according their pregestational BMI [498] and their weight gain during pregnancy category following the recommendations of the Institute of Medicine [438] for each pre-gestational BMI.

Maternal microbiota clustering was generated at genus level as described elsewhere [499] using the phyloseq [500], cluster [501], MASS [502], clusterSim [503] and ade4 R packages [504]. Briefly, Jensen-Shannon distance and partitioning around medoid (PAM) clustering were used. Optimal number of clusters was calculated by the Calinski-Harabasz (CH) index.

RStudio (R v. 3.6.1) was used to perform the PERMANOVA (Adonis) multivariate analysis in maternal and neonatal microbiota by the vegan package [505] based on Bray-Curtis distance. Vegan package was also used for alpha diversity by Shannon (diversity)

and Chao1 indexes (richness) and Kruskal-Wallis/Mann-Whitney test with false discovery rate (FDR) adjustment was performed to test significance between variables. Partial Spearman correlation adjusted by BMI was used to assess the relation between weight gain over pregnancy and maternal microbiota at delivery.

Discriminant analysis of Principal Components (DAPC) and Adonis test were also achieved based on Bray-Curtis distance in Calypso online platform v. 8.84 [443]. Boxplots showing differences in microbial genera were generated in GraphPad software with the log10 transformation to facilitate the visualization. Calypso online platform was also used to test significant differences in microbiota composition according to studied variables. All comparisons were adjusted by FDR. Spearman correlations in both delivery modes were conducted in SPSS v. 25 [444] software to describe the association between salivary cortisol concentration and maternal microbiota genera. A multivariate model adjusted by pre-pregestational BMI, weight gain during pregnancy and breastfeeding duration was performed on maternal weight variations according to delivery mode during the post-partum period using SPSS v.25. The maternal post-partum weight recovery (PPWR) evolution was studied based on maternal weight (Kg) for 4 months post-partum and the difference with pre-gestational weight was also calculated. In the weight recovery analysis, mothers were considered to recover the pre-gestational weight when had a difference between pre- and post-partum weight below mean difference + 1SD (standard deviation).

RESULTS

Study participants characteristics

Characteristics of participating mothers in the microbiota study presented a median age of 35 years old and showed a pre-gestational body mass index (BMI) of 22.58 kg/m2 which was within the normal weight range (BMI<25 Kg/m^2) (**Table II-1**).

The 54.6% of the mothers undergone a vaginal labour (VAG) while the 36.1% had an elective C-section (CS) and the rest (9.3%), an emergency C-section (ECS). VAG and ECS pregnancies showed a slightly higher gestational age than elective C-section ($p<0.001$). Besides this, no differences were found in any of the other studied factors between the three groups. Furthermore, no differences in nutrients intake were found between maternal groups according to delivery mode (**Table II-1**). Characteristics of

the population used in the study of the effect of delivery mode on the postpartum weight retention (PPWR) were listed in **Supplementary file II-1**.

Table II-1. Characteristics of population participant in the microbiota study (n=97).

	Total (n=97)	Vaginal (n=53)	Emergency C-section (n=9)	Elective C-section (n=35)	P
Pregnancy data					
Maternal age (years)	35 [31-37]	33 [29.50-36.50]	35 [32.50-36]	35 [31-38]	*0.200*
Weight gain (Kg)	12 [10 – 15]	12 [10-14]	15 [9.85-20]	12.5 [10.5-15.30]	*0.242*
Low	21 (21.7)	16 (30.2)	3 (33.3)	6 (17.1)	***<0.001****
Recommended	51 (52.6)	30 (56.5)	2 (22.2)	19 (54.3)	
High	25 (25.8)	7 (13.2)	4 (44.4)	10 (28.6)	
Pre-gestational BMI (Kg/m2)	22.58 [20.43 – 25]	21.63 [20.14-24.06]	20.32 [19.42-23.49]	23.42 [21.01-25.88]	*0.070*
NW	68 (70.1)	39 (73.6)	7 (77.8)	22 (62.9)	***0.001****
OW	23 (23.7)	10 (18.9)	1 (11.1)	12 (34.3)	
LW	6 (6.2)	4 (7.5)	1 (11.1)	1 (2.9)	
Antibiotic at pregnancy	40 (41.2)	23 (43.4)	4 (44.4)	13 (37.1)	*0.525*
Gestational age (weeks)	39 [38 - 40]	40 [39-40] [b]	39 [38-40] [ab]	38 [38-40] [a]	***<0.001****
Siblings	35 (36.1)	18 (34)	1 (11.1)	16 (45.7)	***<0.001****
Delivery data					
O'Sullivan index	124.7 ± 28.78	122.5 ± 27.66[8]	127.1 ± 33.87	121.5 [107-154.3] [7]	*0.821*
Salivary cortisol (µg/dl)	0.55 [0.23 – 1.32]	0.93 [0.42-2.61][26 b]	0.77 [0.23-1.65][2 ab]	0.35 [0.11-0.61] [9 a]	***0.003****
Intrapartum antibiotic	7 (13.2)	7 (13.2)	9 (100)	35 (100)	-
Episiotomy	18 (34) 8	18 (34) 8	-	-	-
Instrumentalization	12 (22.7)	12 (22.7)	-	-	-
Rupture of membranes					
Spontaneous	33 (62.3)3	33 (62.3)3	-	-	-
Artificial	17 (32.1)	17 (32.1)	-	-	-
Dietary data					
Energy (Kcal)	2770 [220-3275]	2817 [2313-3382]	3040 [2103-4157]	2309 [2115-3051]	*0.092*
Total proteins (g)	113 [94-130]	116 [94-127]	107 [97-162]	107 [87-103]	*0.485*
Animal proteins (g)	74 [58-89]	73 [57-87]	75 [64-103]	74 [55-96]	*0.690*
Vegetable proteins (g)	39 [30-49]	41 [33-51]	32 [28-73]	32 [25-29]	*0.077*
Lipids (g)	137 [111-170]	142 [111-171]	149 [101-205]	133 [111-148]	*0.380*
SFA (g)	28 [28-49]	38 [27-49]	42 [27-58]	38 [28-49]	*0.902*
MUFA (g)	69 [52-80]	71 [56-80]	79 [54-95]	68 [47-74]	*0.174*
PUFA (g)	22 [17-30]	23 [18-29]	18 [13-39]	22 [17-30]	*0.986*
Cholesterol (mg)	390 [332-482]	403 [331-486]	393 [357-508]	370 [305-465]	*0.643*
Carbohydrates (g)	264 [205-346]	287 [222-353]	249 [76-364]	210 [160-288]	*0.062*
Fiber (mg)	32 [23-45]	39 [25-47]	34 [29-53]	26 [21-39]	*0.122*

Significance differences were tested in the three studied groups. Normally distributed data was presented as mean ± SD and non-normal data as median [IQR]. Categorical variables were expressed as positive cases (percentage). Data no sharing letters was significantly different between studied groups. Number of missing values for each variable was represented as a superscript. NW (Normoweight), OW (Over weight), LW (Low weight).

Maternal microbiota composition at delivery time

Maternal microbiota at delivery was characterized by a dominance of Firmicutes and Bacteroidetes phyla with relative abundances of 68.07% and 18.59%, respectively followed by Actinobacteria (5.75%) and Proteobacteria (3.50%) phyla (**Supplementary file II-2**).

We performed a clustering of mothers enrolled in the study according to their microbiota at delivery. Results revealed two distinct microbial clusters (**Fig. II-1, A-B**). Cluster I was characterized by higher relative abundances of *Blautia*, *Lachnospira*, *Bacteroides* and *Faecalibacterium* genera. However, Cluster II was predominantly dominated by *Ezakiella*, *Peptoniphilus*, *Campylobacter* and *Porphyromonas* genera. (**Fig. II-1, C**). Higher relative abundance of *Blautia* ($p<0.001$), *Rombustia* ($p<0.001$), *Roseburia* ($p<0.001$), *Lachnospira* ($p<0.001$) and several groups from *Ruminococcaceae* family were found in cluster I compared to cluster II. However, cluster II mothers had higher relative abundance of *Prevotella* ($p<0.001$), *Mobiluncus* ($p<0.001$), *Lawsonella* ($p<0.001$), *Murdochiella* ($p<0.001$), *Corynebacterium* ($p=0.001$), *Lactobacillus* ($p=0.003$), among others.

At phylum level, only Epsilonbacteraeota ($p<0.001$) phylum that represent less than 1% of total reads showed significant differences between clusters (**Supplementary file II-2**). Furthermore, differences in the relative abundance of Euryarchaeota ($p=0.015$) was also found between clusters. In terms of alpha-diversity (**Fig. II-1, D-E**), mothers classified in Cluster I showed higher microbial diversity and richness measured as Shannon ($p<0.001$) and Chao1 ($p=0.001$) index, respectively.

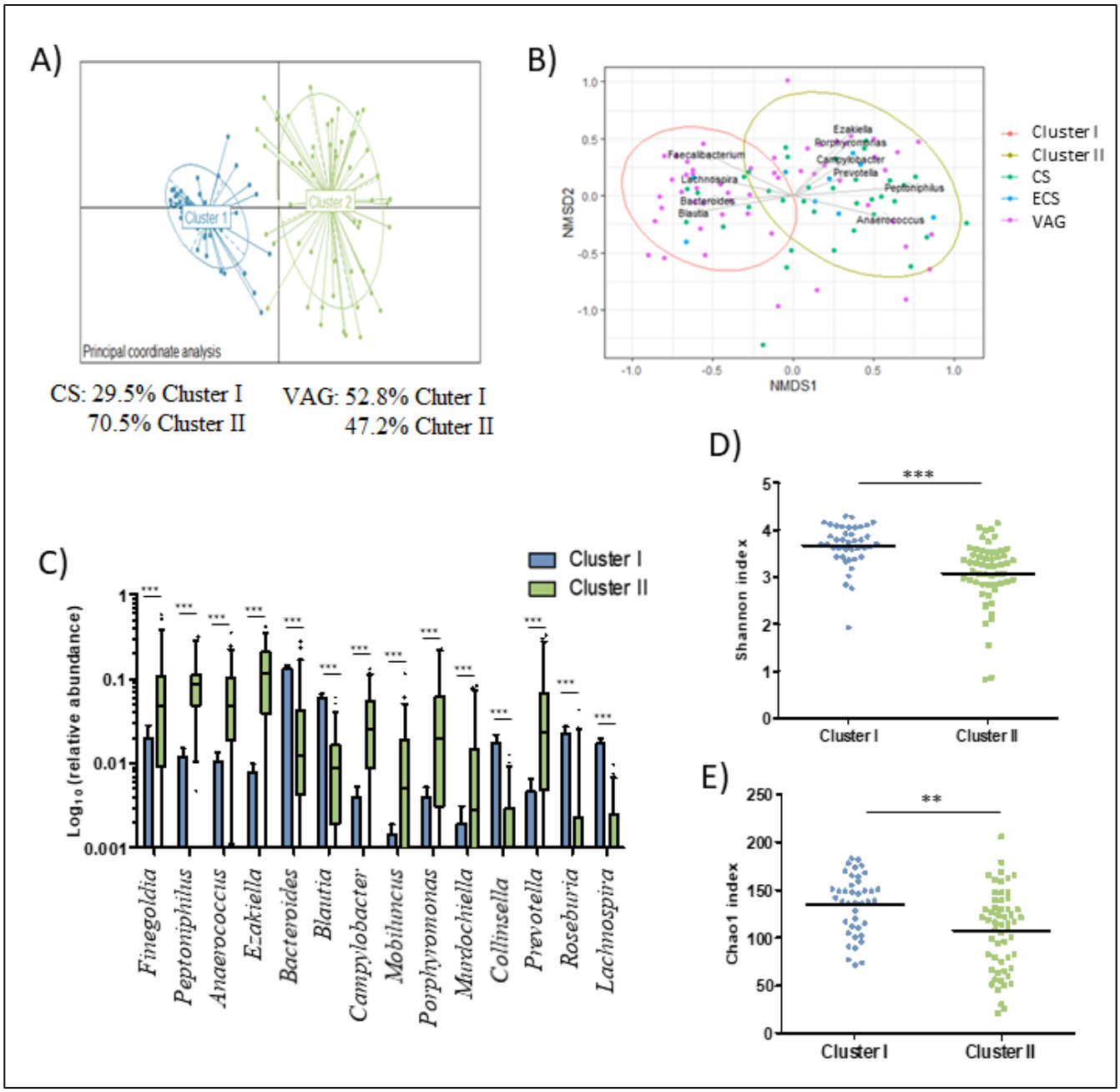


Figure II-1. Maternal microbiota is clustered in two groups based in their composition and diversity at delivery. A) Principal coordinate analysis (PCoA) of maternal microbiota at delivery according to cluster at genus level. **B)** Non-metric multidimensional scaling (NMDS) of maternal microbiota at delivery time at amplicon sequence variant (ASV). Arrows showed the genus loadings for each cluster. Colour of the points expressed the delivery mode group of the mother. **C)** Boxplot of the main genera that marked the difference of maternal microbiota composition between both clusters. Data was transformed by log10 of relative abundance of each genus for plotting. Whiskers represented 5-95 percentile interval. D-E) Differences in diversity (**D**) and richness (**E**) of maternal microbiota based on Shannon index according to cluster. Line represented the median of each group. * *p<0.05*. ** *p<0.01*. *** *p<0.001*.

Factors affecting maternal microbiota at delivery

We performed a permutational multivariate analysis of variance (PERMANOVA) based on Bray-Curtis distance to clarify the relevance of the perinatal factors and maternal microbiota composition at delivery (**Fig. II-2, A**). Delivery mode (*p=0.003*) significantly affected the maternal microbiota composition at delivery followed by the rupture of membranes mode (*p=0.07*) which was only analysed in vaginal deliveries since all CS were carried out during the intervention.

At genus level (**Fig. II-2, B**) (**Supplementary file II-3**), CS women showed higher relative abundance of *Lawsonella* (*p<0.001*), *Finegoldia* (*p=0.001*), *Corynebacterium* (*p=0.004*) genera, among others compared to VAG deliveries. However, women with VAG showed an enrichment in *Christensenellaceae_R7_group* (*p=0.002*) and some groups from Ruminococcaceae groups including *Ruminococcaceae_UCG002* (*p=0.053*), *Ruminococcaceae_UCG009* (*p=0.053*) and *Ruminiclostridium_5* (*p=0.053*). While we found significant differences in microbiome composition between VAG and CS mothers, no differences were found between VAG and ECS mothers. Moreover, no differences in terms of alpha-diversity were observed between three studied delivery modes, neither in diversity nor richness index (**Fig. II-2, C-D**).

We found a microbial core which genera prevalence was not affected by delivery mode that include several groups from *Ruminococcaceae* family, *Romboutsia*, *Roseburia*, *Prevotella*, *Faecalibacterium*, among others (**Supplementary file II-3**). Venn diagrams revealed that ECS and VAG shared higher number of genera than elective CS (**Fig. II-2, E**). Mothers that had an emergency CS were characterized by the prevalence of *Corynebacterium* and *Clostridiales_Family XII* while those that had an elective CS were characterized by higher prevalence of *Prevotella* and *Veillonella* genera. Microbiota from VAG mothers were overrepresented by *Akkermansia* and *Coprococcus* genera.

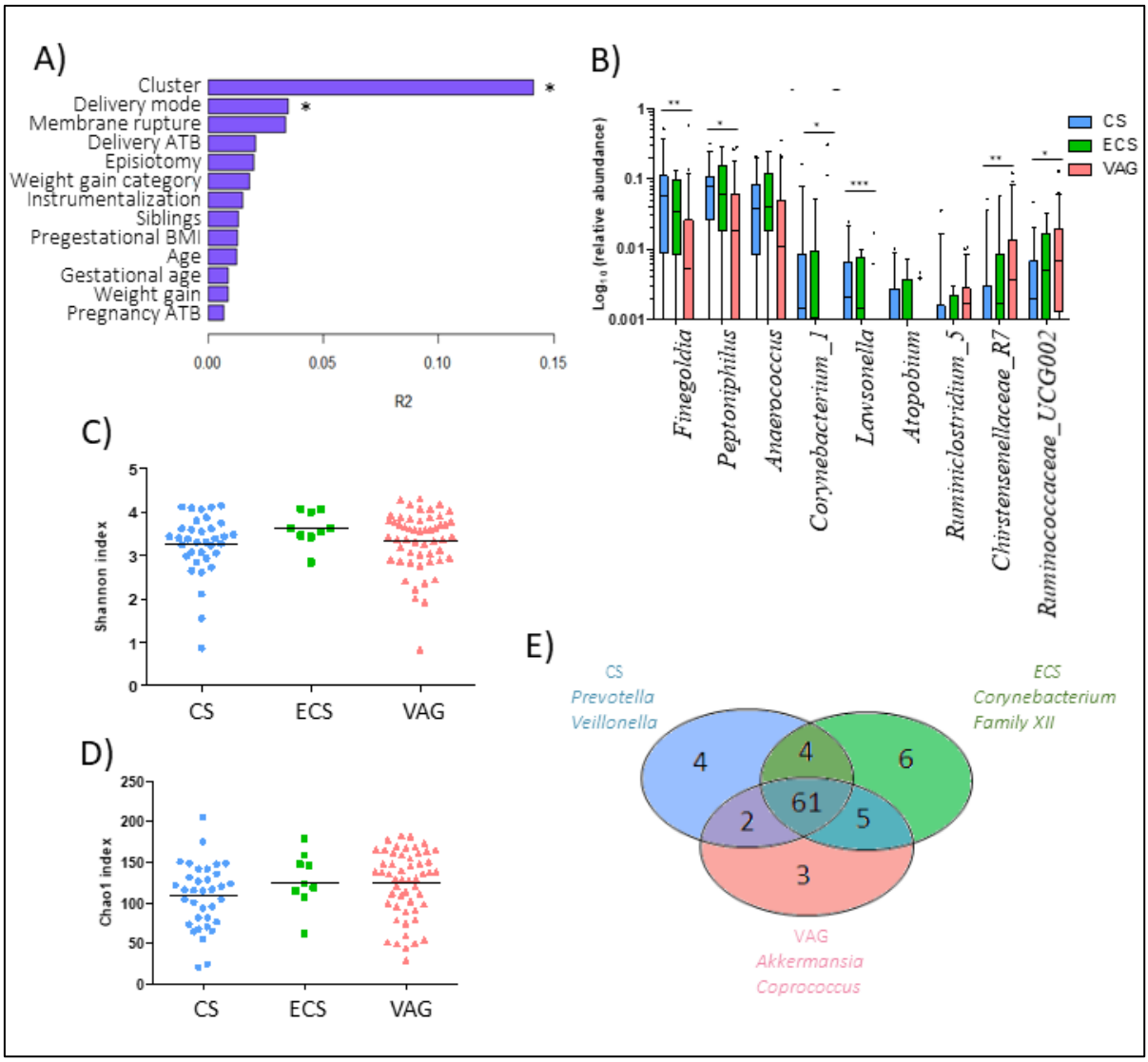


Figure II-2. Maternal microbiota composition is influenced by delivery mode. A) Multivariate analysis of the effect of studied perinatal factors on maternal microbiota composition at delivery based on Bray-Curtis. Membrane rupture. antibiotic at delivery and episiotomy were only studied in mothers who had vaginal delivery. **B**) Boxplot of the main genera that marked the difference of maternal microbiota composition between delivery mode. Data was transformed by log10 of relative abundance of each genus for plotting. Whiskers represented 5-95 percentile interval. **C**) Core group of maternal microbiota composition at genus level performed by Venn diagram. **D-E**) Differences in diversity (**D**) and richness (**E**) of maternal microbiota based on Shannon index according to delivery mode. Line represented the median of each group. CS (Elective C-section). ECS (Emergency C-section). VAG (Vaginal delivery). * *p<0.05.* ** *p<0.01.* *** *p<0.001.*

While mothers who gave birth by VAG were equally distributed in both clusters, Cluster II were enriched in mothers that had a CS (55.4% of prevalence in Cluster II vs 31.7% in Cluster I). Since results suggested that could have interference between both factors, we decided to study their possible effect on maternal microbiota, independently.

We observed that mothers classified in Cluster II were more sensitive to alterations related to delivery mode (*p<0.001*) (**Fig. II-3, A-B**). Indeed, Bray-Curtis dissimilarity index showed that mothers from Cluster I had a microbiota more similar between both delivery modes than those mothers from Cluster II (*p=0.004*) (**Fig. II-3, C**). Within Cluster II, CS mothers showed higher relative abundance of *Lawsonella* (*p=0.014*) and *Finegoldia* (*p=0.082*) while VAG mothers were enriched in *Corynebacterium* genera (*p=0.029*) (**Fig. II-3, B**).

In terms of alpha-diversity (**Fig. II-3, D-E**) and within VAG mothers, those belonging to Cluster I had higher richness than both groups of mothers in Cluster II, those from CS (*p=0.009*) and vaginal birth (*p=0.017*). Also, mothers from Cluster I from both CS and VAG groups showed higher diversity than those from Cluster II (*p<0.01*) (**Fig. II-3, D**).

Regardless other studied factors, we found significant relations between weight gain over pregnancy and maternal microbiota at delivery. Weight gain during pregnancy was negatively associated with *Escherichia/Shigella* genus (rho= -0.51, *p<0.001*) from Proteobacteria phylum (rho= -0.46, *p<0.001*) as well as *Christensenellaceae_R7_group* (rho= -0.30, *p=0.023*). No signficiant differences were found among VAG mothers according to antibiotic administration at delivery.

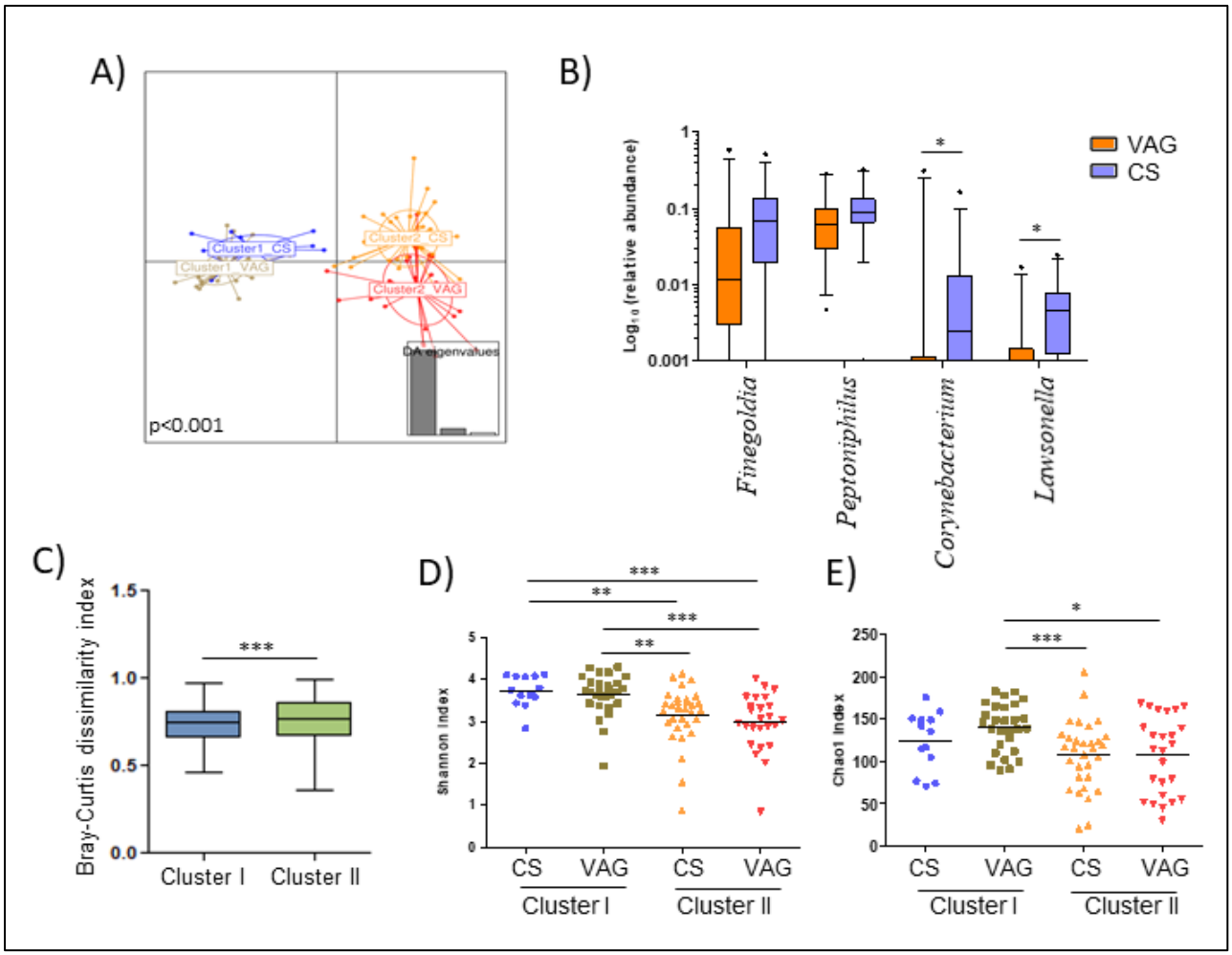


Figure II-3. Maternal microbiota at delivery was dissentingly shaped by delivery mode. A) Discriminant Analysis of Principal Components (DAPC) of the maternal microbiota according to a variable resulted of the combination of cluster and delivery mode. **B**) Boxplot of the main genera that marked the difference of maternal microbiota composition between delivery mode in mothers classified as Cluster II. Data was transformed by log10 of relative abundance of each genus for plotting. Whiskers represented 5-95 percentile interval. **C**) Bray-Curtis dissimilarity index within mothers that had both vaginal and C-section according to the cluster classification. **D-E**) Differences in maternal microbiota diversity (**D**) and richness (**E**) based on Shannon and Chao1 index respectively. according to the variable resulted of the combination of cluster and delivery mode. Line represented the median of each group. * *p<0.05*. ** *p<0.01*. *** *p<0.001*.

Delivery mode was related to saliva cortisol concentrations

Cortisol concentrations in maternal saliva at delivery was 0.55 [IQR: 0.24 – 1.26] µg/dl. Significant differences were found in saliva cortisol concentration according to delivery mode (*p=0.003*) (**Table II-2**). While ECS mothers showed no differences in terms of cortisol concentration with VAG mothers, those from elective CS group presented lower cortisol concentrations compared to VAG mothers (*p=0.001*) (**Fig. II-4, A**). Furthermore, saliva cortisol concentration was related to maternal microbiota

composition (**Table II-2**). Saliva cortisol was negatively associated to *Finegoldia* (rho= -0.43, *p=0.024*), *Peptoniphilus* (rho= -0.36, *p=0.062*), *Corynebacterium* (rho= -0.66, *p<0.001*) and *Lawsonella* (rho= -0.54, *p=0.003*) independently of delivery mode effect. Indeed, salivary cortisol concentration showed a positive correlation with *Christensenellaceae_R7_group* (rho=0.37, *p=0.033*) and genera from *Lachnospiraceae* and *Ruminococcaceae* groups, including *Ruminococcus_1* (rho= 0.42, *p=0.031*) and *Lachnoclostridium* (rho= 0.44, *p=0.023*), and *Lachnospiraceae_UCG_010* (rho= 0.41, *p=0.017*).

Table II-2. Spearman correlation between salivary cortisol concentration and maternal microbiota at delivery.

Genus	rho	P-value	Rel. abund.
Peptoniphilus	-0.36	*0.062*	3.47 [0.49-9.04]
Finegoldia	-0.43	*0.024*	2.9 [0.14-13.42]
Lachnoclostridium	0.44	*0.023*	0.27 [0.05-0.72]
Christensenellaceae_R_7_group	0.37	*0.033*	0.23 [0.01-0.90]
Corynebacterium_1	-0.66	*<0.001*	0.17 [0-0.27]
Lawsonella	-0.54	*0.003*	0.06 [0-0.28]
Staphylococcus	-0.60	*0.001*	0 [0-0.42]
Lachnospiraceae_UCG_010	0.41	*0.017*	0 [0-0.13]
Ruminococcus_1	0.42	*0.031*	0 [0-0.10]
Arcanobacterium	-0.47	*0.013*	0 [0-0.02]

Spearman correlations were performed separately in vaginal or C-section deliveries in order to avoid its effect on both cortisol concentrations and maternal microbiota. Relative abundance was expressed as the median of percentage [interquartile range] of total reads obtained for each faecal samples.

Delivery mode was associated with post-partum maternal weight retention

Multivariate analysis adjusted by covariates including pre-gestational BMI, weight gain during pregnancy and breastfeeding duration showed a significant effect of delivery mode in the weight loss over the 4 months post-partum (**Fig. II-4, B**) (*p<0.001*). Mothers who gave birth by vaginally showed lower difference between their weight at 4 months post-partum and their pre-gestational weight, compared to those CS group mothers (*p<0.001*) (**Fig. II-4, C**). Indeed, a higher number of mothers who

recovered their pre-gestational weight status was found within VAG mothers (67.28%) than in the group of CS mothers (16.13%) (**Fig. II-4, D**).

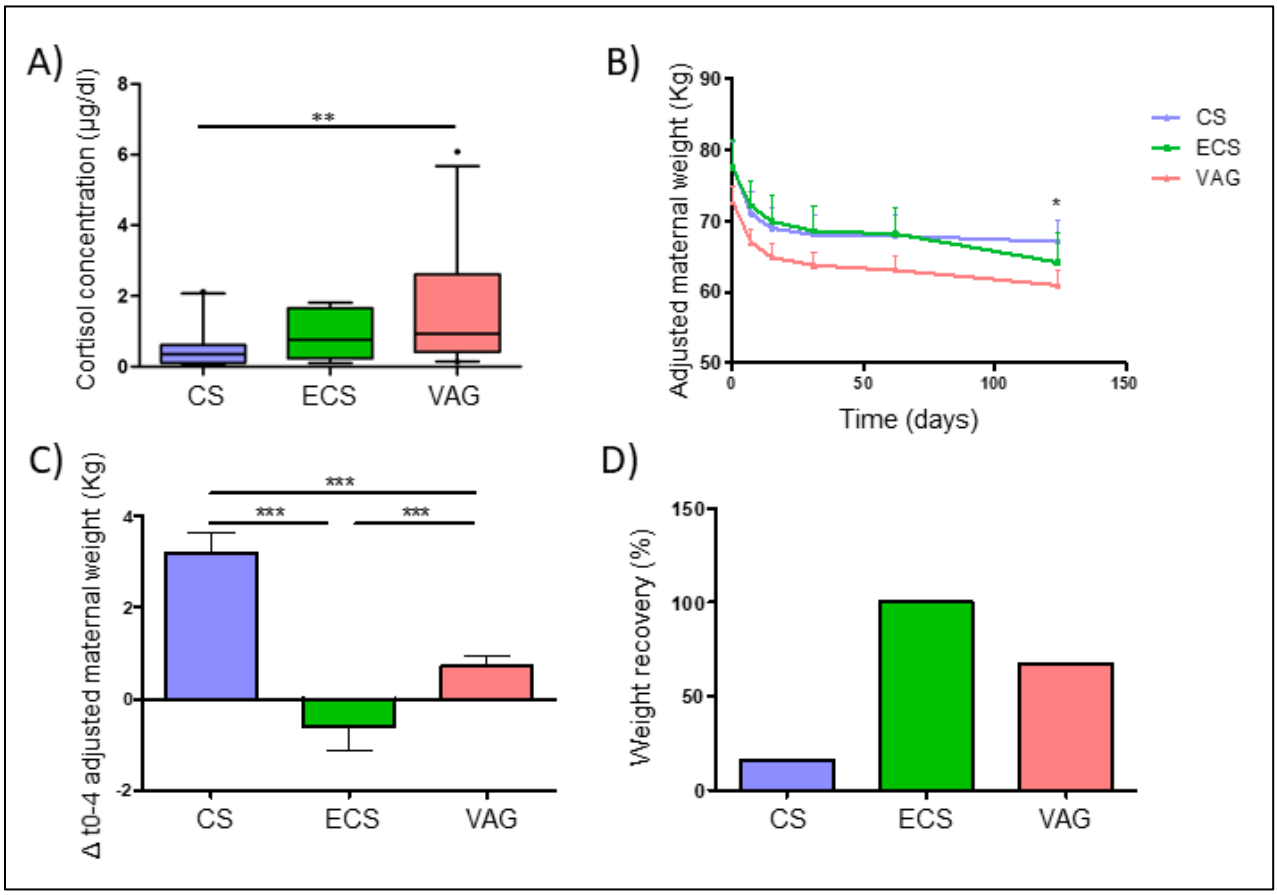


Figure II-4. Delivery mode affected the saliva cortisol concentration and weight gain recovery four month post-partum. A) Differences in saliva cortisol concentration (µg/dl) according to delivery mode. **B**) Evolution of adjusted maternal weight by breastfeeding during this time, pre-gestational weight and weight gain over pregnancy from delivery to 4 months post-partum. Data was expressed as mean and 95% CI. **C**) Differences in the increment of maternal weight from pre-gestational weight to 4 months post-partum. **D**) Percentage of mother that had a recovery of their pre-gestational weight 4 months post-partum. Mother was considered to recovered its pre-gestational weight if they showed a difference between pre and post-partum weight below mean difference + 1SD (standard deviation). * *p<0.05*. ** *p<0.01*. *** *p<0.001*.

DISCUSSION

Pregnancy is an extremely demanding process that affects all the systems of the body, including the maternal microbiota [131]. Several authors have described the variations that occur during gestation, mainly in the oral [506], vaginal [143], and intestinal microbiomes [138]. Furthermore, this period is recognized as a unique time in which health care designed to improve the mother's well-being can affect both maternal and neonatal health. Thus, interventions during pregnancy may provide an opportunity

to positively impact the long-term health of the mother and her offspring. The present study aimed to describe the effects of various perinatal factors on the maternal microbiome at the time of delivery as well as its associations with post-partum maternal weight retention.

Several prior studies have highlighted the importance of the maternal microbiome in relation to the initial colonization of the neonatal microbiota [129,130,153,490,491]. However, only a few studies have evaluated the effects of pregnancy and delivery-related factors on maternal physiology and body composition after birth. In particular, the influence of the metabolic, immunological, and hormonal shifts that occur during delivery on the maternal microbiota are only poorly understood. It is important to recognize that birth represents the first contact between the newborn and a significant bacterial challenge, and further, that the maternal microbiota determines the nature of that initial bacterial exposure [152,507].

We identified two bacterial patterns in the maternal microbiota composition and richness at the time of delivery. One of the clusters (Cluster I) was composed of mothers with a higher microbial richness and diversity, and a microbiota characterized by the presence of health-related bacteria such as *Blautia, Roseburia*, *Lachnospira*, and *Faecalibacterium* genera. The other group of mothers (Cluster II) presented less rich and less diverse microbiota, which were dominated by *Finegoldia*, *Peptoniphilus*, *Campylobacter*, *Prevotella*, *Porphyromonas*, and *Lawsonella*, among others.

The bacteria that dominated in Cluster I are commonly related to the health status of the non-pregnant human microbiota [50,508], mainly due to their ability to produce short-chain fatty acids (SCFA) [50], which is derived from their capacity to metabolize certain dietary compounds, including fiber [180,509]. Propionate, acetate, and butyrate are all associated with multiple functions in the gut and other host systems [510], and they are considered to be the metabolites that control the interplay between the diet, microbiota, and host metabolism [29]. Indeed, these molecules, which are derived from the maternal microbiota, are also known to be associated with metabolic parameters in neonates [511], even participating in immune system development [512]. Additionally, in the MAMI cohort, García-Mantrana et al. [490] identified an association between a cluster represented by the same health-related genera and a higher intake of plant-derived components, including total dietary fiber, omega-3 fatty acids, and polyphenols. Thus, the gut microbial profiles observed at the time of delivery could be partially modulated by the maternal diet during gestation.

Although several prior studies have demonstrated the effect of the mode of delivery on the neonatal microbiota at the time of birth and thereafter [217], little is currently known about the effect of the mode of birth on the maternal microbiota. In the present study, we found that the mode of delivery significantly affected the composition of the maternal microbiota. More specifically, CS delivery was found to be associated with higher relative abundances of *Corynebacterium* and *Lawsonella* genera as well as the gram-positive anaerobic cocci (GPAC), including *Finegoldia* and *Peptoniphilus* genera, while VAG delivery was associated with higher abundances of the *Christensenellaceae* and *Ruminococcaceae* families. Interestingly, the mothers who underwent an emergency CS (ECS) showed an intermediate pattern regarding the microbial composition of their microbiota, which was somewhere between the patterns associated with VAG delivery and elective CS delivery. It is important to recognize that an ECS is normally performed after the hormonal and metabolic processes associated with delivery have been triggered [449,450]. In addition, mothers who undergo an ECS do not usually follow the practice of pre-fasting, which is required for a scheduled CS [513,514]. Thus, our results are in accordance with the hypothesis that hormonal and clinical differences related to the mode of delivery could induce shifts in the maternal gut microbiome, which could have consequences in relation to mother–infant transference during delivery.

Although prior research has focused on just a few factors, such as the use of antibiotics or the delivery mode, the host–microbiota interactions during the perinatal period could be more complex than initially thought, which means that other potential factors, such as diet or hormonal status, should be considered. In the present study, the mothers in Cluster II, which was characterized by less diversity, presented higher rates of CS delivery. However, the VAG delivery mothers were equally distributed among both clusters. Indeed, while the Cluster I mothers had microbiota that were less influenced by the mode of delivery, the identified birth-related shifts in the gut microbiota were more pronounced in the Cluster II mothers.

Several authors have proposed diversity, stability, and resilience to be characteristics associated with a healthy microbiota [53,54]. A resilient ecosystem is capable of remaining stable and resistant in relation to perturbations over time without losing its equilibrium [515]. Both species and functional diversity are the principal features that promote the resilience of the bacterial community. High diversity would allow minor species to fill a niche if the more abundant species were diminished by a

disturbance. In the present study, the mothers from Cluster I, which was characterized by richer and more diverse microbiota, also presented more robust microbiota, which led to less shifts being induced by the delivery mode when compared with those seen in Cluster II. This probably reflects the higher stability and resilience of their gut microbial communities.

The triggering of labor induces several biological processes, including hormonal and immunological system responses, which culminate in childbirth [449,450]. In this study, the saliva cortisol concentration was found to be increased in the mothers who underwent VAG delivery. Cortisol determination has previously been proposed as a stress marker in several studies [516] involving pregnant populations [517]. Further, pregnancy has been suggested to be associated with an increased cortisol concentration throughout the gestation period [518,519]. Indeed, labor is widely assumed to be a stressful process. Miller et al. found the salivary cortisol concentration to gradually increase from the latent to the active phase in low-risk VAG deliveries, with the maximum concentration being observed within two minutes of the onset of labor [520]. In line with our results, some authors have described how VAG deliveries are associated with higher serum cortisol concentrations and more intense inflammation than elective CS deliveries, although the inflammatory response period is shorter than in the case of CS [521–523].

Aside from cortisol, other bioactive molecules have been observed to a greater extent in the maternal plasma obtained from VAG deliveries than in that obtained from CS deliveries [524]. Despite evidence suggesting that the maternal host systems could interact with and alter the human microbiota [60], the possible effects of the physiological response that occurs during labor, as well as during the immediate pre- and post-labor periods, have not yet been adequately investigated. Our results suggest a possible relation between the salivary cortisol concentration and the composition of the maternal gut microbiota at delivery. Even in mothers who have undergone a VAG delivery, the cortisol concentration has been found to be negatively related to those genera associated with CS delivery, including the above-mentioned GPAC (*Finegoldia, Peptoniphilus,* and *Anaerococcus*). However, the genera known to be enriched in VAG delivery mothers, including genera from the *Christensenellaceae* and *Ruminococcaceae* families, were observed to be positively associated with the salivary cortisol concentration. These results suggest that the cortisol concentration may play a role in the delivery-mode-related shifts observed in the maternal microbiota. The possible

mechanisms by which the circulating cortisol concentration could modify the maternal gut composition include bile acid and cholesterol regulation, acidic gut secretion and motility alterations, and gut barrier function disruption [525]. Other perinatal labor-related physiological changes could also influence the observed clustering of mothers based on their gut microbiota.

Furthermore, both VAG delivery and the cortisol concentration are known to be associated with health-related genera, including members of the *Christensenellaceae* [287], *Lachnospiraceae*, and *Ruminococcaceae* families [50]. In fact, Vojinovic et al. found these groups to be correlated and involved in several metabolic pathways, including SCFA production and bile acid metabolism [38]. In particular, the *Christensenellaceae* family has been observed to be inversely associated with the BMI [286,526] of lean individuals [453], as well as with a healthy condition, in studies investigating inflammatory bowel disease (IBD) [527] and other inflammatory gut-related disorders [58,528]. For instance, Papa et al. reported lower levels of *Christensenellaceae* in fecal samples obtained from pediatric and young adult IBD patients when compared with samples obtained from healthy controls [529]. In the present study, we identified negative correlation between weight gain during pregnancy and this bacterial group. Furthermore, some prior studies have suggested that this family could be among the most hereditable families [286,530], which suggests that it might exert an influence on neonatal health.

In this study, more VAG delivery mothers were found to have returned to their pre-pregnancy weight after four months post-partum than CS delivery mothers. Post-partum weight retention (PPWR) has been identified as an important challenge facing public health systems worldwide due to its negative association with both maternal recovery and neonatal development [531]. The use of CS is commonly associated with longer recovery times, a special post-surgical intervention diet [532,533], and delayed breastfeeding [227,534], which could also influence PPWR.

The mother's pregestational BMI and gestational weight gain have been found to be related to increased PPWR during the post-partum period [188]. Our model, when adjusted according to these covariables, indicated the VAG delivery mothers to exhibit significantly lower PPWR. However, the mother's pregestational BMI and gestational weight gain have also been proposed as risk factors in relation to CS intervention [189]. Thus, although several epidemiological studies have identified higher PPWR in mothers who have undergone CS deliveries, there remain contradictions in terms of the overall

results [234], possibly due to the complex interactions that occur between all these factors.

As mentioned above, metabolites other than cortisol may have influenced the delivery-mode-related differences we observed regarding both the microbiota composition and post-partum weight loss. Recently, Koren et al. demonstrated that the progesterone concentration could influence the relative abundance of *Bifidobacterium* in the maternal gut microbiota [451]. Further, Rebelo et al. identified differences in the adiponectin concentration according to the mode of birth, with CS delivery being associated with a lower adiponectin concentration than VAG delivery at 30–45 days post-partum, which was likely mediated by the long-term inflammation associated with the CS procedure [535].

It is important to recognize that the present study had a few limitations, including the low sample size, which might have influenced the power analysis of the observed results. Additionally, the lack of a microbiota profile during the post-partum period represents another weakness of the study. However, the main aim of the analysis was to describe the effects of delivery-related factors on the maternal microbiota at the time of birth. Among the strengths of this study, we wish to highlight the detailed information gathered regarding the maternal clinical and anthropometrical characteristics, which allowed us to evaluate multiple factors within the same population. Our contact with hospitals and community health centers greatly helped in reducing the rate of data collection mistakes and also decreased the amount of missing data.

CONCLUSION

In summary, the results of this study suggest the existence of a complex relation between hormonal delivery-related changes, the gut microbiota, and maternal health outcomes in the post-partum period, which might impact on neonatal development during this important period and so have long-term consequences. However, the link between these factors has been underexplored in the prior maternal health research. Our results support the hypothesis that the mode of delivery and codominant hormonal changes could influence the maternal microbiota at the point of delivery. Further research is required to comprehensively explain the molecular mechanisms that mediate

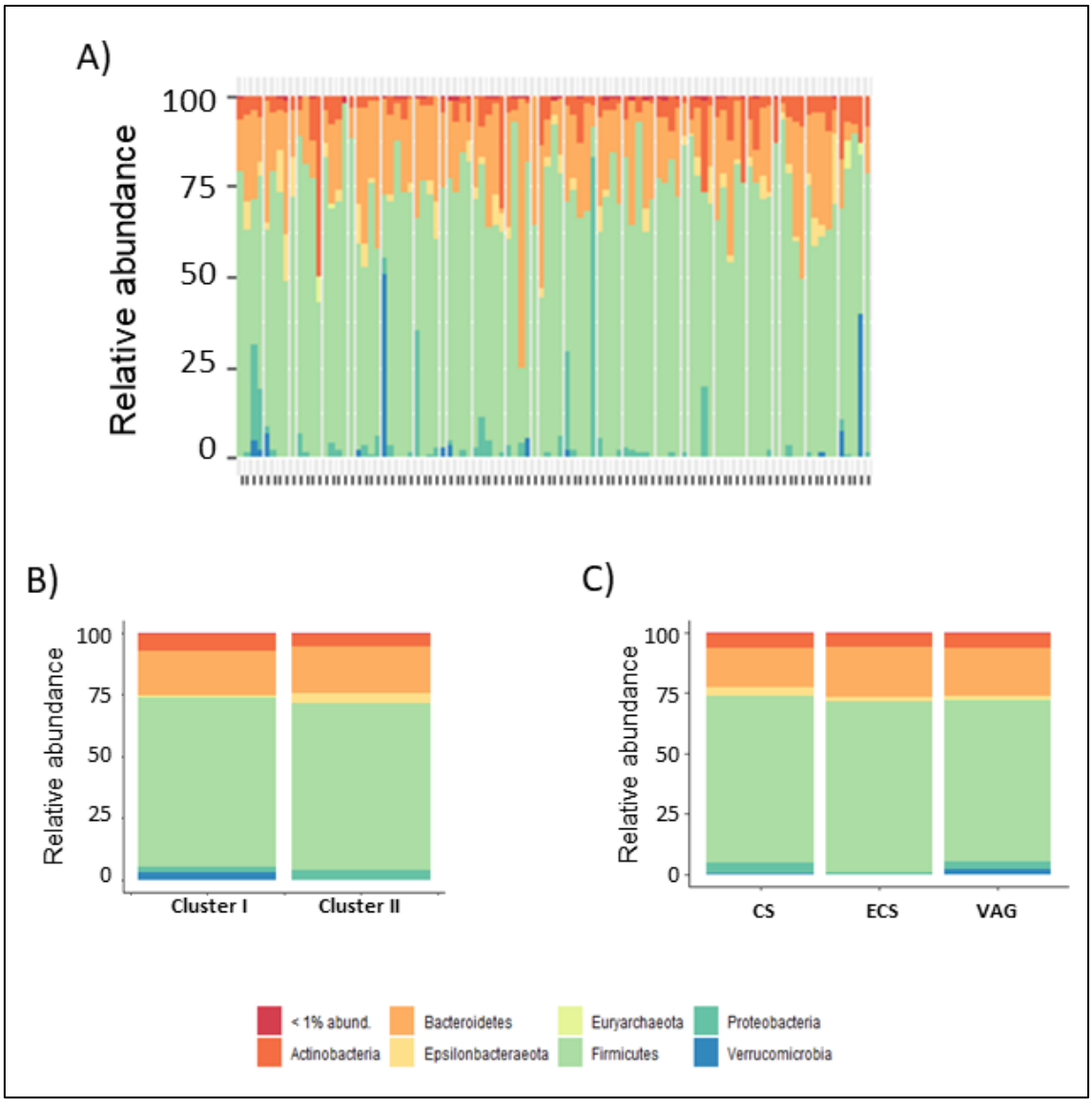
the host–microbiota interplay at birth in order to design clinical strategies for improving maternal and neonatal health during the post-partum period and beyond.

CHAPTER II - SUPPLEMENTARY DATA

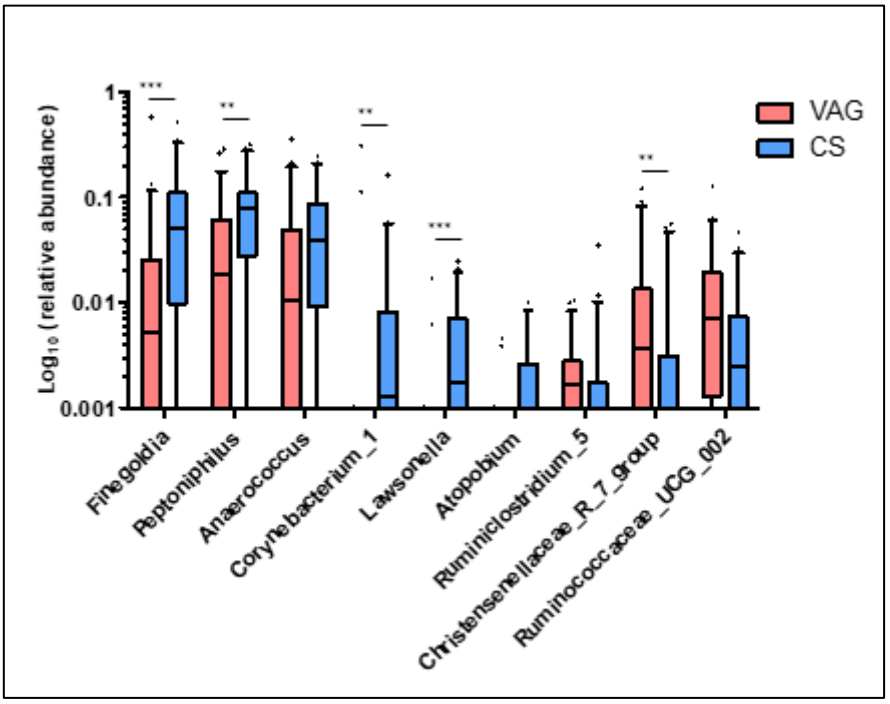
Supplementary file II-1. Characteristics of population participant in the post-partum weight retention study

	Weight retention study (n=167)
Weight gain (Kg)	12 [10-15]
Low	58 (34.7)
Recommended	73 (43.7)
High	36 (21.6)
Pre-gestational BMI (Kg/m^2)	22.31 [20.44-24.46]
NP	118 (70.65)
OW	38 (22.8)
LW	11 (6.6)
Antibiotic pregnancy (positive cases)	57 (33.9)
Gestational age (weeks)	40 [39-40]
Delivery mode	
Vaginal	111 (66.5)
Emergency C-section	25 (14.9)
Elective C-section	31 (18.5)
Anthropometric data (maternal weight)	
Pregestational (Kg)	60.50 [54.5-66]
Delivery (Kg)	72 [67.5-80]
7 days (Kg)	67.50 [61-73.88]
15 days (Kg)	65.7 [60-71.8]
31 days (Kg)	64 [58-70.5]
2 months (Kg)	63 [57.50-70.13]
4 months (Kg)	61.50 [56-69]

Normally distributed data was presented as mean ± SD and non-normal data as median [IQR]. Categorical variables were expressed as positive cases (percentage). In the case of microbiota study population. only a subset of participants had anthropometric data during post-partum period (n=40).



Supplementary file II-2. Maternal microbiota composition at phylum level at delivery time. Bar plot at phylum level of maternal microbiota according to individual (**A**). cluster (**B**) and delivery mode (**C**).



Supplementary file II-3. Differences in maternal microbiota at delivery time according to delivery mode. Boxplot of the main genera that marked the difference of maternal microbiota composition between delivery mode grouping both C-section modes. Data was transformed by log10 of relative abundance of each genus for plotting. Whiskers represented 5-95 percentile interval. * p<*0.05*, ** p<*0.01*, *** p<*0.001*.

Supplementary file II-4. Occurrence of genera from maternal microbial core at delivery according to delivery mode.

Taxa	CS (%)	ECS (%)	VAG (%)
UBA1819	54	56	77
Subdoligranulum	91	100	89
Streptococcus	91	100	91
S5A14a	57	67	51
Ruminococcus_2	57	44	62
Ruminococcaceae_UCG014	43	67	60
Ruminococcaceae_UCG013	63	78	77
Ruminococcaceae_UCG005	66	67	77
Ruminococcaceae_UCG004	57	56	57
Ruminococcaceae_UCG003	40	56	60
Ruminococcaceae_UCG002	69	78	89
Ruminococcaceae_NK4A214_group	57	56	62
Ruminiclostridium_9	60	78	72
Ruminiclostridium_5	86	78	89
Roseburia	63	89	81
Romboutsia	71	78	77
Prevotella_6	40	67	60
Prevotella	86	100	79
Porphyromonas	83	89	77
Peptoniphilus	91	89	91
Parasutterella	40	67	55
Parabacteroides	77	89	89
Oscillibacter	66	89	74
Odoribacter	71	78	79
Murdochiella	69	78	57
Mobiluncus	74	89	62
Lawsonella	89	89	60
Lactobacillus	71	78	55
Lachnospiraceae_NK4A136_group	66	78	87
Lachnospiraceae_ND3007_group	63	67	66
Lachnospiraceae_FCS020_group	57	44	70
Lachnospira	69	89	77
Lachnoclostridium	77	89	87
Intestinibacter	51	89	58
Fusicatenibacter	69	89	81
Flavonifractor	43	78	58
Finegoldia	94	100	83
Family_XIII_AD3011_group	46	78	72
Faecalibacterium	86	78	96
Ezakiella	83	89	89
EscherichiaShigella	83	100	98
Erysipelotrichaceae_UCG003	69	67	74
Dorea	94	89	89
Dialister	83	100	75
Corynebacterium_1	74	78	42
Coprococcus_3	80	56	79
Coprococcus_1	74	67	72
Collinsella	83	78	81
Clostridium_sensu_stricto_1	49	56	68
Christensenellaceae_R7_group	60	67	92
Campylobacter	89	78	75
Butyricicoccus	69	89	75
Blautia	97	89	94
Bilophila	49	56	66
Bifidobacterium	94	100	89
Bacteroides	97	100	98
Anaerostipes	77	89	81
Anaerococcus	91	100	85
Alistipes	83	89	94
Agathobacter	91	78	81

Occurrence was expressed as percentage of samples with presence of each genera within a delivery mode group. VA (vaginal delivery), ECS (emergency C-section), CS (elective C-section)

CHAPTER III

MATERNAL DIET DURING PREGNANCY AND INTESTINAL MARKERS ARE ASSOCIATED WITH EARLY GUT MICROBIOTA.

CHAPTER III - MATERNAL DIET DURING PREGNANCY AND INTESTINAL MARKERS ARE ASSOCIATED WITH EARLY GUT MICROBIOTA.

Selma-Royo, M.; García-Mantrana, I.; Calatayud, M.; Parra-Llorca, A.; Martínez-Costa, C.; Collado, M.C. Maternal diet and intestinal homeostasis influence early gut microbiota. Eur. J. Nutr. 2020.

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ABTRACT

Diet has an important role in host-microbiome interplay, which may result in intestinal permeability changes and physio pathological effects at a systemic level. Despite the importance of maternal microbiota as the main contributor of the initial microbial seeding, little is known about the effects of maternal diet during pregnancy on maternal-neonatal microbiota.

To ascertain the possible associations between maternal dietary intake during pregnancy and neonatal microbiota at birth, and to evaluate the relation with maternal intestinal markers.

In a nested cross-sectional study in the longitudinal MAMI cohort, maternal-neonatal microbiota profiling at birth (n = 73) was assessed by 16S rRNA gene sequencing. Maternal intestinal markers as zonulin, intestinal alkaline phosphatase (IAP) activity, and faecal calprotectin were measured in faeces. Furthermore, maternal-neonatal clinical and anthropometric data as well as maternal nutrient intake during pregnancy obtained by FFQ questionnaires were collected.

Maternal diet is associated with both maternal and neonatal microbiota at time of birth, in a delivery mode dependent manner. The existing link between maternal diet, intestinal makers, and neonatal gut microbiota would be mainly influenced by intake of saturated (SFA) and monounsaturated fatty acids (MUFA). Firmicutes members in the neonatal microbiota were positively associated with maternal fat intake, especially SFA and MUFA, and negatively correlated to fibre, proteins from vegetable sources, and vitamins.

Maternal diet during pregnancy, mainly fat intake (SFA and MUFA), was related to intestinal biochemical markers, thus likely shifting microbial transmission to the neonate, and priming the neonatal microbial profile with potential health outcomes.

Keywords: maternal diet, intestinal permeability, microbiota.

INTRODUCTION

Maternal microbiota represents the most important microbial source for the neonatal microbiota colonization process. Increasing evidence suggests that maternal diet during pregnancy shapes the offspring's microbiota composition and intestinal development in the short and long term [194]. Studies of animal models have highlighted the role of the maternal intake of specific nutrients, such as fibre, which drive neonatal microbiota and suppress intestinal permeability [536].

During pregnancy, the female body adapts its metabolism, hormonal cascades, and immunological events to optimize the growth of the foetus and prepare the body for lactation [132], leading to similar markers to those observed in metabolic syndrome [132,537]. Gut microbiota is also modified by these changes, which have several consequences including increasing the energy extraction efficiency from the diet [132]. An increased intestinal permeability has also been reported in pregnant women [538], which may relate to low-grade systemic inflammation in mothers and neonates [539]. Higher gut permeability was observed in overweight pregnant women [540] and those with gestational diabetes mellitus (GDM) risk [541,542] compared to healthy pregnant women.

Most studies of microbiota and dietary patterns have evaluated extreme diets (e.g., Western diet vs. non-Western diet) or diseased individuals (e.g., obese) [543,544]. However, the impact of diet and intestinal permeability during pregnancy on female microbiota and the possible subsequent health consequences are still not fully understood, specifically in healthy cohorts (non-obese) following a healthy standard diet. Few publications have shown that maternal diet can modulate the gut microbiome of the mother or infant [198]. Thus, more information is required to address how nutrient patterns influence maternal and neonate gut microbiota.

This study assesses the association of maternal nutrients intake during gestation on maternal intestinal functional markers and determines its relation to maternal-neonatal gut microbiota. The aim is to shed light on the potential factors related to the vertical mother–infant transmission affecting the critical initial contact of the neonate with maternal microbiota and to identify possible "window" for dietary intervention promoting health-associated taxa in the gut of the mother and their offspring.

MATERIALS AND METHODS

Study design and volunteers

A total number of 116 mother-infant dyads from the observational study MAMI birth cohort recruited during 2015-2017 were included in the study. MAMI cohort consist in a prospective mother-infant cohort in the Spanish-Mediterranean area as detailed previously [420]. Samples at birth time were selected to assess the maternal diet association with the neonatal and maternal microbiota.

Clinical, anthropometric, and obstetric characteristics

Nutritional, anthropometrical and clinical parameters of mother-infant pairs were recorded at birth time. Available data included antibiotic consumption during pregnancy, pre-gestational body mass index (BMI) and weight gain during pregnancy, gestational age, as well as mode of birth, intrapartum antibiotic exposure, neonatal weight and length at birth.

None of the participating volunteers have been diagnosed of any disease; neither were under drug treatment or prebiotics administration, except for antibiotics use during pregnancy and/or at delivery.

Assessment of maternal dietary intake

Women were asked to fill out in a 140-item validated food questionnaire (FFQ) about their diet during the pregnancy [421] within first week after delivery. FFQ information was analysed using the Nutrients Food Composition Tables developed by the Centro de Enseñanza Superior de Nutrición Humana y Dietética (CESNID), Spain [494]. FFQ has been validated previously [422]. For further analysis, the caloric contribution (in percentage) of each macronutrients to total energy intake, including total protein, those from both animal and vegetable sources, lipids, SFA, mono- and poly-unsaturated fatty acids (MUFA and PUFA), total carbohydrates, polysaccharides and fibre, was calculated. Proteins and carbohydrates, including polysaccharides, was considered to contribute in 4 kcal/g, lipids 9 kcal/g and fibre, as special carbohydrate, was estimated to have 2 kcal/gr contribution.

Maternal-neonatal biological samples

Maternal-neonatal rectal swabs were collected by trained personnel at delivery room at the hospital [420]. Maternal gut samples were obtained by swabbing a sterile, cotton-tipped swab in the rectum before giving birth in the delivery room. Neonatal sample collection was also obtained by introducing a sterile, cotton-tipped swab in the neonatal rectum after birth on the resus-A-cradle in the delivery room. After collection, samples were immediately stored at -20ºC and transported in dry ice within the next 24 hours to the laboratory for storage at -80ºC until further analysis.

Faecal samples were homogenized in PBS 1X, vortexed 1 min and centrifuged at 13,000g for 10 min. Supernatants were collected for the intestinal markers quantification while the resulted pellet was used for the total DNA extraction procedure.

Maternal intestinal markers

Faecal supernatants were used to determine the following intestinal markers: zonulin, Intestinal alkaline phosphatase (IAP) and calprotectin.

Zonulin measurement was performed by commercial ELISA test (MyBioSource, San Diego, CA) according to manufacturer protocol and using a 1:2 dilution sample. Final concentrations were calculated based on each standard curve values, including in duplicate in each batch. IAP activity was quantified by the Alkaline Phosphatase Diethanolamine Activity Kit following the manufacturer's instructions (Sigma-Aldrich, USA) and faecal calprotectin was determined by ELISA assay (Wuhan Fine Biotech, Wuhan, China) according to manufacturer's instruction with a 1:5 dilution of maternal faecal supernatants in sample dilution buffer from the kit reagents.

DNA Extraction and 16S rRNA amplicon sequencing

Total DNA was isolated from the maternal-neonatal faecal swabs using the Master-Pure DNA Extraction Kit (Epicentre, Madison, WI, United States) by the manufacture's instruction with some modifications. Cell lysis was performed by mechanical disruption using 3-µm diameter glass beads 1 min at 6 m/s and a FastPrep 24-5G Homogenizer (MP Biomedicals), followed by a chemical treatment with lysis buffer from the extraction kit, supplemented with lysozyme (20 mg/ml) and mutanolysine (5U/ml) (60 min at 37ºC). DNA Purification Kit (Macherey-Nagel, Duren, Germany) was used to the DNA purification. The resulting DNA was quantified

and normalized to 5ng/ul by Qubit 2.0 Fluorometer (Life Technology, Carlsbad, CA, United States).

The microbial composition was determined by sequencing of the V3-V4 variable region of the 16S rRNA gene. The region was amplified using the Illumina adapter overhang nucleotide sequences following Illumina protocol. Nextera XT Index Kit (Illumina, CA, USA) was used for the multiplexing step and a Bioanalyzer DNA 1000 chip (Agilent Technologies, CA, USA) for checking the PCR product quality. Libraries were sequenced using a 2x300 pb paired-end run (MiSeq Reagent kit v3) on a MiSeq-Illumina platform (FISABIO sequencing service, Valencia, Spain).

Quality filtering, sequence joining, and chimera removal were achieved using DADA2 pipeline [545]. Taxonomic assignment was performed using Silva v132 database [440] with the addition of the specie level classification. Samples with low relative abundance (<0.01%) and those present in less than 5 times in at least 20% of the samples were filtered. Similarly, sequences from contaminants and those classified as Cyanobacteria and Chloroplast, considered ingested plant material were also removed. Samples with less than 1000 reads (n=5) were also removed from the final data analysis. Bacterial counts were normalized by Total Sum Scaling (TSS).

Statistical analysis

Microbial sequences were analysed by use of Calypso online platform (v. 8.72) [443], SPSS software [444] and Rstudio environment [445] were used. RStudio was used to perform the PERMANOVA (Adonis) multivariate analysis based on Bray-Curtis distance in maternal and neonatal microbiota by the vegan R package [505]. This R package was also used for microbial alpha diversity by Shannon (diversity) and Chao1 indexes (richness). The heatmaps between bacterial taxonomic groups and dietary components were performed through "ggplot" package [446]. Indeed, spearman rank correlations between maternal diet and microbiota composition were adjusted by delivery mode. Multivariate analysis of maternal-neonatal microbiota, including Discriminant analysis of Principal Components (DAPC) and Adonis test (PERMANOVA) were also achieved based on Bray-Curtis distance in Calypso online platform.

SPSS software v.25 was used for the spearman correlations and for T-test and Mann-Whitney analysis according to data normality assessed by Shapiro-Wilk test. Chi-

Squared test (2x2) was performed to assess the significance of the differences in the population characteristics and nutrients intakes in categorical variables.

In order to group the mothers according to their diet, we performed a classification of the mothers for each nutrient according whether their consumption was above or below the average of the study population. The differences between the groups were visualized by discriminant of principal components analysis (DAPC) at the OTU level, and statistical significance was assessed using the Adonis test. DEseq2 was conducted in Calypso platform to found significant differences in maternal and neonatal microbiota according to these classifications. All comparisons performed with DEseq2 was adjusted by FDR method.

Ethical aspects

All participants received oral and written information about the study and written consent was obtained. The study was approved by the Hospital ethics committees (HECs) (Hospital Universitario y Politécnico La Fe and Hospital Clínico Universitario de Valencia). The study is registered on the ClinicalTrial.gov platform, with the registration number NCT03552939.

RESULTS

Characteristics of study participants

A total number of 73 mothers-neonates were finally included according to the full availability of biological samples, clinical and dietary data for the cross-sectional study (**Supplementary file III-1**). The maternal population showed a median of 35 years old, a pre-gestational BMI of 21.8 kg/m^2, and a weight gain during pregnancy of around 12.68 ± 4.28 kg, which is in the range recommended by the Institute of Medicine [438] (**Table III-1**).

C-section births represented 38.4% (28/73), and no significant differences in pre-pregnancy BMI and weight gain over pregnancy were found between modes of birth (vaginal vs. C-section). Significant differences in maternal antibiotic treatment at delivery ($p < 0.001$) and gestational age ($p = 0.018$) were found between the vaginal and C-section births. However, neither gestational age nor maternal age showed to influence maternal microbiota (data not shown).

We observed significant correlations between the maternal weight gain during pregnancy and some nutrients consumption (**Supplementary file III-2**). Weight gain over gestation was positively correlated with lipid intake (rho = 0.24, *p =0.041*) and negatively associated with fibre (rho = -0.28, *p =0.016*) and proteins from a vegetable source (rho =0 -0.25, *p =0.035*).

Table III-1. Characteristics of study participants at delivery

	Total (n=73)	Vaginal delivery (n=45)	C-section (n=28) [a]	p-value
Maternal Data				
Age (years)	35 [31-36.25]	34[31-36]	35 [33-37]	*0.107*
Pre-pregnancy BMI (Kg/cm^2)	21.8 [20.43-24.13]	21.51 [20.43-23.88]	21.89 [20.46-24.50]	*0.617*
Weight gain over pregnancy (Kg)	12.68 ± 4.28	12.11 ± 3.52	13.64 ± 5.23	*0.186*
Antibiotic during pregnancy (cases)	29 (39.7 %)	19 (42.2%)	10 (35.7%)	*0.581*
Daily Energy intake (kcal)	2882 ± 789	2937 ± 793	2795 ± 788	*0.458*
Neonate data				
Gestational age (weeks)	40 [39-40]	40 [39-40]	39 [38-40]	***0.018****
Neonatal weight at birth (g)	3323 ± 416	3297 ± 339	3367 ± 524	*0.536*
Neonatal height at birth (cm)	50.16 ± 1.94	50.12 ± 1.63	50.24 ± 2.40	*0.821*
BMI z-score at birth	-0.16 [-0.60-0.50]	-0.28 [-0.66-0.30]	-0.09 [-0.48-0.62]	*0.314*
Intrapartum antibiotic exposure (nº cases)	35 (47.9%)	7 (15.6%)	28 (100%)	***<0.001****
Sex				
Male	40 (54.8%) [b]	22 (48.8%)	18 (64.3%)	*0.199*
Female	33 (45.2 %)	23 (51.2%)	10 (35.7%)	

BMI-z scores were computed using WHO Anthro software (www.who.int/ childgrowth/software/en/). Significant categories were marked in bold. (a) One participant in the CS group with missing delivery associated data. Parametric data were expressed as mean ±SD and non-parametric data as median [IQR].

Relation between maternal diet and maternal gut microbiota composition

The maternal diet was presented as a daily intake for each nutrient (**Table III-2**). The energy intake average was 2,882 ± 789 kcal/day, without differences according to mode of delivery (vaginal vs C-section). Higher lipid and saturated fatty acids (SFA) consumption during pregnancy was significantly associated with higher animal protein intake and with lower vegetable protein, total carbohydrates, polysaccharides, and fibre (**Supplementary file III-3**). However, diets characterized by higher total carbohydrate, including polysaccharides, consumption during pregnancy were related to a higher

intake of vegetable protein and fibre. Therefore, we observed two dietary patterns in our population. The first one was characterized by a high intake of lipids, SFA, and animal protein and by a low intake of vegetable protein and fibre; while the second one was based on higher intakes of total carbohydrate, polysaccharide, and fibre and lower intakes of lipids and SFA.

Table III-2. Maternal diet during gestation according to mode of delivery

Diet	Total	Vaginal	C-section	p-value
Total protein (g/day)	120 ± 40 (16.7)	123 ± 41 (16.7)	116 ± 39 (16.7)	*0.477*
Animal protein (g/day)	80 ± 33 (11.1)	81 ± 33 (11.0)	79 ± 35 (11.3)	*0.820*
Vegetable protein (g/day)	40 ± 3 (5.6)	42 ± 13 (5.8)	37 ± 13 (5.3)	*0.817*
Lipids (g/day)	143 ± 45 (44.5)	142 ± 43 (43.4)	144 ± 49 (46.4)	*0.814*
SFA (g/day)	40 ± 16 (12.2)	39 ± 16 (11.9)	40 ± 18 (12.7)	*0.963*
MUFA (g/day)	67 ± 20 (21.3)	67 ± 21 (20.9)	67 ± 20 (22.0)	*0.502*
PUFA (g/day)	25 ± 12 (7.7)	24 ± 10 (7.36)	26 ± 15 (8.3)	*0.568*
Cholesterol (mg/day)	422 ± 170	431 ± 170	408 ± 173	*0.106*
Carbohydrates (g/day)	277 ± 88 (38.5)	291 ± 86 (39.6)	256 ± 87 (36.7)	*0.168*
Polysaccharides (g/day)	142 ± 47 (19.8)	148 ± 47 (20.3)	131 ± 74 (19.1)	*0.148*
Fibre (g/day)	36 ± 14 (2.5)	38 ± 15 (2.7)	32 ± 11 (2.3)	*0.084*
Vitamin A (mcg/day)	1647 ± 863	1659 ± 896	1629 ± 824	*0.887*
Retinoid (mcg/day)	393 ± 245	410 ± 264	366 ± 212	*0.453*
Carotenoid (mgc/day)	7517 ± 4642	7482 ± 4900	7572 ± 4282	*0.937*
Vitamin D (mcg/day)	3 ± 2	4 ± 3	3 ± 2	*0.156*
Vitamin E (mg/day)	22 ± 9	22 ± 7	23 ± 11	*0.604*
Thiamine (mg/day)	2 ± 0.7	3 ± 0.8	2 ± 0.6	*0.507*
Riboflavin (mg/day)	2 ± 0.8	3 ± 0.9	2 ± 0.7	*0.470*
Niacin (mg/day)	28 ± 9	29 ± 10	27 ± 8	*0.331*
Vitamin B6 (mg/day)	3 ± 1.2	4 ± 1.4	3 ± 0.9	*0.208*
Folic acid (mcg/day)	653 ± 282	672 ± 315	623 ± 221	*0.478*
Vitamin B12 (mcg/day)	10 ± 8	10 ± 8	10 ± 8	*0.955*
Vitamin C (mg/day)	347 ± 168	356 ± 190	333 ± 125	*0.573*

Each nutrients value was extracted by the food frequency questionnaire using the Centro de Enseñanza Superior de Nutrición Humana y Dietética (CESNID) tables [494]. The percentage of caloric contribution of each nutrient to total energy intake was presented in brackets. Saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA).

Maternal diet during gestation was associated with the maternal microbiota composition (**Fig III-1**). In the multivariate analysis, the results showed that MUFA intake significantly affects the overall structure of maternal microbiota at delivery (*p =0.03*, PERMANOVA) at the ASV level (**Fig III-1, A**). We found a positive correlation between lipid intake and Firmicutes phylum (rho = 0.30, *p =0.014*). Mothers classified

as having high lipids ($p < 0.001$) and consumers of SFA ($p = 0.011$) had lower Proteobacteria relative abundance in their microbiota (**Fig III-1, B-E**). However, mothers classified as having higher carbohydrate consumption showed a microbiota enriched by this phylum ($p = 0.003$). A similar pattern was found with Bacteroidetes phylum, which was found to be enriched in microbiota of mothers with higher carbohydrates intake ($p = 0.052$) and depleted in lipids consumers ($p = 0.011$). Besides this relation, total protein intake, especially those from an animal source (rho = -0.32, $p = 0.009$), was negatively associated with Actinobacteria phylum genera.

At the genus level, several correlations were found between nutrient intake and maternal microbiota (**Supplementary file III-4**); a higher relative abundance of *Lachnospira* was associated with lower total carbohydrates intake (rho = -0.27, $p = 0.040$) and showed a positive correlation with total lipid intake (rho = 0.28, $p = 0.039$), including SFA and MUFA but not PUFA. Similarly, a pattern was found in *Rombustia* genus, which was associated with MUFA intake (rho = 0.28, $p = 0.025$) but negatively linked with total carbohydrates (rho = -0.25, $p = 0.049$) intake, especially polysaccharides (rho = -0.28, $p = 0.22$). No significant effect of any nutrient consumption was observed in the alpha diversity of maternal microbiota at delivery.

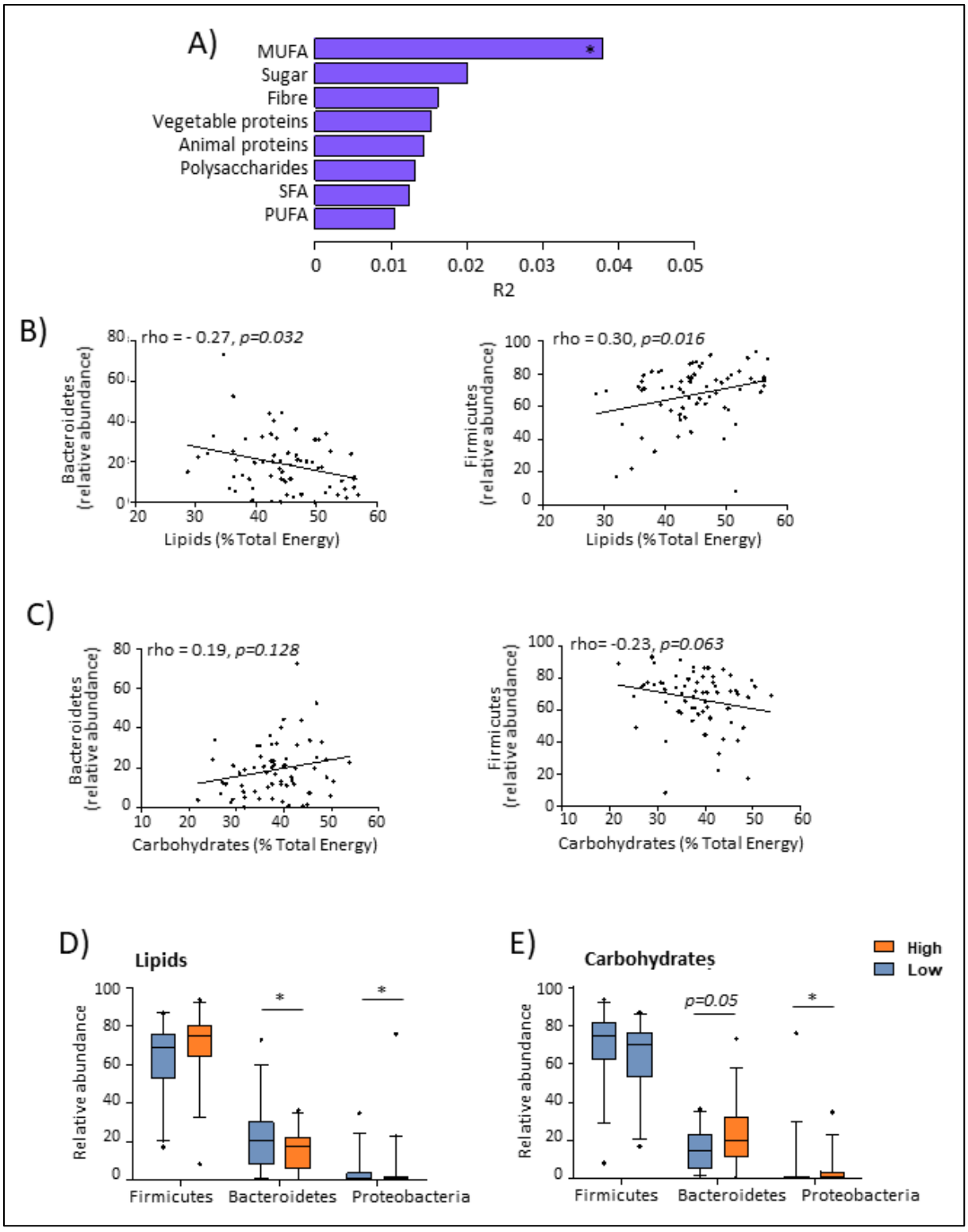


Figure III-1. Maternal diet was associated with maternal microbiota. **A)** Multivariate analysis of the effect of studied nutrients on maternal microbiota composition at delivery based on Bray-Curtis. **B)** Maternal lipids intake was associated to the relative abundances of Bacteroidetes and Firmicutes (Spearman's rank correlation). **C)** Maternal total carbohydrates intake was associated to maternal relative abundances of Bacteroidetes and Firmicutes (Spearman's rank correlation). **D-E)** Comparison of principal phyla of maternal microbiota between mothers classified as high/low consumers of total lipids **(D)** and carbohydrates intakes **(E)**. Nutrients consumption was expressed as caloric contribution to total energy intake in percentage.

Maternal diet and intestinal markers

Maternal diet was associated with the intestinal zonulin concentrations (42.05 ± 9.9 ng/ml faecal supernatant) at birth. A lower zonulin concentration was related to a higher total carbohydrate intake (rho = -0.37, *p = 0.012*), including polysaccharide (rho = -0.31, *p =0.035*) intake during pregnancy. However, a higher consumption of lipids (rho = 0.32, *p = 0.031*), including MUFA intake (rho = 0.29, *p = 0.05*), was associated with higher zonulin concentrations (**Fig III-2, A-D**). Neither protein nor fibre consumption presented any significant correlation with zonulin concentration on maternal stool samples.

We found some positive relations between excreted zonulin concentrations and some genus of maternal microbiota. A higher zonulin concentration showed a tendency to lower Proteobacteria phylum (rho = -0.28, *p = 0.067*). Zonulin was positively correlated with relative *Rombustia* (rho = 0.32, *p = 0.033*) and *Turicibacter* (rho = 0.31, *p =0 .041*) genera.

Furthermore, higher zonulin concentrations were significantly associated (*p = 0.021*) with higher intestinal alkaline phosphatase (IAP) activity, considered as a measure of intestinal function (0.06 [0.03 – 0.21] U/ml faecal supernatant) (data not shown). We also found a positive correlation between IAP concentration and excreted zonulin (rho = 0.349, *p =0.019*). Although no significant correlations were observed between IAP activity and any nutrient intake, IAP activity was observed to be related to some genera of maternal microbiota (**Supplementary file III-4**). Similarly to the zonulin associations, IAP was also positively linked to some Firmicutes members, including the genus *Rombustia* (rho = 0.31, *p = 0.031*) and *Turicibacter* (rho = 0.35, *p = 0.015*, but also some Ruminococcaceae groups including Ruminococcaceae_UCG013 (rho = 0.39, *p =0.006*), *Roseburia* (rho = 0.37, *p =0.009*), and *Intestinibacter* (rho = 0.38, *p =0.007*). Additionally, lower abundance of Proteobacteria phylum was associated with higher IAP activity (rho = -0.27, *p =0.062*).

Intestinal inflammation, measured by calprotectin concentrations (10.73 [IQR=2.38-20.71] µg/g), was not found to be related to maternal nutrient intake during pregnancy. Calprotectin faecal concentrations were also not affected by the mode of delivery (*p =0.084*) and intrapartum antibiotic exposure (*p =0.668*). Moreover, calprotectin concentration was negatively associated with *Rombustia* genus (rho = -0.32, *p = 0.029*), that it was also related to zonulin and IAP concentrations.

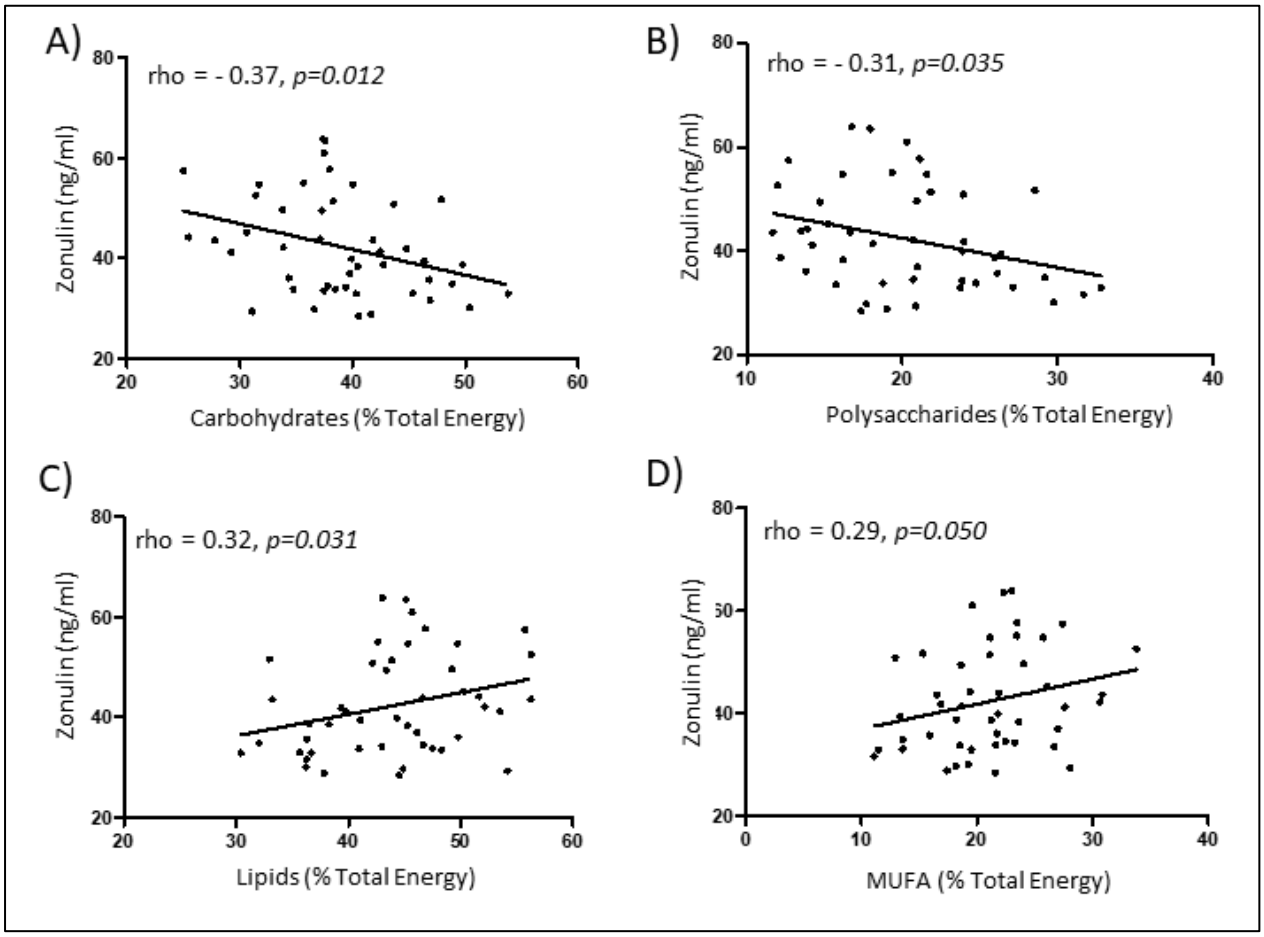


Figure III-2. Maternal diet was associated with intestinal markers. Spearman's rank correlation between total energy contribution (in percentage) of total carbohydrates (**A**), polysaccharides (**B**), lipids (**C**) and MUFA (**D**) and zonulin concentration in maternal stool samples.

Relation between maternal diet, intestinal markers, and neonatal gut microbiota composition in a birth-dependent manner

A significant difference was found between the composition of maternal and infant microbiota (**Supplementary file III-5**). Neonatal microbiota was characterized by lower alpha-diversity, measured using the Chao indices ($p < 0.001$) and Shannon index ($p = 0.06$), and a significant enrichment in genera from Proteobacteria (18.9% vs. 0.8% in neonates and mothers, respectively, $p < 0.001$) and Actinobacteria (15.4% vs. 4.07% in neonates and mothers, respectively, $p < 0.001$) phylum compared to maternal microbiota.

In terms of alpha-diversity, the results showed that maternal diet during gestation was associated with the diversity and richness of neonatal microbiota (**Fig III-**

3). Neonatal microbiota richness, measured using the Chao index, was positively correlated to SFA (rho = 0.24, *p = 0.047*) and proteins from animal sources (rho = 0.25, *p = 0.038*) and negatively associated with maternal vegetable protein intake (rho = -0.25, *p = 0.045*). Similarly, the neonatal bacterial diversity measured by Shannon index was negatively correlated to vegetable proteins (rho = -0.30, *p =0.013*) and fibre (rho = -0.26, *p = 0.037*) intakes.

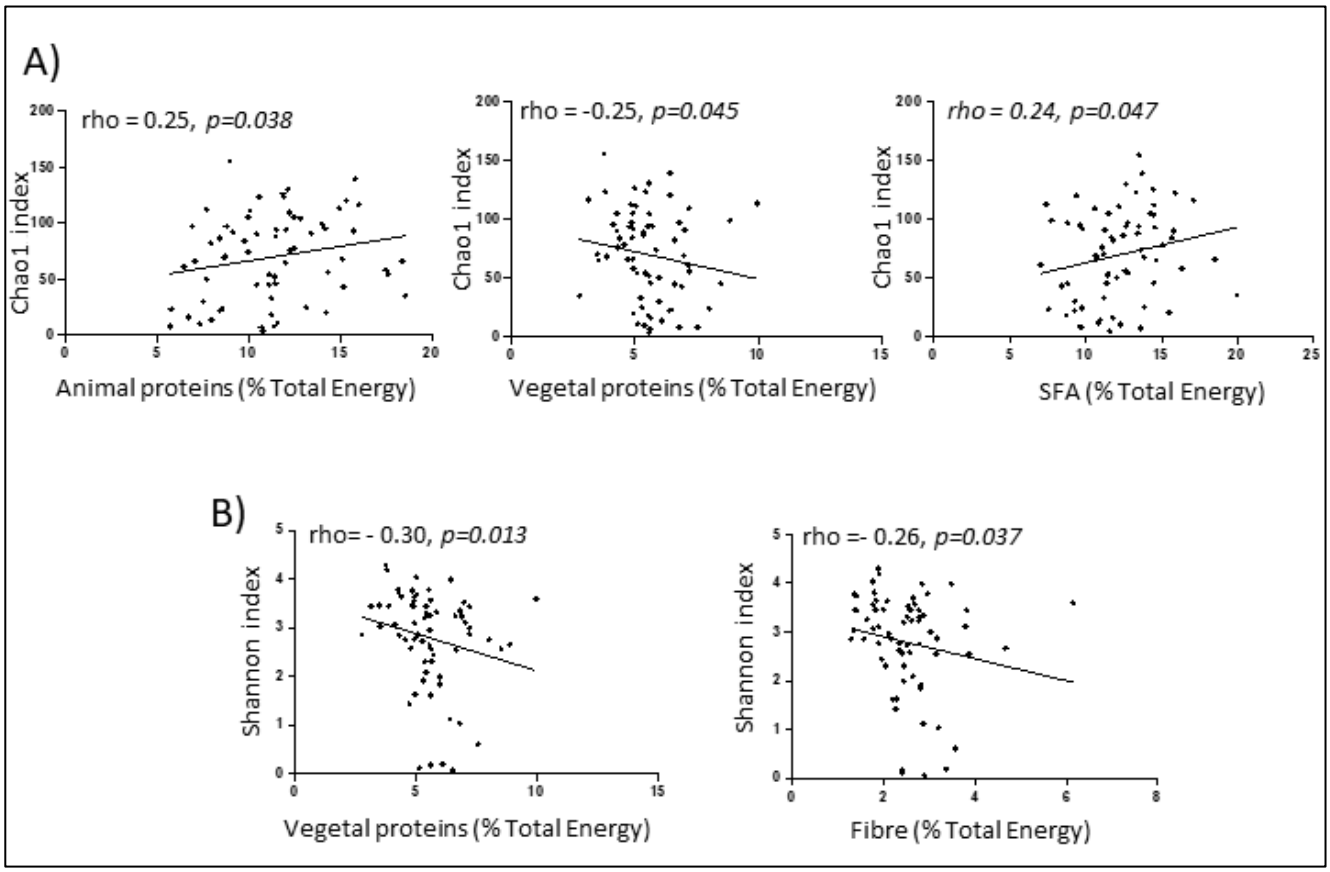


Figure III-3. Maternal diet was associated with the neonatal microbial richness and diversity. A) Significant associations (Spearman's rank correlation) between neonatal microbial richness (measured as Chao index) and maternal intake of proteins from animal and vegetable sources and saturated fatty acids (SFA). **B)** Correlations between vegetal proteins and fibre consumption and neonatal microbiota diversity measured by Shannon index.

Specific neonatal bacterial groups were associated with nutrient patterns from the maternal diet. At the phylum level, we observed that fat-related nutrients intake by mothers, including total lipids, SFA, and MUFA, but not PUFA, showed enrichment in Firmicutes phylum genera and a depletion in Proteobacteria phylum genera in the offspring microbiota (**Supplementary file III-7-8)**. Thus, a negative correlation was

found between the total lipids (rho = 0.27, *p =0.026)*, SFA (rho = 0.29, *p =0.016*), MUFA (rho = 0.32, *p = 0.008*), and Proteobacteria phylum.

At the genus level (**Fig III-4**), a cluster composed of several vitamins, proteins from plant-based sources, and fibre was negatively correlated with the relative abundance of the genus *Coprococcus*, *Blautia*, *Roseburia*, and several groups from the *Ruminococcaceae* and *Lachnospiraceae families* in neonatal faecal samples. Most of these genera were enriched and had a positive correlation with the maternal intake of lipids, MUFA, and animal protein, being especially significant the maternal intake of SFA. The only link of maternal zonulin and neonatal gut microbiota was found for Bacteroidetes phylum. Higher maternal zonulin was associated with a higher relative abundance of Bacteroidetes phylum (*p = 0.042*), including ASVs from the *Bacteroides* (*p = 0.019*) genus.

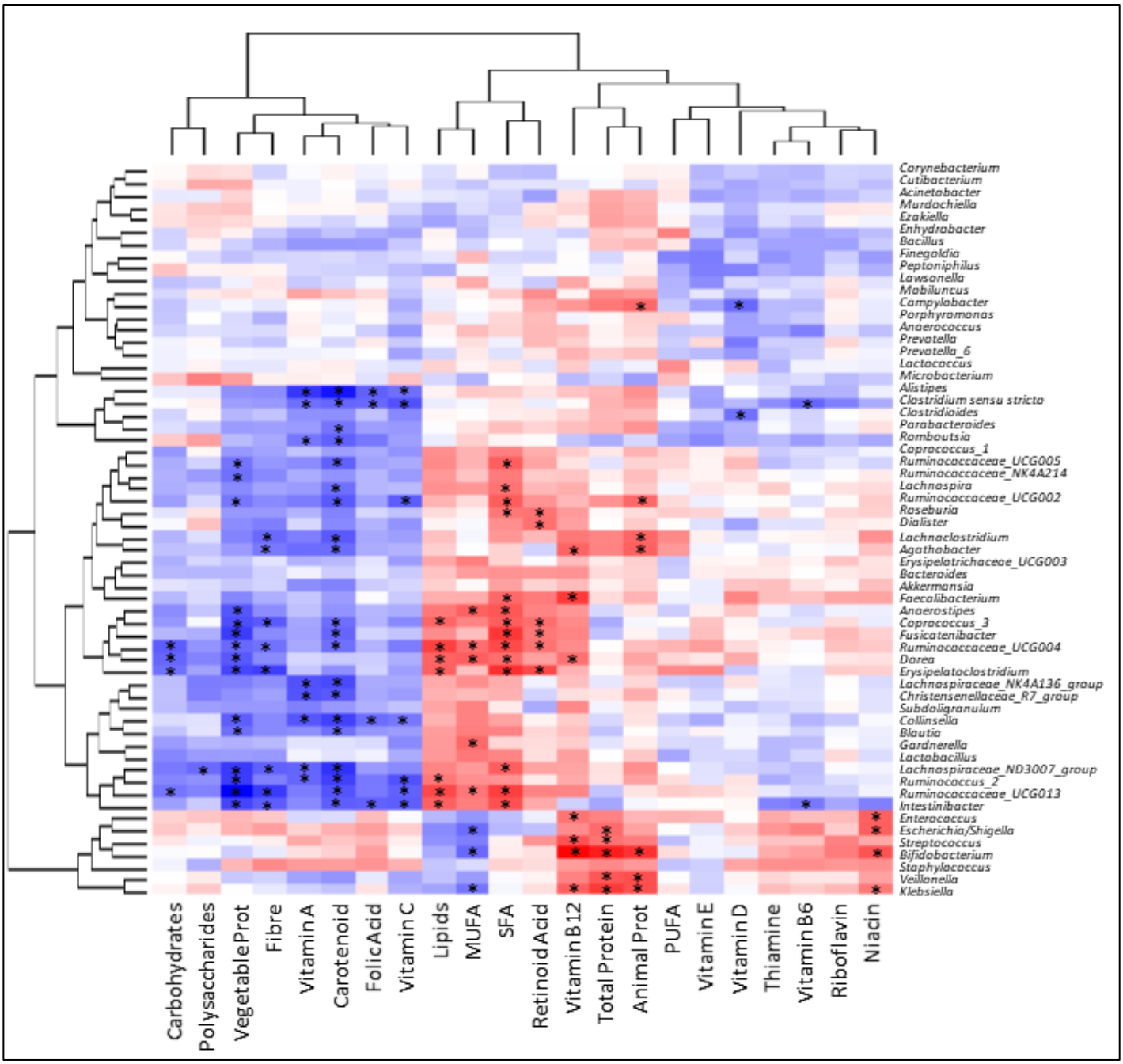


Figure III-4. Maternal diet was associated with neonatal microbiota at birth. Spearman correlations between maternal nutrients intake during pregnancy and neonatal bacterial at genus level at birth. Significant correlations ($p<0.05$) were marked by an asterisk (*). Red colour represents the positive correlations whereas blue colour shown negative relations. The most abundant genera were showed in the graph. Bacterial genera and dietary components are grouped at the square edges

When stratifying the infants according to birth mode (vaginal or C-section) and the maternal intake of total protein ($p = 0.001$), plant-based protein ($p = 0.001$), lipids ($p = 0.006$), total carbohydrates ($p = 0.007$), and fibre ($p = 0.003$), we observed that these nutrients have different effects on neonatal microbiota according to the mode of

delivery (**Supplementary file III-6**). Further, the maternal intake of SFA and fibre reduced the microbiota shifts related to delivery mode (**Fig III-5**). The Bray-Curtis similarity index between vaginal and C-section born infants was lower in offspring from mothers who had higher intakes of SFA ($p < 0.001$) and fibre ($p < 0.001$) (**Fig III-5, C-D**).

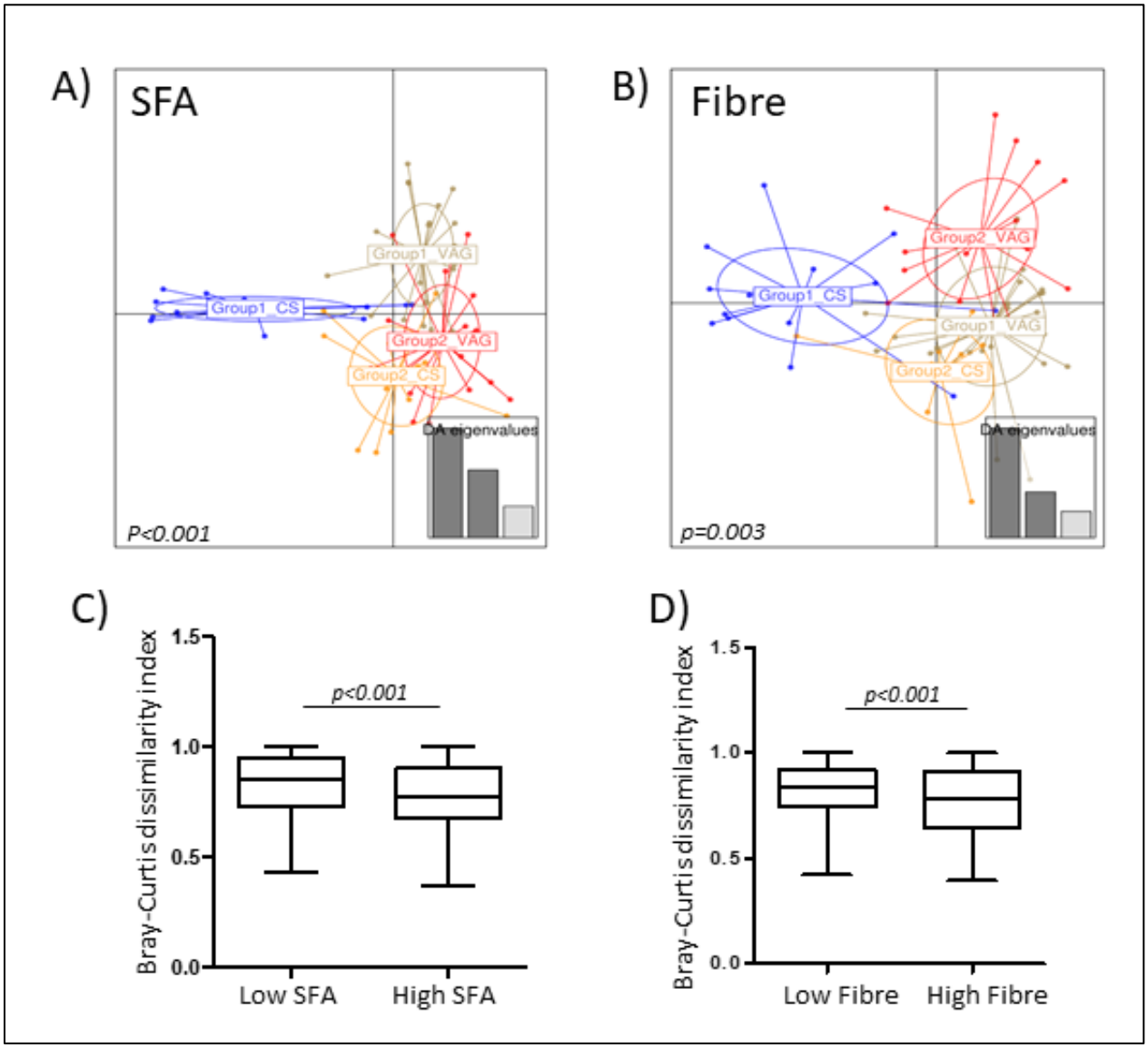


Figure III-5. Neonatal microbiota was distinctly affected depending on mode of birth and maternal diet. Discriminant Analysis of Principal Components (DAPC) of the neonatal microbiota according to mode of delivery and maternal nutrients intake classification. VAG (Vaginal delivery), CS (C-Section), Group1/2: maternal consumption of the each nutrient lower (1)/higher (2) than average intake of the study population, including saturated fatty acids (**A**) and fibre (**B**). Bray-Curtis dissimilarity index within both vaginal and C-section born infants according to their mothers had high or low consumption of SFA (**C**) and fibre (**D**).

In general, we observed a higher effect of maternal diet in vaginal delivered infants than CS-born neonates, especially of fat-related nutrients and vegetable source proteins and fibre. The positive correlations were maintained in the independent analysis of the vaginal delivered neonates **(Supplementary file III-7)**. Total lipids were negatively linked to *Escherichia/Shigella* genus (rho = -0.38, $p = 0.014$) and positively associated with Firmicutes phylum, including genera from the Ruminococcaceae groups and from the *Blautia*, *Roseburia*, *Rombustia*, and *Faecalibacterium* genera. Similarly, these genera also showed a negative correlation with fibre and vegetable source proteins. The total carbohydrates (rho = -0.34, $p = 0.028$), vegetable source proteins (rho = -0.31, $p = 0.046$), and fibre (rho = -0.30, $p = 0.054$) were negatively linked with Firmicutes phylum genera. Similarly, we observed a lower relative abundance of Proteobacteria phylum in the maternal microbiota SFA ($p < 0.001$) and MUFA ($p = 0.012$) intake.

However, in the CS-born infants (**Supplementary file III-8**), we found less relation between maternal diet and neonatal microbiota. Thus, we observed that the offspring of mothers classified as higher fibre consumers showed a higher relative abundance of Proteobacteria phylum genera ($p = 0.046$). Similarly, the maternal PUFA intake was positively correlated with this phylum (rho = 0.44, $p = 0.028$) and negatively associated with Firmicutes phylum (rho = 0.40, $p = 0.046$).

At the genus level, we found correlations of neonatal microbiota and maternal protein intake, especially those from animal sources, including *Veillonella* (rho = 0.54, $p = 0.005$), *Escherichia/Shigella* (rho = 0.40, $p = 0.045$), *Klebsiella* (rho = 0.43, $p = 0.032$), or *Clostridium sensu stricto* (rho = 0.40, $p = 0.047$) genera.

DISCUSSION

This study shows that maternal diet during pregnancy is associated with the maternal intestinal markers, including gut microbiota, and the offspring microbiota composition at delivery.

It is well known that diet is a main driver of the human gut microbiome that can modulate state of health. Pregnancy is a sensitive period related to the health programming of offspring [546,547]. Recently, a systematic review found associations between the maternal diet and the maternal microbiota (five papers) or infant gut

microbiome (two papers) [198], but no studies have examined both maternal and infant microbiota simultaneously. In our study, the mothers followed a conventional healthy diet, without any intervention, and they were not obese or diabetic. The maternal consumption of different nutrients showed a homogenous pattern and was in concordance with other studies performed on a Spanish general population [548,549] and on pregnant women [550]. Therefore, the associations between nutrients and maternal or infant gut microbiota are likely caused by subtle changes in nutritional patterns, which better represents the general population than extreme diets (e.g., high fat vs. high fibre).

We found relations between maternal microbiota and specific nutrients, such as fat-related nutrients intake and Firmicutes phylum, including *Lachnospira* genus and a reduction of Proteobacteria phylum. Several studies have shown the increase in the relative abundance of Firmicutes in intestinal microbiota with increased body mass index [237] and in response to a high fat diet [551]. Furthermore, Koren et al. found that maternal microbiota was altered during late pregnancy, increasing the Proteobacteria and Actinobacteria phyla, which contribute with a positive feedback loop to host adiposity and a low grade of inflammation commonly observed in normal pregnancy development [132]. Our results suggest that maternal diet could modify the progress of microbiota remodelling that has been observed during pregnancy and affect the mother-infant transmission.

Several studies have shown how maternal diet determines the variations of infant microbiota [198]. In a primate model, the maintenance of a high-fat diet through pregnancy resulted in a significant variation in intestinal commensal taxa in the offspring, with an enrichment of *Ruminoccoccus* and *Dialister* genera and a reduction of the *Campylobacter* genus [193]. These results were partially confirmed by the same authors in humans [194]. Infants born from mothers with a higher fat consumption during gestation had a relative depletion of the *Bacteroides* genus in their gut microbiota, observable up to six weeks [194]. In 2019, Lundgren et al. observed that maternal diet had an effect on infant gut microbiota that could differ by delivery mode [195]. Mandal et al. reported that fat and some fat-soluble vitamins influenced the maternal gut microbiota changes during pregnancy [196]. Notably, we found a cluster of vitamins with a significant association with neonatal microbiota, including vitamins D, A, and B12. In 2019, Boran et al. observed positive correlations between vitamin B12 status and different genera of Firmicutes phylum, while negative associations were

reported for *Clostridiales* and *Prevotella*, among others, in infants' bacterial gut population [197]. However, the effect of these relations and whether they are cause or consequence of the microbiota alterations observed remain unclear. Therefore, the link between maternal diet during pregnancy and gut microbiota may be involved in maternal intestinal metabolic function and in the vertical transmission of specific genera impacting early neonatal colonization.

Studies have shown that maternal intestinal permeability is affected during gestation. Pregnant woman had higher permeability compared to non-pregnant woman [552]. It is unknown whether this could have implications for maternal-infant microbial transference. In our study, lipids, total carbohydrates, and polysaccharides showed the greatest influence on the maternal intestinal function, assessed by faecal zonulin, calprotectin, and IAP. These markers of inflammation and functional maturation [308,540,553–556] have been previously reported as responsive to dietary interventions in the human population [557].

It is recognized that the consumption of excess dietary fats can enhance intestinal permeability [558,559]. Kim et al. showed that high fat diet (HFD) increased endotoxin concentrations in the intestinal lumen and plasma through an altered permeability that leads to systemic inflammation [560]. In our study, zonulin was increased in mothers with higher fat intake. Altered upregulation of zonulin secretion from the intestinal epithelial cells into the lumen can increase paracellular permeability [561,562] and previous research showed that some nutrients, especially those related to fats, can increase faecal zonulin concentrations [557,563]. Although intestinal permeability cannot be directly linked to zonulin levels in faeces, increased faecal zonulin is described in professional athletes [564], Crohn disease patients [565] or individuals with metabolic disorders [308,566], all physiological or pathological conditions related with impaired intestinal homeostasis.

While other studies linked high-fat diets and IAP expression [567], we could not confirm this observation in our data. However, in our population, fat-related nutrients were related to maternal microbiota, thus increasing some Firmicutes genera that are also associated with higher IAP concentrations. Thus, the link fat-zonulin-IAP-microbiota could be a relation between fat metabolism and maternal microbiota at delivery with possible consequences in the mother-infant transference. We have shown that this link is also observable in first-pass infant microbiota. Most previous studies that focused on IAP were conducted on animals using an experimental design that

specifies extremely different feeding groups; however, our study did not permit the control of all variables affecting IAP expression. The faecal concentration of calprotectin in the mothers of our study was in the range of the reported values in other studies [554]. Our results suggested that the link of diet on maternal faecal zonulin and IAP is not related to an increment of inflammatory status in the gut, since no differences were observed in terms of calprotectin concentration. In that sense, one weakness of our study is the lack of data on intestinal markers in the offspring, which would allow for establishing associations of maternal nutrients and effects on infant permeability and will ensure further research.

Beyond maternal shifts in microbiota, gestational diet and intestinal functionality may be associated with offspring microbiome shifts, especially induced by lipid intake. Based on previous research, pre-natal exposure to bacterial metabolites (e.g., short chain fatty acids), endotoxins or lipopolysaccharide, and actual bacteria may occur in healthy pregnancies, tightly regulated by intestinal permeability (reviewed in Walker et al. in 2017) [568]. Lipids, especially SFA and MUFA, and fibre had a significant impact on neonatal microbiota. Neonates born from mothers with a higher fat consumption, including lipids and SFA, showed enrichment of bacterial taxa from Firmicutes phylum—such as *Lachnospira*, *Roseburia,* and *Coprococcus* genera, and some Ruminococcaceae groups—and a depletion of other taxa typically found on neonatal meconium, especially those from Proteobacteria phylum such as *Escherichia/Shigella* genus. Shifts on microbiome acquisition may have unknown consequences for priming the immune system during a critical window of immune development.

The effect of maternal diet on infant microbiome structure was more evident in vaginally delivered infants than in those born by C-Section, possibly influenced by labour and birth-related factors affecting microbiome transference (e.g., contact with vaginal and faecal microbiota, hormonal cascades [130,153], pre-surgical intervention fasting [569]). C-section born infants have less contact with maternal gut microbiota during delivery and are inevitably exposed to antibiotics at birth [163,164], which could modify the effect of perinatal factors, such as maternal diet. Since almost all of C-sections in our study were elective, the mothers did not have any labour process. The C-section procedure is associated with antibiotic administration; therefore, we cannot discriminate between the effects of antibiotics or the method of birth, and we consider both as concomitant factors affecting the microbial transmission from the mother to the neonate.

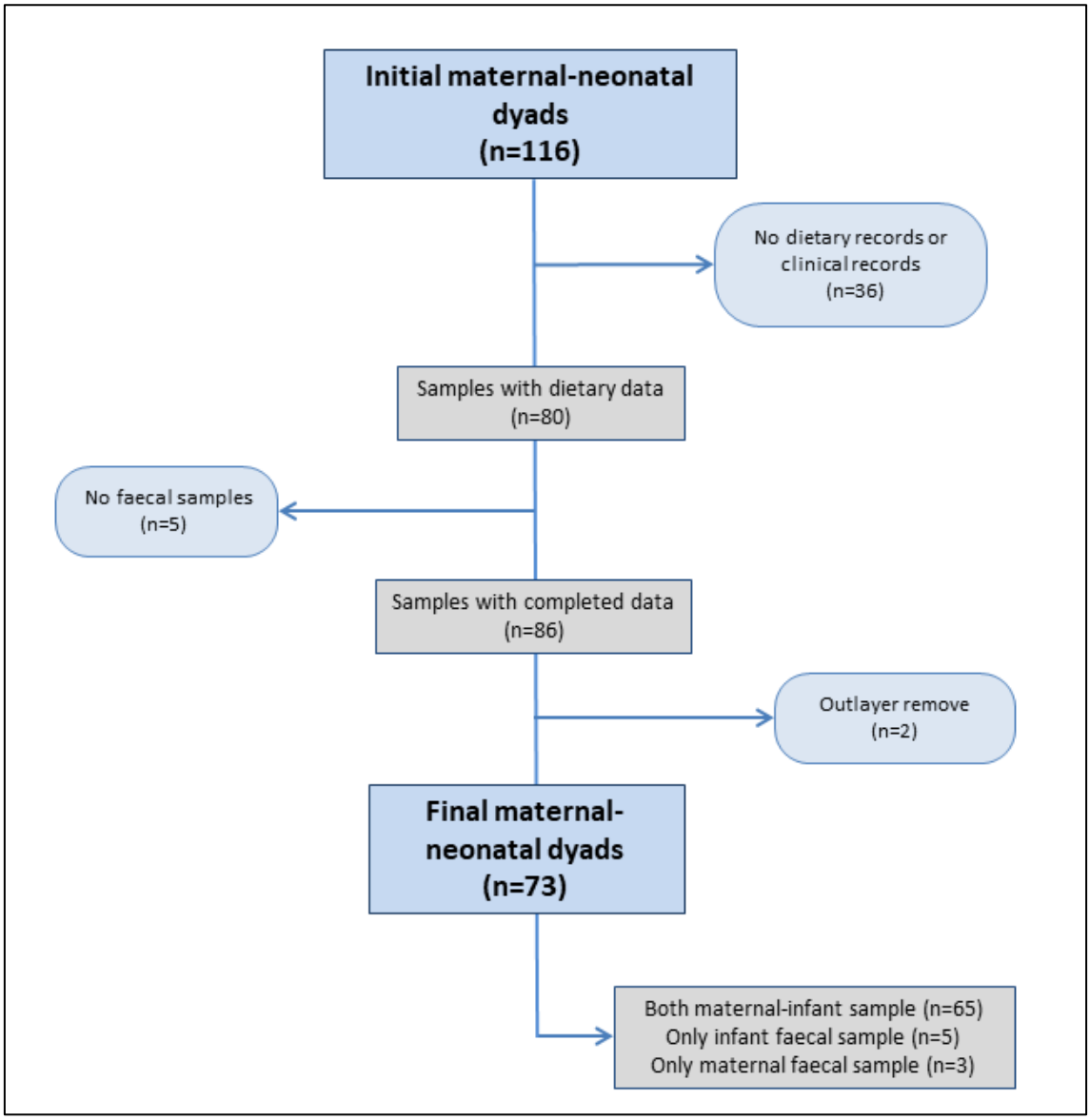
Remarkably, we analysed 16S configurations at delivery, which may not necessarily correlate well with the 16S configurations that rapidly establish themselves very early in life. However, growing evidence suggested that the composition of the meconium or first-pass faeces microbiota could have a relevant influence in the colonization process in the later days and months, but also in the immune priming and epigenetic programming of the foetus and neonate [570].

Among the limitations of the study, FFQ-Dietary information is subject to memory bias as well as the lack of perception on the food proportion sizes. Therefore, the use of specific 24h recall and/or 3 days recall questionnaires including portions would be needed for future studies. Furthermore, our sample size was limited and further studies with higher number of participants and also, with the follow-up of the participants would be needed. Despite all those limitations, our study demonstrated how maternal diet during pregnancy, especially related to lipid intake, had a significant effect on maternal intestinal function and neonatal microbiome, potentially contributing to an adequate transmission of initial colonization and health-programming. Therefore, specific dietary programs targeting pregnant women may be a cost-effective intervention factor to promote adequate vertical mother–infant exposition.

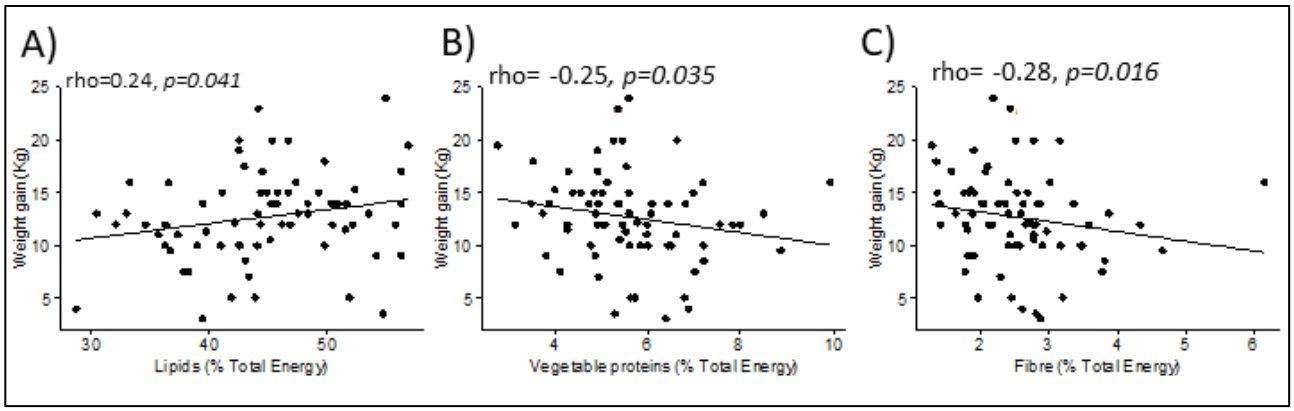
CONCLUSION

Fat intake during pregnancy is associated with maternal microbiome and intestinal permeability, which could affect the initial microbial exposure and acquisition of the neonate. The importance of the priority events on the interplay host-microbiome is crucial. Our results shed light on the unexplored mechanisms, whereby maternal diet can influence the neonatal development and highlight the crucial role of maternal diet in infant colonization. These findings highlight the importance of dietary counselling for a correct mother–infant microbial transmission.

CHAPTER III - SUPPLEMENTARY DATA



Supplementary file III-1. Flow-chart of participants in the study

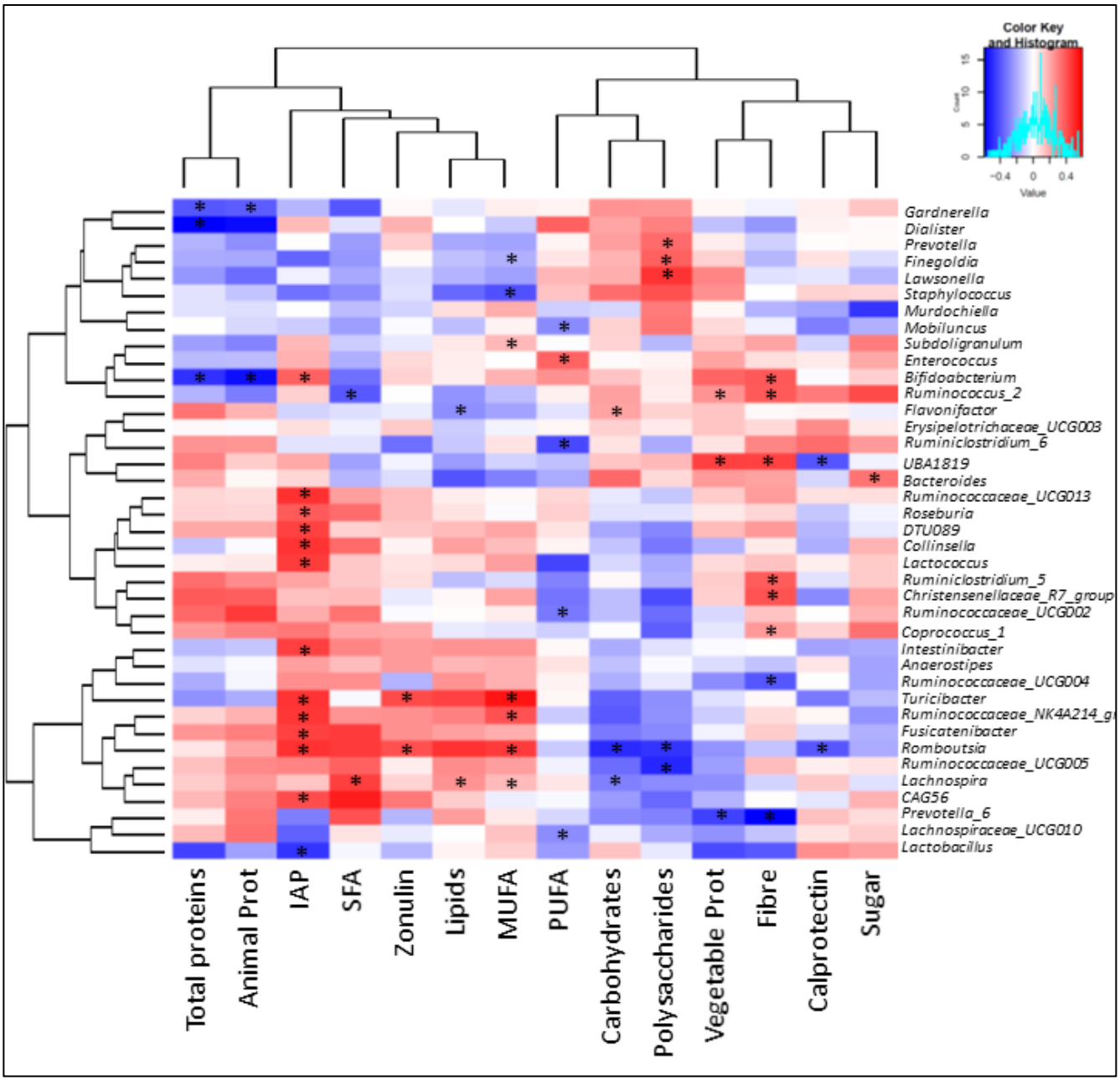


Supplementary file III-2. **Maternal diet was related to the weight gain during pregnancy.** Spearman correlation between total energy contribution (in percentage) of total lipids intake (**A**), proteins from vegetable source (**B**) and fibre (**C**) and maternal weight gain during pregnancy (Kg).

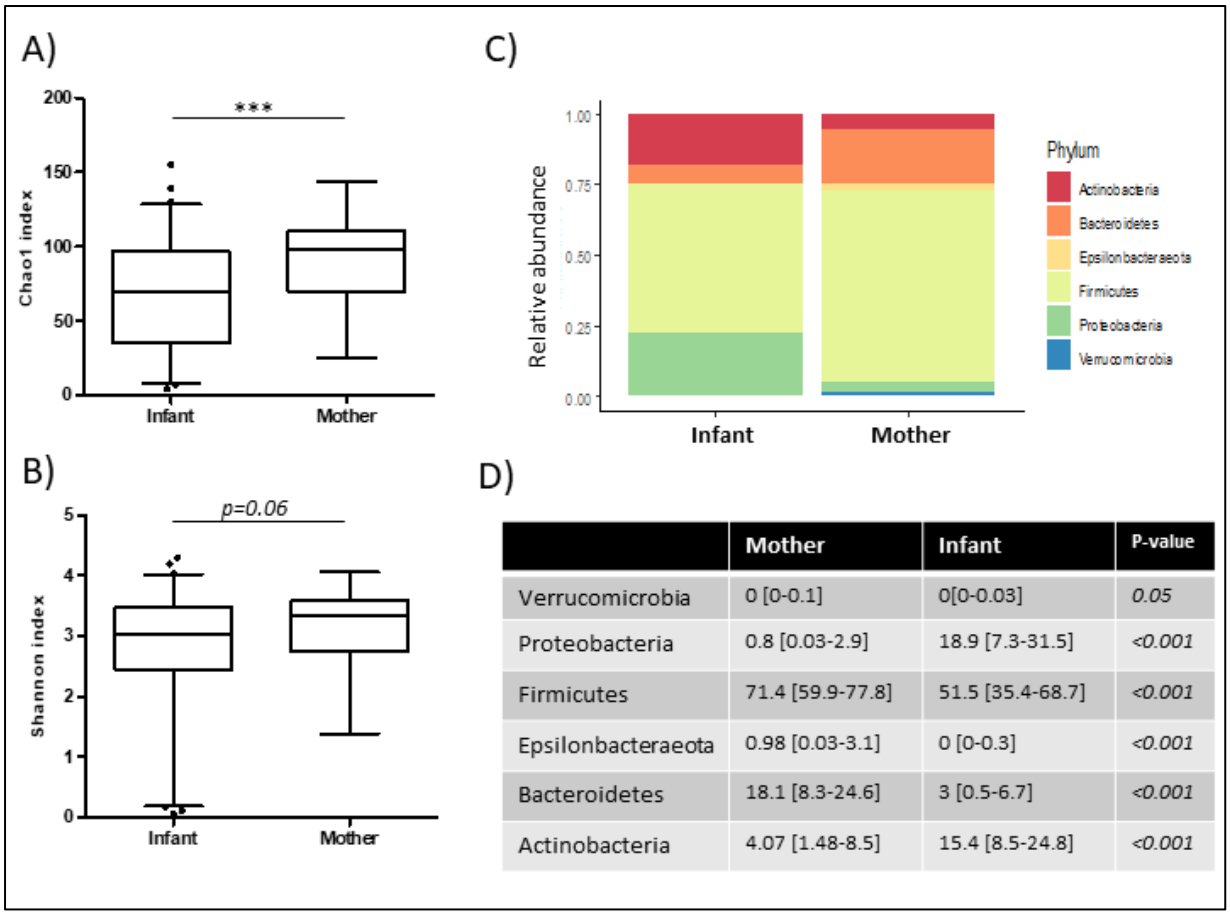
Supplementary file III-3. Spearman correlations between reported nutrients intake of maternal diet during pregnancy.

Nutrient		rho	p-value
Animal protein			
	SFA	0.338	*0.003*
	Carbohydrates	-0.461	*<0.001*
	Polysaccharide	-0.305	*0.009*
Vegetal protein			
	Lipids	-0.644	*<0.001*
	SFA	-0.642	*<0.001*
	MUFA	-0.454	*<0.001*
	Carbohydrates	0.547	*<0.001*
	Polysaccharide	0.573	*<0.001*
	Fibre	0.832	*<0.001*
Lipids			
	Carbohydrates	-0.896	*<0.001*
	Polysaccharides	-0.743	*<0.001*
	Fibre	-0.533	*<0.001*
Saturated Fatty Acids			
	Carbohydrates	-0.647	*<0.001*
	Polysaccharides	-0.489	*<0.001*
	Fibre	-0.507	*<0.001*
Monounsaturated fatty acids			
	Carbohydrates	-0.623	*<0.001*
	Polysaccharides	-0.510	*<0.001*
	Fibre	-0.297	*0.011*

Only macronutrients were included in the table. Saturated (SFA) and mono- (MUFA) unsaturated fatty acids.

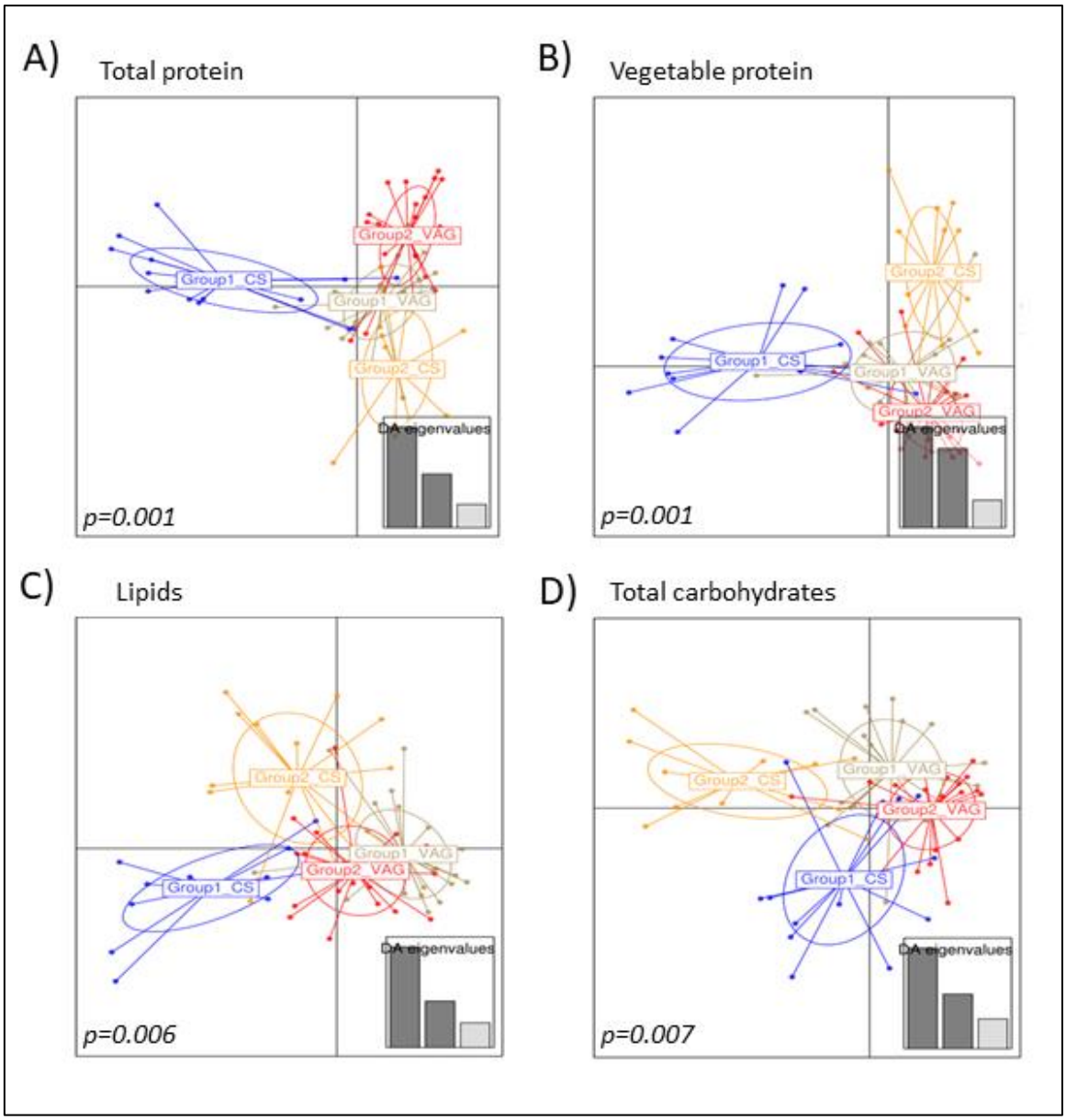


Supplementary file III-4. **Maternal diet and intestinal markers were associated with the maternal microbiota at delivery.** Spearman correlations between maternal nutrients intake during pregnancy and maternal bacterial at genus level at birth**.** Significant correlations ($p<0.05$) were marked by an asterisk (*). Red colour represents the positive correlations whereas blue colour shown negative relations. Bacterial genera and dietary components are grouped at the square edges. Only genera with significant relations were shown.

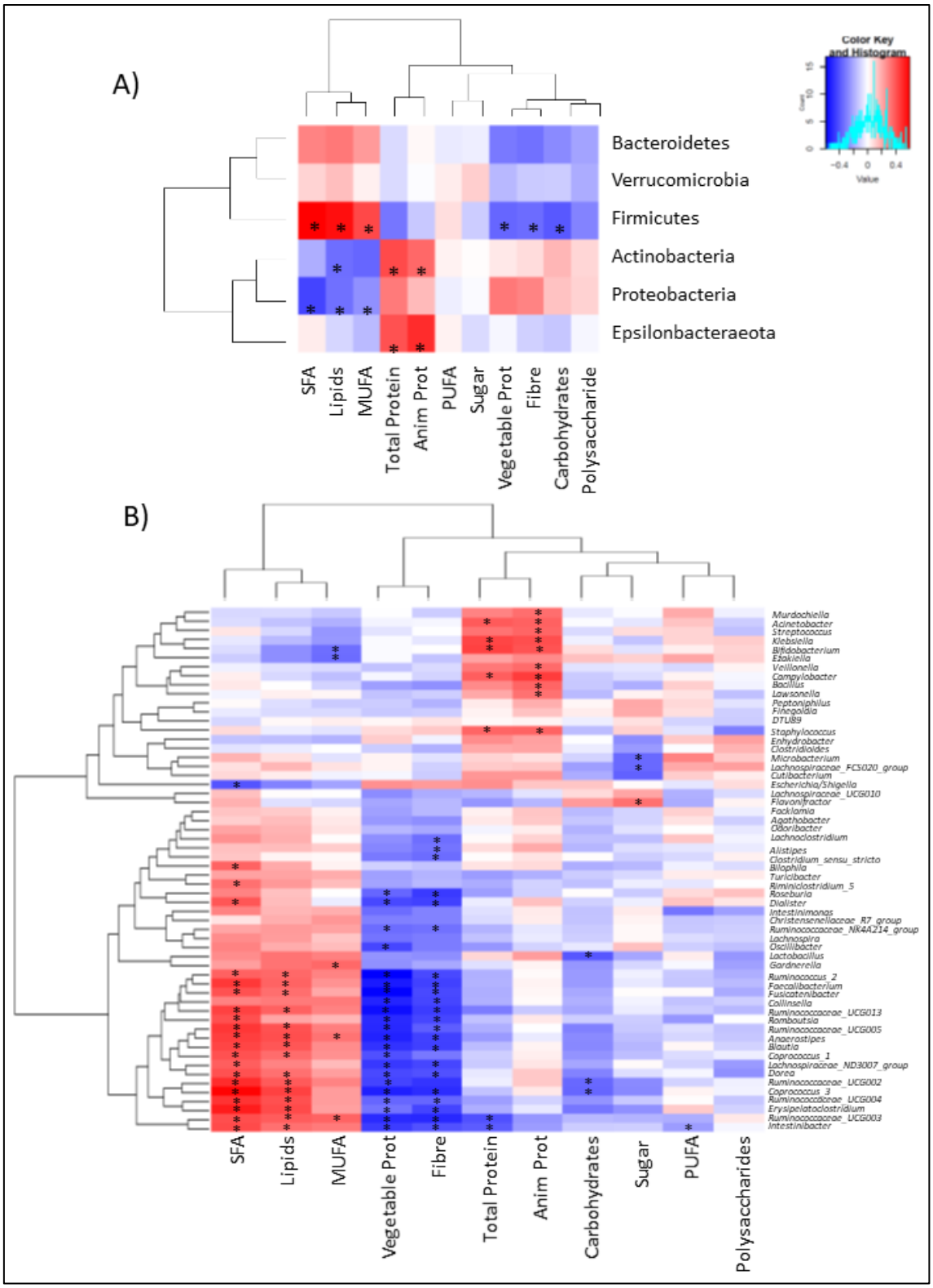


	Mother	Infant	P-value
Verrucomicrobia	0 [0-0.1]	0[0-0.03]	*0.05*
Proteobacteria	0.8 [0.03-2.9]	18.9 [7.3-31.5]	*<0.001*
Firmicutes	71.4 [59.9-77.8]	51.5 [35.4-68.7]	*<0.001*
Epsilonbacteraeota	0.98 [0.03-3.1]	0 [0-0.3]	*<0.001*
Bacteroidetes	18.1 [8.3-24.6]	3 [0.5-6.7]	*<0.001*
Actinobacteria	4.07 [1.48-8.5]	15.4 [8.5-24.8]	*<0.001*

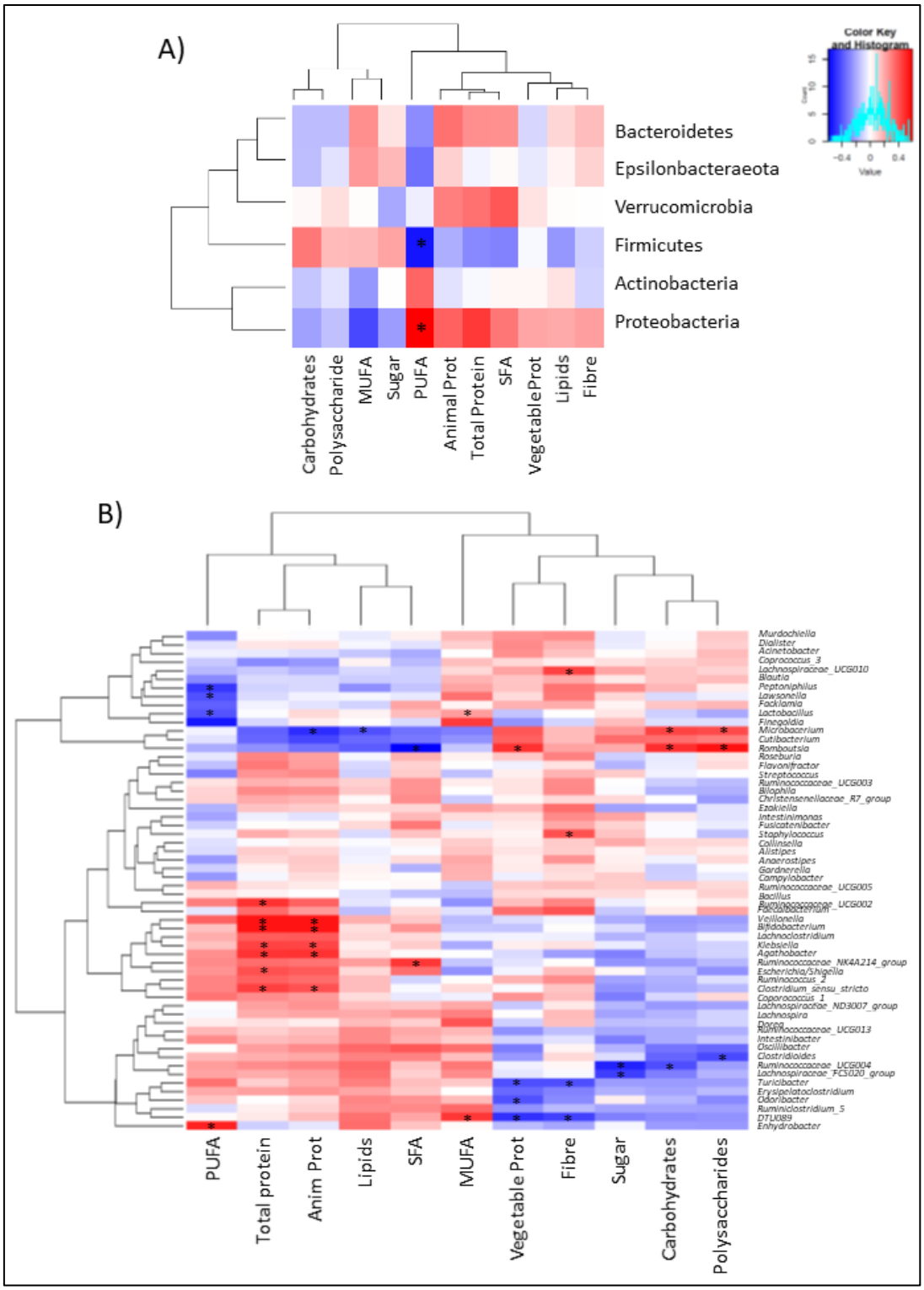
Supplementary file III-5. Comparison of bacterial population from maternal intestinal microbiota and infant faecal swabs microbiota. **A-B**) Alpha diversity measured by Chao1 (**A**) and Shannon (**B**) index of infant and maternal microbiota at delivery. **C**) Bar-plots at phylum level of maternal and neonatal microbiota at delivery. **D**) Relative abundance of each phylum was expressed as median and interquartile range between brackets. Mann-Whitney test with FDR adjustment was used to test the significance of the differences between maternal stool and infant microbiota and for diversities analysis. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.



Supplementary file III-6. Neonatal microbiota was distinctly shaped depending on mode of birth and maternal diet. Discriminant Analysis of Principal Components (DAPC) of the neonatal microbiota according to mode of delivery and maternal nutrients intake classification. VAG (Vaginal delivery), CS (C-Section), Group1/2: maternal consumption of the each nutrient lower (1)/higher (2) than average intake of the study population, including total protein (**A**) those from vegetal source (**B**), lipids (**C**) and total carbohydrates (**D**).



Supplementary file III-7. Maternal diet was associated with neonatal microbiota at birth in vaginal born infants. Spearman correlations between maternal nutrients intake during pregnancy and neonatal bacterial composition at phylum (**A**) and genus level (**B**) at birth**.** Significant correlations ($p<0.05$) were marked by an asterisk (*). Red color represents the positive correlations whereas blue color shown negative relations. The most abundant genera were showed in the graph. Bacterial genera and dietary components are grouped at the square edges. Genera with some significant relation with maternal diet were shown.



Supplementary file III-8. Maternal diet was associated with neonatal microbiota at birth in C-section born infants. Spearman correlations between maternal nutrients intake during pregnancy and neonatal bacterial composition at phylum (**A**) and genus (**B**) level at birth**.** Significant correlations ($p<.05$) were marked by an asterisk (*). Red color represents the positive correlations whereas blue color shown negative relations. The most abundant genera were showed in the graph. Bacterial genera and dietary components are grouped at the square edges. Genera with some significant relation with maternal diet were shown.

CHAPTER IV

DISTINCT MATERNAL MICROBIOTA CLUSTERS ARE ASSOCIATED WITH DIET DURING PREGNANCY: IMPACT ON NEONATAL MICROBIOTA AND INFANT GROWTH DURING THE FIRST 18 MONTHS OF LIFE

CHAPTER IV - DISTINCT MATERNAL MICROBIOTA CLUSTERS ARE ASSOCIATED WITH DIET DURING PREGNANCY: IMPACT ON NEONATAL MICROBIOTA AND INFANT GROWTH DURING THE FIRST 18 MONTHS OF LIFE

García-Mantrana, I.; Selma-Royo, M.; González, S.; Parra-Llorca, A.; Martínez-Costa, C.; Collado, M.C. Distinct maternal microbiota clusters are associated with diet during pregnancy: impact on neonatal microbiota and infant growth during the first 18 months of life. Gut Microbes 2020, 1–17, doi:10.1080/19490976.2020.1730294.

Impact factor: 7.823

ABSTRACT

Nutrition during pregnancy plays an important role in maternal–neonatal health. However, the impact of specific dietary components during pregnancy on maternal gut microbiota and the potential effects on neonatal microbiota and infant health outcomes in the short term are still limited.

A total of 86 mother–neonate pairs were enrolled in this study. Gut microbiota profiling on maternal–neonatal stool samples at birth was carried out by 16S rRNA gene sequencing using Illumina. Maternal dietary information and maternal–neonatal clinical and anthropometric data were recorded during the first 18 months. Longitudinal body mass index (BMI) and weight-for-length (WFL) z-score trajectories using the World Health Organization (WHO) curves were obtained. The maternal microbiota was grouped into two distinct microbiota clusters characterized by *Prevotella* (Cluster I) and by the *Ruminococcus* genus (Cluster II). Higher intakes of total dietary fiber, omega-3 fatty acids, and polyphenols were observed in Cluster II compared to Cluster I. Higher intakes of plant-derived components were associated with a higher presence of the Christensellaceae family, *Dehalobacterium* and *Eubacterium*, and lower amounts of the *Dialister* and *Campylobacter* species. Maternal microbial clusters were also linked to neonatal microbiota and infant growth in a birth-dependent manner. C-section neonates from Cluster I showed the highest BMI z-score at age 18 months, along with a higher risk of overweight. Longitudinal BMI and WL z-score trajectories from birth to 18 months were shaped by maternal microbial cluster, diet, and birth mode. Diet was an important perinatal factor in early life that may impact maternal microbiota; in particular, fiber, lipids, and proteins, and exert a significant effect on the neonatal microbiome and contribute to infant development during the first months of life.

Keywords: maternal nutrition, pregnancy, microbiota, early colonization, obesity

INTRODUCTION

The first 1,000 days of life comprise a sensitive, critical period in which nutrition has a pivotal impact [571,572], influencing the risk of non-communicable diseases (NCDs) such as metabolic disorders, cardiovascular diseases, allergies and obesity [573]. Maternal nutrition during this period is considered crucial for supporting health status and promoting adequate neonatal growth and development [574,575], as the diet is one of the most powerful factors driving the microbiota. Recent studies have highlighted the impact of maternal diet on maternal microbiota; most of these studies focused on maternal obesity and gestational diabetes (GDM) [196,576–579]. In addition, some studies have reported the effects of maternal diet on the offspring's microbiota [580]. Despite the evidence of the effects of maternal diet on the microbiome, little is known about the potential effects on the infant's health outcomes. It is well known that the maternal microbiota represents the most important microbial source for the development of the neonatal microbiota. Shifts in infant microbiota development have been linked to alterations at the immunological and metabolic levels, leading to an increased risk of NCDs [130,254]. Early exposure to antibiotic use, unbalanced diets, and the increased ratios of C-section deliveries could disrupt this adequate early infant colonization, which have been linked to the risk of childhood obesity [86,581]. Maternal overweight and obesity have an impact on the infant microbiota and can influence the risk of obesity later in life [487,582,583].

In this scenario, the relevance of maternal dietary patterns and the role of specific nutritional compounds on the gut microbiota during pregnancy has not been fully understood. Moreover, little is known about its influence on infant health outcomes in the short and long term. Thus, the aim of this study is to assess whether maternal gut microbiota is shaped by diet and specific nutritional components during pregnancy and to evaluate the potential impact on infant development during the first 18 months of life.

MATERIALS AND METHODS

Study design and volunteers

The study included 116 mother–neonate pairs from the prospective and observational MAMI birth cohort, recruited from 2015–2017. MAMI (The power of

MAternal Microbes on infant health) is a prospective mother–infant birth cohort in the Spanish-Mediterranean area, as detailed by García-Mantrana et al. [420]. The following nutritional and clinical parameters were collected: maternal antibiotic exposure, BMI and weight gain during pregnancy, mode of delivery, birth weight, birth length, infant feeding, infant BMI, and WFL z-scores.

None of the participating volunteers were diagnosed with any disease; none were under drug treatment or prebiotics administration, apart from antibiotics use during pregnancy and/or at delivery. All participants received oral and written information about the study and written consent was obtained. The study was approved by the Hospital Ethics Committees (HECs) (Hospital Universitario y Politécnico La Fe and Hospital Clínico Universitario de Valencia). The study is registered on the *ClinicalTrial.gov* platform, with registration number NCT03552939.

Nutritional assessment

Dietary records were collected during the first week after birth by a nutritionist using a 140-item Food Frequency Questionnaire (FFQ) about their regular diet during the pregnancy [421]. FFQ information was analyzed for the energy and daily intake of macro- and micronutrients by using the nutrient Food Composition Tables developed by the Centro de Enseñanza Superior de Nutrición Humana y Dietética (CESNID) [584]. The intake of specific dietary fiber, as soluble and insoluble fiber types, was completed from the Marlett food composition tables [585]. Polyphenol content was obtained from the Phenol-Explorer database [586]. Data were normalized by 2500 kcal/day.

Furthermore, adherence to the Mediterranean Diet (MD) was also calculated by the use of the PREDIMED validated test.[423] The MD score ranged from 0 (minimal adherence) to 14 (maximal adherence). A score of nine or more points meant good adherence to the Mediterranean diet.

Child growth development

Length and weight were registered at birth, 1, 6, 12, and 18 months. Z-scores of anthropometric measures were electronically computed using WHO Anthro software (www.who.int/ childgrowth/software/en/). The WHO Child Growth Standards provide child growth measures standardized by age and sex using z-score. Longitudinal BMI and WFL z-score trajectories from birth to 18 months were obtained. For children under 5 years old, the WHO defines the risk of overweight when BMI and/or WLF z-score are

greater than 1 standard deviation (SD), overweight when they are greater than 2 SD, and obesity when they are greater than 3 SD according to the WHO Child Growth Standards median [587].

Faecal samples and DNA extraction

Maternal–neonatal samples were collected in the delivery room by trained clinical personnel, in order to avoid the potential impact of the environment during the first 24 h after birth. Maternal gut samples were obtained by swabbing a sterile, cotton-tipped swab in the rectum before birth in the delivery room. Neonatal sample collection was also obtained by introducing a sterile, cotton-tipped swab in the neonatal rectum just after birth in the delivery room.

Both swabs were stored in pre-numbered sterile containers to avoid errors. After collection, all samples were immediately transported to the specimen biobank within 1 hour of collection and placed in sterile, pre-numbered cryovials at -80° C under specific standardized protocols at Biobanco para la Investigación Biomédica y en Salud Pública de la Comunidad Valenciana (Fisabio Public Health Biobank IBSP-CV) until further analysis. Once all samples were collected and placed in the biobank, aliquots were transported in dry ice to IATA-CSIC for the analysis.

Total DNA was isolated from the fecal samples using the Master-Pure DNA Extraction Kit (Epicentre, Madison, WI, USA) according to the manufacturer's instructions; modifications included physical and enzymatic treatments. In brief, cell lysis was performed by mechanical disruption using 3-µm diameter glass beads in a FastPrep 24-5G Homogenizer (MP Biomedicals, CA, USA), followed by an enzymatic treatment for 60 min at 37° C with the lysis buffer supplemented with lysozyme (20 mg/ml) and mutanolysine (5U/ml). A DNA Purification Kit (Macherey–Nagel, Duren, Germany) was used and DNA was quantified using Qubit 2.0 Fluorometer (Life Technology, Carlsbad, CA, United States). Meconium is a low-microbial biomass sample that would be affected by potential contaminants from the environment and DNA extraction kits reagents (kitome). Then, to rule out potential contamination, controls during DNA extraction and polymerase chain reaction (PCR) amplification were also included and sequenced.

16S rRNA amplicon sequencing

Gut microbiota composition and diversity were determined by the V3-V4 variable region of the 16S rRNA gene sequencing, following Illumina protocols. A Nextera XT Index Kit (Illumina, CA, USA) was used for the multiplexing step and a Bioanalyzer DNA 1000 chip (Agilent Technologies, CA, USA) was used to check the PCR product quality. Libraries were sequenced using a 2x300 pb paired-end run (MiSeq Reagent kit v3) on a MiSeq-Illumina platform (FISABIO sequencing service, Valencia, Spain), according to manufacturer´s instructions (Illumina).

Quality filtering, sequence joining, and chimera removal were obtained using an ad-hoc pipeline written in an RStatistics environment; data processing was performed using a QIIME pipeline (version 1.9.0) [387]. Operational Taxonomic Units (OTUs) were constructed by an open-reference OTU picking method with 99% as a threshold of identity. Representative sequences were taxonomically assigned based on the RDP database. Samples with a relative abundance of less than 0.05% and sequences classified as Cyanobacteria and Chloroplast were removed from the dataset as they represent ingested plant material.

Statistical analysis

Microbiota data was analyzed in the Calypso online platform (v8.84) (http://cgenome.net/wiki/index.php/Calypso/) and data was normalized by the Total-Sum Scaling (TSS) method. Alpha diversity indexes (Chao1, Shannon, and inverse Simpson indexes) were determined and beta diversity based on Bray–Curtis distance was obtained.

Maternal microbiota clustering was performed at the genus level, as described elsewhere [499]. Briefly, Jensen-Shannon distance and partitioning around medoid (PAM) clustering were used and the optimal number of clusters was calculated by the Calinski-Harabasz (CH) index. The clusters were generated using the phyloseq, cluster, MASS, clusterSim, and ade4 R packages [500,501,503,588,589].

Linear discriminant analysis effect size (LEfSe) was used to identify microbial genera enriched in maternal clusters. An LDA score (log10) > 3 was considered significant. Multivariate analysis, including Redundancy Analysis (RDA), Principal Component Analysis (PCA) as well as Principal Coordinate Analysis (PCoA), and permutational multivariate analysis of variance (PERMANOVA) based on Bray–Curtis distance was also achieved on Calypso. Specific correlations, general linear models

(GLM) for fixed factors and/or covariate analysis were assessed by SPSS software v26 and Graphpad Prims v5.04 [444]. P-values were corrected with Benjamini-Hochberg's false discovery rate (FDR) method. RStudio was also used to represent the heatmaps between bacterial groups at the genus level and dietary components through the ggplot package [445,446]. Selection of covariates for model adjustment were performed using the direct acyclic graph (DAG) method (**Supplementary file IV-I**) using the online tool DAGitty [590].

RESULTS

Maternal microbial clusters at birth and clinical data

Microbial relative abundance-based clustering using Jensen Shannon distances revealed two clusters of maternal gut microbiota at birth (**Fig. IV-1, A-B**). The clustering was validated using the Calinski Harabasz Index (CHI) and prediction strength, which uses a cross-validation approach to validate the robustness of clustering.

From a total number of 116 mother–infant dyads, we excluded the pairs lacking biological infant samples and/or clinical data as well as maternal and infant data. Then a total of 86 mother–infant pairs were analyzed based on the matched biological samples as well as dietary information and clinical data availability up to 18 months of life.

No significant differences in maternal clinical and anthropometric data were found between the maternal microbiota clusters (**Table IV-1**). The prevalence of C-section as well as the administration of antibiotics due to the mode of delivery was significantly higher for Cluster I than for Cluster II. Cluster II presented higher rates of exclusive breastfeeding. Neonates belonging to Cluster II presented significantly lower BMI and WFL z-scores at birth, 1 month, and 18 months of age.

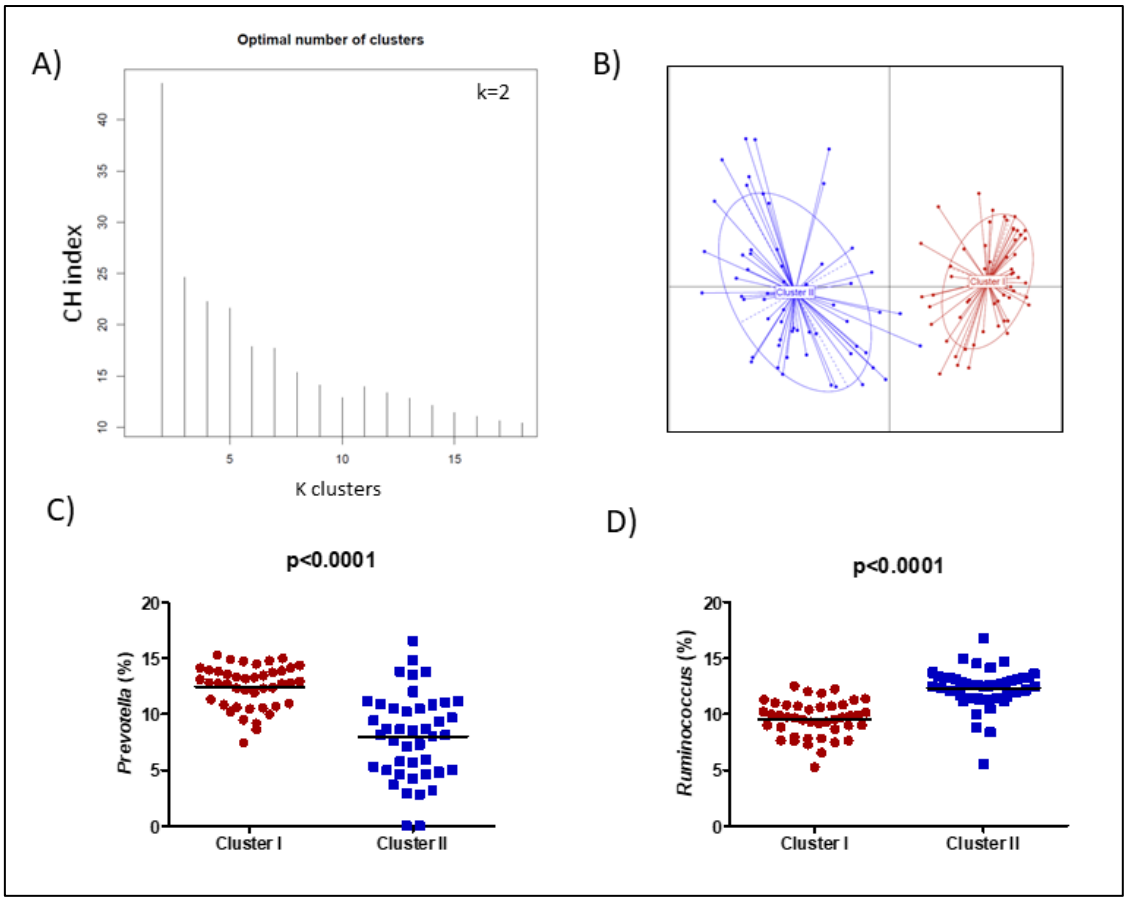


Figure IV-1. Maternal gut microbial clusters and representative genera. A-B) The PAM method shows that participants are separated into two clusters and Principal Coordinate Analysis (PCoA) showed the two differential clusters (**B**). **C-D**) The relative abundances (%) of the representative bacterial genus in each cluster are represented, *Prevotella* in Cluster I (red circles) (**C**) and *Ruminococcus* in Cluster II (blue squares) **(D**). The line represents the media of all values.

Table IV-1. Characteristics of mothers-neonates

		All (n=86)	Cluster I (n=43)	Cluster II (n=43)	*P* value
Maternal characteristics					
Age (years)		31.12 ± 4.12	31.61 ± 4.31	30.65 ± 3.87	*0.39*
Pre-gestational BMI (Kg/m2)		23.14 ± 4.27	23.13 ± 4.79	23.15 ± 3.72	*0.55*
Weight gain during pregnancy		13.05 ± 4.16	13.45 ± 3.96	12.65 ± 4.38	*0.45*
Antibiotic during pregnancy (%)		30 (34.88)	19 (44.19)	11 (25.58)	*0.08*
Gestational age (weeks)		39.39 ± 1.15	39.2 ± 1.03	39.6 ± 1.21	*0.12*
Mode of delivery: Vaginal birth (%)		60 (69.77)	22 (51.16)	38 (88.37)	***<0.0001***
Infant characteristics					
Gender: Female (%)		40 (46.51)	19 (44.19)	21 (48.84)	*0.66*
Birth weight (kg)		3.35 ± 0.42	3.36 ± 0.46	3.33 ± 0.38	*0.69*
Antibiotic at birth		32 (37.21)	22 (51.16)	10 (23.26)	***<0.0001***
Exclusive breastfeeding until 6 month		59 (68.61)	24 (55.81)	35 (81.4)	***0.01***
BMI z-score [1]	at birth	-0.24 ± 1.04	-0.30 ± 1.23	-0.17 ± 0.87	***0.01***
	1 month	-0.47 ± 0.99	-0.46 ± 1.08	-0.49 ± 0.89	***0.02***
	6 months	-0.32 ± 0.95	-0.45 ± 1.04	-0.20 ± 0.86	*0.34*
	12 months	0.06 ± 1.17	0.46 ± 1.28	0.08 ± 1.04	*0.16*
	18 months	0.14 ± 1.16	0.21 ± 1.33	0.07 ± 0.96	***0.04***
WFL z-score [1]	at birth	-0.37±1.23	-0.48±1.42	-0.26±1.01	***0.01***
	1 month	-0.55±1.24	-0.52±1.38	-0.58±1.10	***0.01***
	6 months	-0.19±0.94	-0.28±1.02	-0.09±0.86	*0.44*
	12 months	0.08±1.10	0.06±1.23	0.10±0.97	*0.16*
	18 months	0.13±1.09	0.19±1.25	0.07±0.91	***0.05***
Maternal Dietary intakes					
	MD score	8.8 ± 1.7	8.3 ± 1.7	9.1 ± 1.7	*0.17*
Total protein (g/day)		112.7 ± 18.4	110.4 ± 18.2	115.0 ± 18.6	*0.25*
	Animal protein (g/day)	60.7 ± 18.0	60.9 ± 19.1	60.5 ± 17.1	*0.90*
	Vegetal protein (g/day)	49.2 ± 13.6	46.7 ± 11.6	51.8 ± 15.0	*0.08*
Lipids (g/day)		111.1 ± 17.5	109.9 ± 19.4	112.4 ± 15.5	*0.52*
	Cholesterol (g/day)	295.78 ± 74.7	295.5 ± 72.12	296.0 ± 78.1	*0.98*
	SFA	22.8 ± 5.5	23.34 ± 5.8	22.3 ± 5.1	*0.37*
	TRANS	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	*0.30*
	MUFA	46.4 ± 9.9	45.7 ± 11.7	47.2 ± 7.6	*0.50*
	PUFA	17.6 ± 4.4	18.3 ± 5.3	16.8 ± 3.2	*0.12*
	n-6 CLA	0.004 ± 0.01	0.003 ± 0.01	0.005 ± 0.01	*0.13*
	n-3 ALA	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	*0.33*
	n-3 EPA	0.2 ± 0.1	0.1 ± 0.1	0.2 ± 0.1	*0.07*
	n-3 DPA	0.1 ± 0.05	0.06 ± 0.04	0.08 ± 0.05	***0.04***
	n-3 DHA	0.3 ± 0.2	0.3 ± 0.2	0.4 ± 0.2	***0.04***
Carbohydrates (g/day)		259.34 ± 40.6	264.8 ± 45.4	253.8 ± 34.7	*0.22*
	Polysaccharides (g/day)	137.2 ± 36.8	143.78 ± 42.2	130.5 ± 29.4	*0.10*
Lactose (g/day)		11.3 ±10.4	13.9 ± 10.8	8.7 ± 9.3	***0.02***
Total dietary fiber (g/day)		37.5 ± 12.0	34.1 ± 8.8	40.9 ± 13.8	***0.01***
	Cellulose (g/day)	8.8 ± 3.2	8.1 ± 2.7	9.5 ± 3.6	***0.05***
	Insoluble dietary fiber (g/day)	23.2 ± 8.4	21.4 ± 6.6	25.2 ± 9.6	***0.04***
	Insoluble hemicellulose (g/day)	7.9 ± 3.6	7.1 ± 2.9	8.8 ± 4.0	***0.03***
	Insoluble pectin (g/day)	2.8 ± 1.1	2.5 ± 0.8	3.1 ± 1.3	***0.01***
	Soluble dietary fiber (g/day)	4.6 ± 1.5	4.3 ± 1.2	5.0 ± 1.7	***0.05***
	Soluble hemicellulose (g/day)	2.9 ± 1.0	2.7 ± 0.9	3.1 ± 1.1	*0.10*
	Soluble pectin (g/day)	1.5 ± 0.6	1.4 ± 0.5	1.6 ± 0.6	*0.07*
	Starch (g/day)	29.2 ± 12.5	29.3 ± 12.9	29.1 ± 12.3	*0.94*
	Klason lignin (g/day)	3.45 ± 1.2	3.4 ± 1.1	3.5 ± 1.2	*0.81*
Phytosterols (mg/day)		137.5 ± 37.5	132.8 ± 36.6	142.4 ± 38.2	*0.25*
Polyphenols (mg/day)		1870.7 ± 918.4	1636.7 ± 596.2	2110.5 ± 1117.3	***0.02***

For numerical data, results are shown as media±SD and for the categorical data, results are shown as number of cases (percentage %). Student T-test and Chi-Squared test were performed to assess the significance of the differences in the characteristics between clusters section in numerical variables and categorical variables, respectively. $p<0.05$ was considered statistically significant.

[1] Multivariate ANOVA model adjusted for mode of birth, antibiotic exposure, lactation, and maternal pre-gestational BMI. p<0.05 was considered statistically significant. BMI and WL z-scores were obtained with WHO curves adjusted by age and gender.

Diets were adjusted by total energy intake to 2500 Kcal/day. Each nutrients value was extracted by the food frequency questionnaire using the Centro de Enseñanza Superior de Nutrición Humana y Dietética

(CESNID) tables. Saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), trans-unsaturated fatty acids (TRANS); omega-6 Conjugated Linoleic Acid (CLA); omega-3 alpha-linolenic acid (ALA); omega-3 Eicosapentaenoic acid (EPA); omega-3 Docosapentaenoic acid (DPA); omega-3 docosahexaenoic acid (DHA).

MD: Mediterranean Diet Adherence score according to PREDIMED questionnaires.

Maternal microbial cluster composition and diversity

Microbial structure between maternal microbial clusters was also confirmed by principal coordinates analysis (PCoA) with Bray–Curtis dissimilarity matrices (PERMANOVA R2=0.188, $p < 0.001$) (**Fig. IV-2, A**). A redundancy analysis (RDA) was also consistent with a clear differentiation in the microbiota composition between clusters (variance =31.74, F =4.95 $p = 0.001$) (**Fig. IV-2, B**).

Specifically, the maternal gut microbial in Cluster I was enriched by the genus *Prevotella* ($p< 0.0001$), followed by *Peptoniphilus, Anaerococcus,* and *Porphyromonas;* Cluster II was enriched by *Ruminococcus* ($p <0.0001$) and an unclassified genus from the Ruminococcaceae family ($p<0.001$), followed by the unclassified genus *Clostridiales* ($p <0.001$), an unclassified genus from the Lachnospiraceae family *(* $p <0.001$), *Bacteroides* (p <0.001), *Blautia* ($p <0.001$), and *Bifidobacterium* ($p <0.001$). To further investigate the association of specific microbial taxa with maternal microbiota clusters, a linear discriminant analysis of effect size (LEfSe) was performed. The prevalence of the *Ruminococcus* as well as unclassified Ruminococcaceae, Clostridiales, and Lachnospiraceae reliably identified Cluster II. In addition, the prevalence of *Prevotella* and WAL_1855D (order Clostridiales), *Anaerococcus, Peptoniphilus,* and the *Finegoldia* genus were linked to Cluster I (**Fig. IV-2, C**). Microbiota alpha-diversity values, measured by Shannon, Chao1 and inverse Simpson indexes, also showed significant differences according to clustering stratification ($p < 0.05$). Higher significant microbial diversity and richness were observed in maternal gut microbiota in Cluster I than those observed in Cluster II (**Fig. IV-2, D–F**).

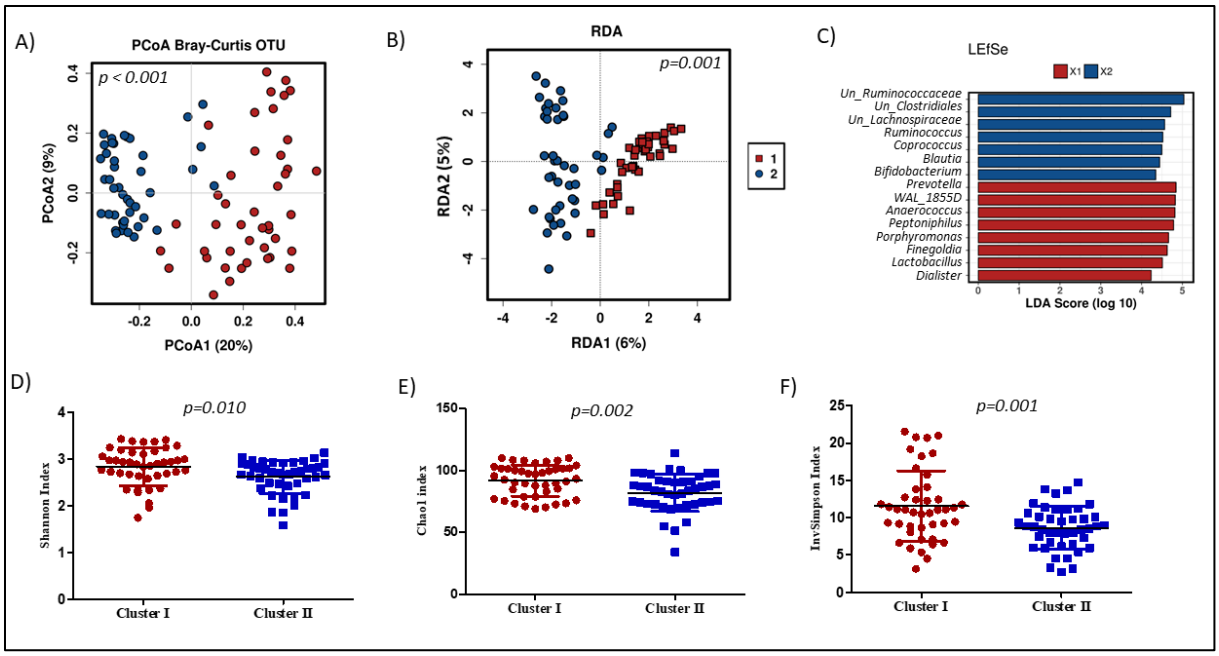


Figure IV-2 Maternal microbial clusters characteristics and alfa and beta diversity. A-B) Principal Coordinate Analysis (PCoA) with Bray–Curtis index (**A**) and Multivariate RDA (**B**) showed significant differences in microbial communities between clusters. **C**) Linear Discriminant Analysis (LDA) Effect Size (LEfSe) plot of taxonomic biomarkers was identified in both clusters. Cluster I (red color) and Cluster II (blue color). **D-F**) Microbial diversity and richness indexes at species level according to each cluster. Mean ±SD and p-values with T-test. Cluster I = red circles and Cluster II = blue squares.

Maternal microbial clusters are linked to specific dietary compounds

The maternal intakes of the major dietary nutrients were significantly different between microbial clusters (**Table IV-1**). A significantly higher intake of total and insoluble dietary fiber (attributable to insoluble pectin), higher intakes of omega-3 fatty acids (mainly of docosahexaenoic acid [DHA] and docosapentaenoic acid [DPA], and polyphenols were observed in mothers from Cluster II compared to Cluster I.

Furthermore, a PERMANOVA test for dietary factors contributing to overall differences in maternal microbiota composition showed that the main contributors were polyunsaturated fatty acids (PUFA (R^2=0.044, *p = 0.010*), animal protein (R^2=0.036, *p = 0.022*), and total fiber (R^2=0.036, *p = 0.023*); no relevance was found for lipids, monounsaturated fatty acids (MUFA), saturated fatty acids (SFA), carbohydrates (CHO), total protein, and vegetable protein. A principal component analysis (PCA) biplot showed that maternal microbiota clusters were associated with a specific microbial genus **(Fig. IV-3, A)** and nutrients **(Fig. IV-3, B)**. Cluster I was associated with CHO, SFA, and proteins (mostly animal protein), and to *Prevotella*, *Peptoniphilus*,

Finegoldia, and *Anaerococcus*, while Cluster II was associated with dietary fiber, vegetable protein, polyphenols, and lipids (mainly, the n-3 fatty acids DHA and DPA) as well as *Ruminococcus* and unclassified *Ruminococcaceae*.

Specific associations between different nutrient intakes and the most predominant gut bacteria at the genus level were found **(Fig. IV-3, C)**. We observed a strong positive association of bacterial groups representative of Cluster II with omega-3 fatty acids such as EPA, DHA, and DPA and several dietary fibers. Moreover, we also demonstrated significant correlations between all these analyzed nutrients and other specific gut bacteria that appeared in lower relative abundances. A significant pattern was detected that associated the consumption of nutrients present in foods of plant-based origin, such as vegetable proteins, dietary fiber, and polyphenols with members of the family *Christensenellaceae*, *Dehalobacterium*, and *Eubacterium* and negatively with bacterial groups such as *Campylobacter* and *Dialister*.

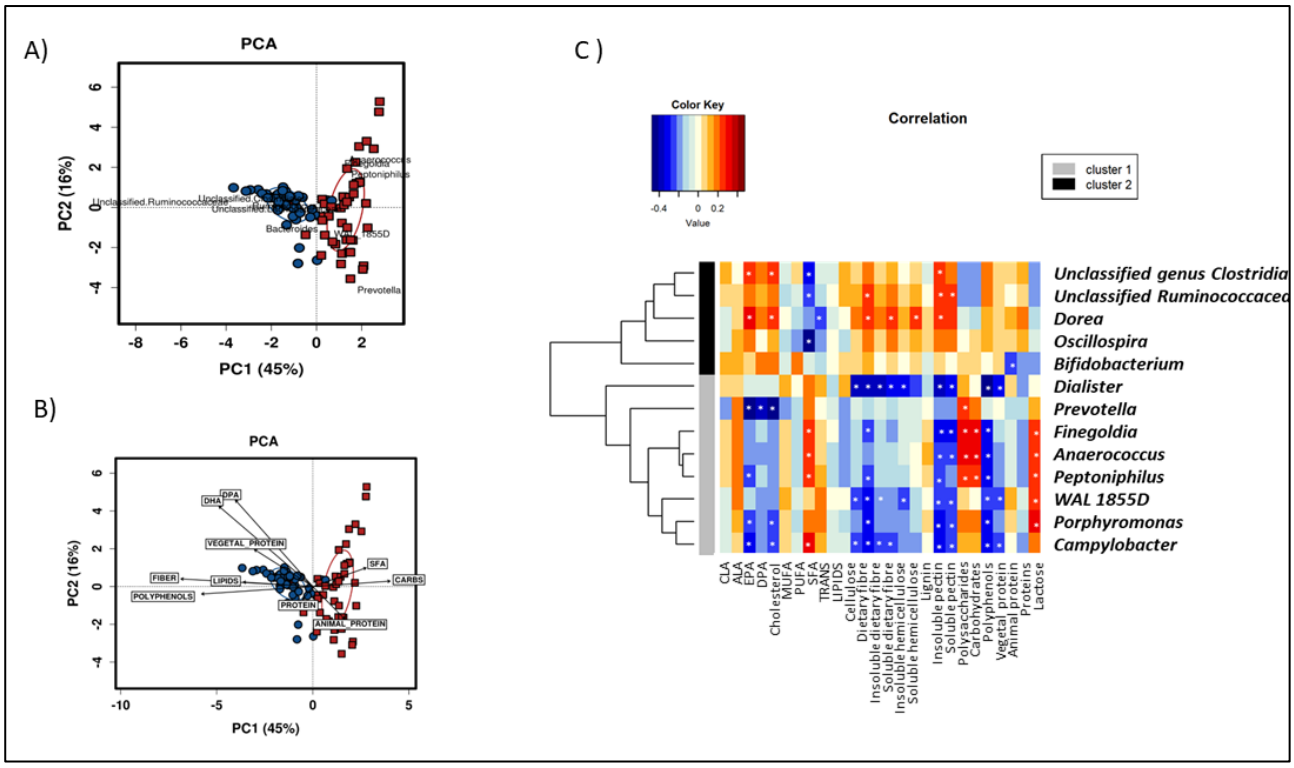


Figure IV-3. Maternal diet shapes the microbial clusters. A-B) Biplots from the principal component analysis (PCA) showed that maternal microbiota clusters were associated with specific microbial genus (**A**) and nutrients (**B**). Cluster I = red squares and Cluster II = blue circles. **C**) Pearson correlations between nutrient intake during pregnancy and bacterial abundance at the genus level at delivery. Association of dietary components with the most abundant bacterial group of each cluster. Significant correlations ($p < 0.05$) were marked by an asterisk (*). Blue squares marked the negative correlations, whereas the red color showed positive correlations as measured by Pearson's correlations.

Maternal microbial clusters are linked to neonatal microbiota at birth

Neonatal microbiota composition at birth was shaped by maternal microbial clusters (PERMANOVA, R^2=0.035, *p = 0.005*). A higher relative abundance of Firmicutes was observed in Cluster I compared to Cluster II **(***p=0.060*) **(Fig. IV-4, A)**. A multivariate analysis (RDA) at the genus level also showed distinct neonatal microbial communities at birth shaped significantly by the maternal microbial cluster **(**F=2.21, *p=0.001*) **(Fig. IV-4, B)**. No differences were found in neonatal alpha-diversity indexes (Shannon, Chao1, and Inverse Simpson indexes) according to maternal clusters. Further, a PERMANOVA model test (Bray-Curtis, *p = 0.050*) for dietary factors contributing to overall differences in neonatal microbiota composition showed that the main contributors were PUFA (R^2=0.028, *p = 0.020*) and animal protein (R^2=0.030, *p = 0.015*). A PCA biplot showed that maternal microbiota clusters were linked to neonatal microbiota, explaining the first two principal components (PC1 and PC2) a 59% of the total variance **(Fig. IV-4, C)**. Cluster I was associated with PUFA and animal protein, while Cluster II was associated with fiber and vegetable protein. Furthermore, specific maternal nutrients were associated with neonatal microbiota (**Fig. IV-4, D**). Higher maternal intake of SFA was significantly associated with lower Proteobacteria and higher Firmicutes, while higher maternal intake of fiber and vegetable protein were associated with lower Bacteroidetes phylum in neonatal gut microbiota.

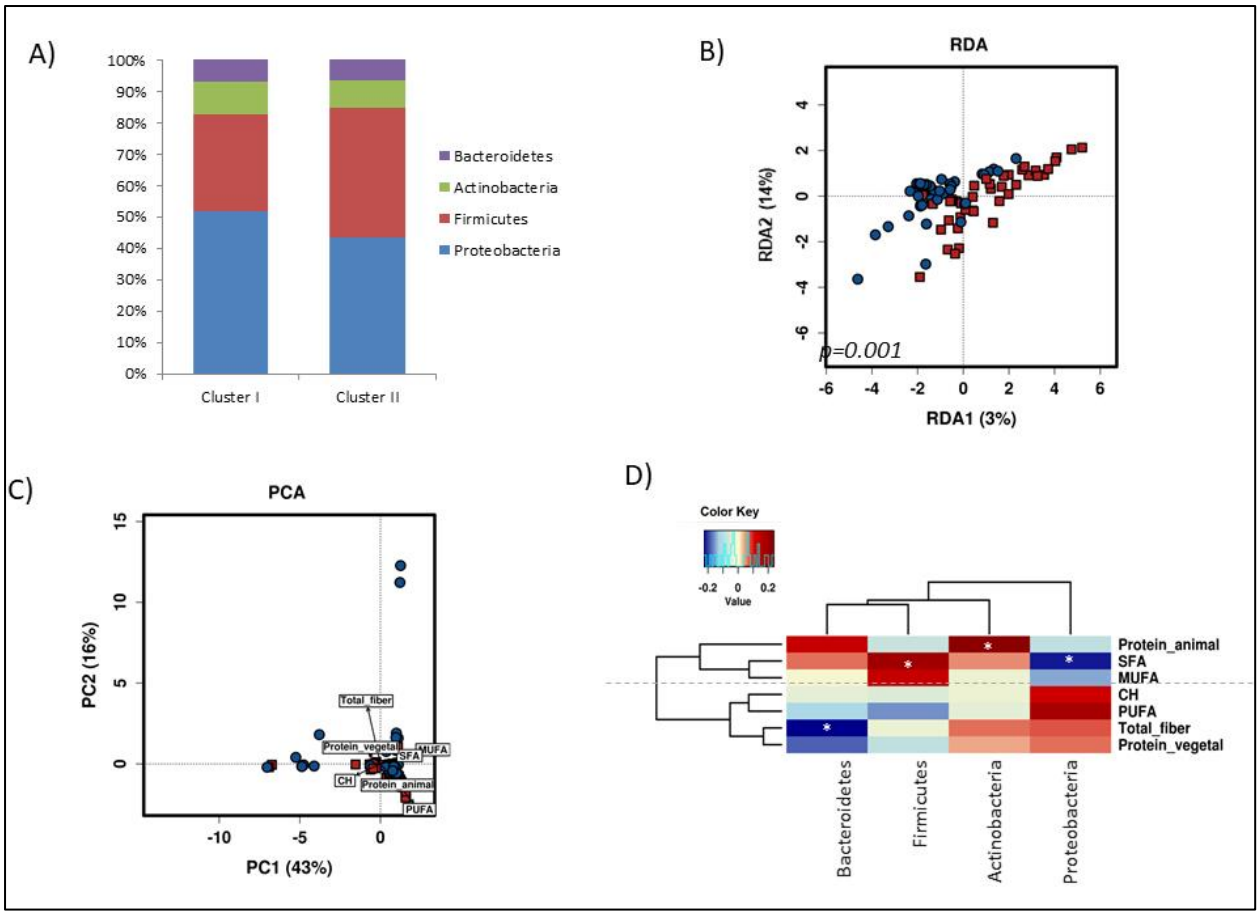


Figure IV-4. Maternal gut microbial clusters and diet drive the neonatal first pass microbiota. A) Relative abundances (%) of the neonatal microbial at the phylum level stratified by maternal microbial cluster. **B**) Multivariate RDA showed significant differences in neonatal microbial communities depending on maternal microbial clusters. **C**) Biplots from the principal component analysis (PCA) showed that neonatal microbiota is shaped by maternal microbiota clusters and they are associated with specific nutrients. **D**) Pearson correlations between maternal intake of nutrients during pregnancy and neonatal first pass bacterial relative abundance at phylum level at birth. Significant correlations ($p < 0.05$) were marked by an asterisk (*). Blue squares marked the negative correlations, whereas red showed positive correlations as measured by Pearson's correlations.

Maternal microbial clusters are linked to infant growth development and risk of overweight

Maternal microbial clusters had a significant impact on the BMI z-scores during the first 18 months (**Table IV-1**). Higher BMI z-scores and WFL z-scores were observed in Cluster I compared to Cluster II. Infants from maternal Cluster I and born by C-section showed significantly higher BMI and WFL z-scores at 18 months than those observed in infants from vaginal births from mothers classified in Cluster II (**Fig. IV-5, A-B**). Furthermore, in stratifying the infants by BMI-for-age z-score cut-points of >1.0 as being at risk of overweight, we found that 52.4% (11/21) of C-section neonates

from maternal Cluster I were at risk compared to 0% (0/5) in neonates from maternal Cluster II (Fisher test *p = 0.052*); no differences were found in vaginal-born neonates. Additionally, a multivariate linear model analysis, adjusted by mode of delivery, antibiotic exposure, pregestational maternal BMI, and feeding method, demonstrated that neonates belonging to maternal Cluster I presented a significantly higher BMI z-score at 1 month of life (*p = 0.020*) and later at 18 months of life (*p = 0.040*).

Infant longitudinal BMI and WFL z-score trajectories from birth to 18 months were shaped by maternal microbial clusters and mode of delivery (adjusted ANOVA repeated measures *p = 0.001*) showed higher BMI z-scores in children born by C-section and, remarkably, in those born from mothers stratified in Cluster I (**Fig. IV-5, C**), which was enriched in the genus *Prevotella*, followed by *Peptoniphilus*, *Anaerococcus*, and *Porphyromonas*, and was linked to lower fiber intake and n-3 fatty acids and higher intakes of animal protein and SFA (**Fig. IV-3**). Similar longitudinal trajectories in WL z-scores from birth to 18 months were observed (**Fig. IV-5, D**)

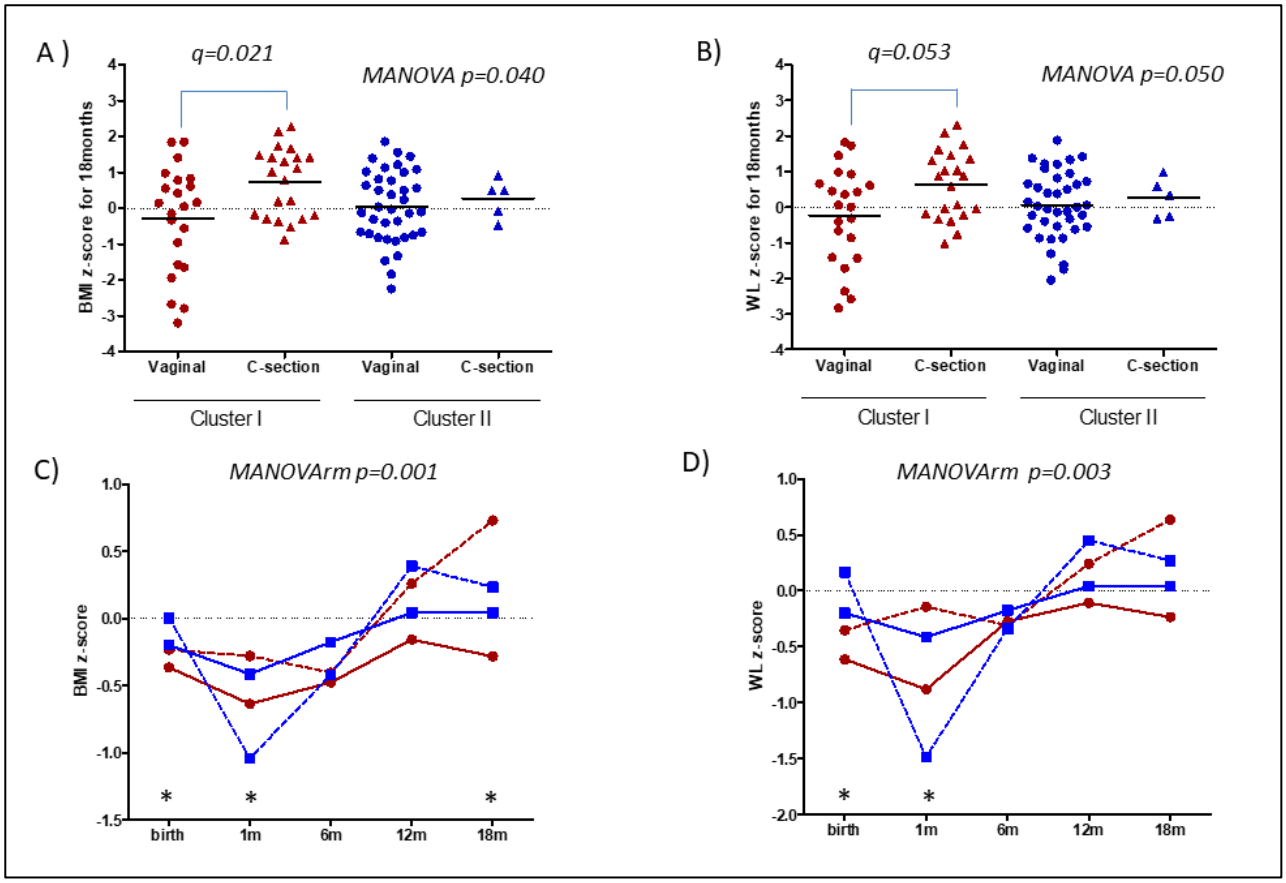


Figure IV-5. Maternal gut microbial clusters and mode of birth impact the infant risk of overweight and obesity. A-B) BMI z-scores (**A**) and WL (**B**) z-scores at 18 months of age adjusted by covariates according to mode of birth and maternal microbial cluster. The middle

line represents the media of all values; a general linear model multivariate ANOVA test adjusted by factors and covariates was done and $p < 0.05$ was considered significant. **C-D**) Infant BMI z-score (C) and WL Z-score (**D**) trajectories from birth to 18 months were stratified by maternal microbial cluster and birth mode. The GLM for repeated measures adjusted for maternal cluster, pregestational BMI, mode of delivery and breastfeeding up to 6 months was done and $p < 0.05$ was considered statistically significant. Points at each time represent the media and lines mark the time series. Cluster I = red and Cluster II = blue; a continuous line = vaginal birth and a dotted line = C-section plus antibiotic. * represents a significant difference in each time point between groups. ***p < 0.05*; ****p < 0.001*; *p < 0.0001*.

DISCUSSION

Our study showed that maternal microbiota was clustered in two distinct microbial groups linked to specific dietary components during pregnancy. We reported that maternal diet modulates maternal microbiota and shapes the neonatal first pass of microbiota at birth, affecting the initial microbial establishment, with potential effects on infant development and health later in life. We observed an association between the maternal gut microbiota composition, neonatal gut microbiota, and WFL and BMI z-score at 1 and at 18 months, depending on birth type. These findings are the first step for the design of dietary strategies targeted at improving the microbial composition of the mother and therefore the newborn microbiota, thus affecting their future health status.

Maternal microbial clusters were predominantly characterized by a higher abundance of *Prevotella* (Cluster I) and a higher abundance of the *Ruminococcus* species (Cluster II), which were closely linked to diets comprising a high content of plant foods and some types of fatty acids.

Maternal microbiota Cluster I was linked to higher intakes of carbohydrates, while Cluster II tended to be associated with higher intakes of vegetable protein and fats. The role of the *Prevotella* genus, mainly *Prevotella copri*, in the response to diet and its influence on human health is still unclear. It has been reported that a dietary intervention using fiber induced an improvement in glucose; this was related to an increase in the relative abundance of *Prevotella copri*, which favors glycogen storage [591]. However, another study reported that *P. copri* induced insulin resistance and affected glucose intolerance [592]. We also found significantly higher intakes of total dietary fiber as well as insoluble dietary fiber and insoluble pectin, polyunsaturated fatty acids (EPA, DHA, and DPA), and polyphenols in mothers in Cluster II, which was

characterized by enrichment of the *Ruminococcus* genus. The *Ruminococcus* species have been reported to be enriched in individuals under a diet rich in plant polysaccharides [593]; in addition, they are cellulolytic bacteria that utilize cellulose and hemicellulose rich in plant material [594]. In fact, Walker et al.[594] also showed that the *Ruminococcus* species were also strongly associated with solid particles, as insoluble dietary fibers present in the human colon compared to *Bacteroides*. Our data showed the presence of *Faecalibacterium prausnitzii* in higher relative abundance in Cluster II. *Faecalibacterium prausnitzii* is a well-known butyrate producer. Considered to be a biomarker of colonic health, it has been associated with anti-inflammatory properties [595,596].

In addition to members of the *Ruminococcaceae* family as main players in Cluster II, we also observed a significantly higher representation of other bacterial groups, such as members of the *Clostridiales* genus, *Lachnospiraceae* family, *Bacteroides*, *Blautia*, *Bifidobacterium*, and *Coprococcus* in Cluster II with respect to Cluster I. Many of these bacterial taxa are also involved in butyrate production [597]. It is well known that fiber intake leads to an increase in the production of short chain fatty acids (SCFA) through microbiota. It has been reported that *Lachnospira*, *Blautia*, *Coprococcus*, and in the case of *Bifidobacterium*, through cross-feeding interactions consume plants to produce SCFA, with important benefits on human health [453]. However, Cluster I showed lower butyrate-producing bacteria but higher presence of oral bacteria *Prevotella corporis* and *Prevotella nigrescens*, *Porphyromonas*, or bacteria related to disease, such as *Peptoniphilus*, *Campylobacter*, and *Dialister* [598–602].

There is growing evidence linking several disorders associated with a translocation of oral bacteria in the intestinal tract [603–605]. It has been suggested that oral bacteria present in the gut might be linked to an increased risk of disease as well as a risk of pregnancy complications [573,606]. In addition, the presence of periodontal pathogenic bacteria at delivery can be transmitted to the neonate, promoting an aberrant early colonization pattern [604,605]. An inverse association has been described between dietary fiber intake and the prevalence and incidence of periodontal disease in large-scale epidemiological studies [607,608]. A recent study, which considered the impact of a dietary pattern on the oral microbiota, demonstrated significant differences in the oral microbiota composition of vegans in comparison with omnivores [601].

Many other works highlight the relevant effect of dietary fiber on gut microbiota composition and diversity, but less is known about the impact of fatty acids such as

omega-3 PUFAs. Omega-3 PUFAs, including DHA, EPA, and DPA, are essential nutrients with potential health benefits during pregnancy and for infant development [609]. Omega-3 PUFAs reduce the risk of preeclampsia and pre-term birth and promote correct neurological and visual development in the neonate [610,611]. Furthermore, omega-3 PUFAs have been shown to decrease inflammatory status, thereby having a positive effect on obesity, diabetes mellitus type-2, and several cardiovascular diseases [612]. Therefore, a diet rich in omega-3 is considered beneficial in several respects; however, the changes in gut microbiota composition associated with PUFAs are poorly understood. In our study, Cluster II showed significantly higher intakes of some of the main fatty acids, such as EPA, DHA, and DPA. The overrepresentation of members of the *Ruminococcaceae* and *Lachnospiraceae* families, the *Blautia*, *Bifidobacterium*, *Coprococcus*, and *Oscillospira* genera in Cluster II, and the association with omega-3 fatty acids is consistent with some previous human studies described by Costantini et al.[613]. Moreover, in some cases, the taxa could differ from previous studies due to the type of food matrix in the diet the mother consumes during pregnancy. These differences in the type of PUFA administration could drive these differential changes in the gut microbiota composition. Furthermore, some phenolic compounds present antimicrobial properties with a potential bacteriostatic or bactericidal role, whereas others, such as flavanols, ellagitannins, or quercetin, promote the growth of beneficial bacterial groups belonging to the *Lactobacillus*, *Bifidobacterium*, and *Blautia coccoides-Eubacterium rectale* groups. Interestingly, bacterial communities that are present in higher relative abundances in Cluster II were characterized by significantly higher intakes of polyphenols [614,615].

Specific relationships between nutrients and maternal microbes were also identified. Higher intake of nutrients present in plant-based foods, such as vegetable proteins, dietary fiber, and polyphenols, was associated with a higher presence of members of *Christensenellaceae*, *Dehalobacterium*, and *Eubacterium*, and negatively associated with *Campylobacter* and *Dialister*. In a previous study, we demonstrated higher levels of *Christensellaceae* in individuals who presented a high-level adherence to the Mediterranean diet (MD) [453]. The MD pattern is characterized by a high intake of fruits, vegetables, legumes, and olive oil as the main source of fats, and foods that contain large amounts of the previously cited nutritional compounds. In addition, *Christensellaceae* has been linked with leanness [286]. *Dehalobacterium* and *Eubacterium* as well as *Anaeroplasma, Roseburia*, and *Oscillospira*, appeared to play

an important role against atherosclerosis and showed significant negative correlation with atherosclerotic plaque size and plasma adipocytes [616]. *Dehalobacterium* and *Eubacterium* produce butyrate and propionate, respectively, and are two of the SCFAs that are most often considered beneficial to health [40,597]. The prevalence of overweight and obesity has nearly tripled in the last 40 years, and the rate among children is also rapidly increasing.[617] Consequently, it is important to identify infants at risk for overweight to avoid short- and long-term health consequences.

For that purpose, various studies have examined specific anthropometrical measurements such as WFL and BMI z-scores to assess the risk of overweight and obesity before age 2. WFL is the current measurement recommended by the American Guidelines, as recommended by the American Academy of Pediatrics (AAP) [618,619]. However, recent studies have suggested that BMI would be a useful tool that also provides information of adiposity and future obesity risk [619–621]. For children under age 5, the WHO defines the risk of overweight when BMI and/or WLF z-score are greater than 1 standard deviation (SD), overweight when they are greater than 2 SD, and obesity when they are greater than 3 SD according to the WHO Child Growth Standards median [587]. In our study, a higher prevalence of children at risk of overweight according to BMI and WFL z-scores at 18 months was found within mothers in Cluster I. Although no significant differences in maternal BMI according to the clusters were observed, a recent study pointed out that *Finegoldia* and *Wal_1885D*, bacterial features associated with maternal Cluster I, were associated with maternal overweight, obesity, and excessive gestational weight gain during pregnancy [492]. Those maternal gut microbial shifts would have health consequences for the child through an early colonization of the infant gut microbiota. It is well known that other factors during early life such as mode of delivery, antibiotic exposure, and infant feeding practices may cause potentially adverse effects on the development of the infant gut microbiota and may have an impact on obesity risk [622,623]. Several studies have shown an increased risk of obesity in infants exposed to antibiotics mainly due to C-section birth [204,254,624,625]. C-section is always performed under antibiotic exposure to avoid infections, and both C-section and antibiotics together alter the natural transmission of microbes from the mother to the child; this affects the correct development of the gut microbiota. In addition, this effect occurs in early life, a period when the gut microbiota is particularly susceptible to perturbations with long-lasting effects on metabolic programming and obesity risk.

Children from maternal Cluster I and born by C-section showed higher BMI and WFL z-scores at 18 months than those observed in vaginal births and in infants from mothers in Cluster II. Additionally, a multivariate linear model analysis, adjusted by mode of delivery, antibiotic exposure, pre-gestational maternal BMI, and feeding method demonstrated that neonates belonging to maternal Cluster I presented significantly higher BMI and WFL z-scores at 1 month and 18 months. Regression models also showed that the maternal microbial cluster and mode of delivery were significant predictors of risk of overweight at 18 months according to the WHO cut-off for BMI z-score. Children's BMI and WFL z-score trajectories from birth to 18 months of age were shaped by maternal microbial clusters and mode of birth, showing higher BMI and WFL z-scores in neonates born by C-section and, remarkably, in those born from mothers in Cluster I, which was enriched in the genus *Prevotella*, followed by *Peptoniphilus*, *Anaerococcus*, and *Porphyromonas*, was linked to lower fiber intake and n-3 fatty acids, and had higher intakes of animal protein and SFA. This suggests that maternal diet is also a relevant factor during early life due to the potential role in mother–infant microbial transference, which would affect infant growth and development. Although C-section birth limits maternal microbial transfer due to antibiotic treatment together with reduced exposure to the intestinal maternal microbiota, maternal diet would modulate microbial pioneers that could be relevant for colonization of the neonatal microbiota and have a potential effect on infant health. The direct relationship between maternal diet–microbiota and C-section with infant development and the risk of overweight and obesity later in life warrants further investigation.

Study limitations

Our study has some limitations related to its observational nature, the sample size and power analysis, and the collection of dietary information. The limited power size may have hampered our ability somewhat to detect other significant associations; large-scale prospective longitudinal studies are required. However, this would be mitigated by the difficulty of including mother–infant pairs in the study. Dietary information recorded by the Food Frequency Questionnaire (FFQ) would introduce bias caused by errors in memory and the lack of perception of food proportion sizes. Therefore, the quantification of the nutritional compound intake is limited and 24 h recall and/or 3 d recall, including portions, would be desirable for future studies.

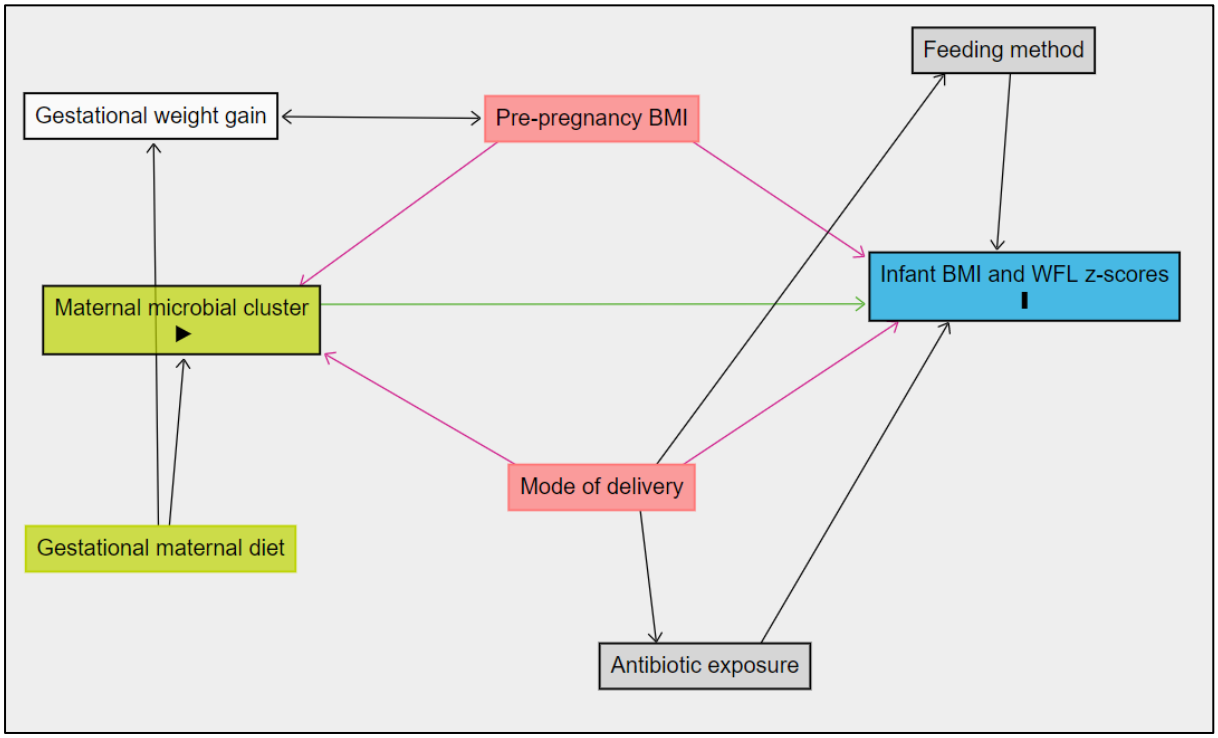
Additionally, there is increasing evidence suggesting that dietary patterns are usually associated with other lifestyle routines such as exercise or chronic stress, which are not considered in this study. Furthermore, our study considered some perinatal factors, but it is known that during the first 18 months of life, many other environmental factors play a role in infant development and risk of diseases.

Despite all these limitations, our study demonstrated how diets during healthy pregnancies have an influence on the maternal–neonatal microbiota, with potential effects on infant growth. Furthermore, the microbiota composition was analyzed using DNA methods; microbiota activity measured by SCFA or metabolomics would provide novel perspectives to understand the diet–microbiota interactions and their effects on host health. Further studies with a larger number of infants as well as a longer follow-up period are needed.

CONCLUSION

Maternal diet during pregnancy, together with other perinatal factors such as mode of delivery, antibiotic exposure, and breastfeeding, represent potential factors to consider for infant microbial colonization. The maternal microbiota is shaped by diet, especially with regard to fiber, lipids, and proteins, and may have a significant effect on the establishment of neonatal microbiota, as well as a potential contribution on infant development and the risk of overweight during the first months of life. C-section neonates from mothers with more adequate diets shaping specific microbiota presented a lower risk of overweight at age 18 months. Diet matters for maternal–neonatal microbiota and health outcomes. Therefore, specific dietary programs targeting pregnant women may be a cost-effective intervention factor to promote adequate vertical mother–infant exposition.

CHAPTER IV- SUPPLEMENTARY DATA



Supplementary file IV-1. Direct acyclic graph (DAG) method for covariate selection adjustment. DAG method revealed that minimal sufficient adjustment set for estimating the total effect of maternal microbial cluster on infant BMI and WFL z-scores were mode of delivery, pre-pregnancy body mass index (BMI), antibiotic exposure and feeding method (not included in the original paper).

CHAPTER V

PERINATAL ENVIRONMENT SHAPES MICROBIOTA COLONIZATION AND INFANT GROWTH: IMPACT ON HOST RESPONSE AND INTESTINAL FUNCTION

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ABSTRACT

Early microbial colonization triggers processes that result in intestinal maturation and immune priming. Perinatal factors, especially those associated with birth, including both mode and place of delivery are critical to shaping the infant gut microbiota with potential health consequences. Gut microbiota profile of 180 healthy infants (n=23 born at home and n=157 born in hospital, 41.7% via caesarean section [CS]) was analyzed by 16S rRNA gene sequencing at birth, seven days and one month of life. Breastfeeding habits, infant clinical data, including length, weight, and antibiotic exposure, were collected up to 18 months of life. Long-term personalized *in vitro* models of the intestinal epithelium and innate immune system were used to assess the link between gut microbiota composition, intestinal function, and immune response.

Microbiota profiles were shaped by the place and mode of delivery, and they had a distinct biological impact on the immune response and intestinal function in epithelial/immune cell models. Bacteroidetes and *Bifidobacterium* genus were decreased in C-section infants, who showed higher z-scores BMI and W/L during the first 18 months of life. Intestinal simulated epithelium had a stronger epithelial barrier function and intestinal maturation, alongside a higher immunological response (TLR4 route activation and pro-inflammatory cytokine release), when exposed to home-birth fecal supernatants, compared with CS. Distinct host response could be associated with different microbiota profiles.

Mode and place of birth influence the neonatal gut microbiota, likely shaping its interplay with the host through the maturation of the intestinal epithelium, regulation of the intestinal epithelial barrier and control of the innate immune system during early life, which can affect the phenotypic responses linked to metabolic processes in infants.

Keywords: Microbiota, environment, mode of birth, antibiotics, epithelial barrier, immune system, early programming.

INTRODUCTION

Microbial colonization plays an important role in numerous functions, including digestion, metabolic reactions and trophic effects, and it also influences the development and maturation of the host's innate and adaptive immune system [10,30,626]. The mode of birth is a key factor shaping early microbial colonization [217,281,627]. Vaginally born (VAG) infants acquire microbial communities resembling the maternal vaginal and gut microbiota, whereas infants born via C-section acquire environmental-like bacteria such as *Staphylococcus*, *Corynebacterium* and *Propionibacterium* spp. [130]. In addition, CSs are associated with lower microbial diversity, delayed colonization of *Bacteroides* and *Bifidobacterium* spp. and reduced immune responses [223]. The CS rate increased by an annual increment of 3.7% from 2000 to 2015 worldwide [211,212]. In Europe, the average CS rate is 28% [214], although the World Health Organization (WHO) recommends a rate of 10–15% [213]. Epidemiological studies have linked CSs with a higher risk of non-communicable diseases such as obesity [91,431] and allergy [628]. Indeed, the CS procedure is characterized by pre- and intra-partum antibiotic exposure and other medical practices, which may affect early gut colonization and predispose the infant to developing immune-related disorders later in life, including asthma [629–631], allergy [93,182,303], obesity [427,488] and diabetes [632,633]. Hospital interventions during birth are critical for pioneer microbial colonizers and proper immune system maturation [634,635], which may impact adult health [35,68]. However, neonatal microbiota colonization in the absence of hospital interventions remains underexplored. Furthermore, the hospital environment (high-level disinfection and antibiotic therapy) has an impact on microbial exposure, thereby extending the hygiene hypothesis to the time of birth [636,637].

Home births (HBs) increased by 77% between 2004 and 2017 in the United States, while the rate of birth-center deliveries doubled during the same period [229]; however, in Europe, such births account for less than 1% of all deliveries, except in the Netherlands, where HBs represent 16.3% of births [638]. Recently, a distinct microbiota profile has been reported in VAG neonates born at home or in hospital [232], although the impact on microbiota development and the potential effects on neonatal health are not fully understood.

In this study, we investigate the influence of birth-related factors, including the place and mode of delivery on early colonization during the first month of life and on infant growth during the first 18 months. Furthermore, to understand the potential biological mechanisms involved, *in vitro* gut models are used to study the impact of distinct microbiota patterns on intestinal function and innate immune system maturation.

MATERIALS AND METHODS

Subjects and Sampling

A prospective cohort study was conducted to compare the intestinal microbiota of delivered infants born at the hospital (VAG, n=92 and CS, n=65) and at home (HB, n=24). Infants with available biological samples at birth, 7 days and 1 month, together with clinical data, were included (**Supplementary file V-1**).

Ethical approval for the study was obtained from the Ethics/Bioethics Committee for Clinical Research of Hospital La Fe, Hospital Clinic, Parc de Salut MAR and CSIC (Consejo Superior de Investigaciones Científicas) [ClinicalTrial.gov NCT03552939].

Parents gave written informed consent before enrollment. Methods were performed in accordance with the relevant guidelines and regulations published previously [420]. Women had healthy pregnancies with non-declared pathology and neonates were delivered at term without complications during labor or CS intervention. Neonatal fecal samples were obtained within the first 24 hours after delivery and stored in sterile conditions. A sterile, cotton-tipped swab was used for sampling neonatal fecal samples by trained clinical personnel at delivery room as detailed previously [420]. Subsequently, fecal samples were collected in sterile containers by the parents at home using detailed instructions at 1 week and 1 month after birth. Samples were immediately stored at -20°C, transported within 24h after collection and stored at -80 °C until analysis.

Pregnancy, intrapartum variables, and anthropometric data were recorded (**Supplementary file V-2**). Maternal age, maternal pre-pregnancy weight, weight gain over the pregnancy, maternal smoking status, mode of delivery, place of birth (home or hospital), birth weight and length, sex of the neonate, birth instrumentalization, maternal antibiotic exposure during pregnancy and maternal/infant antibiotic use at birth were also collected.

Infant length and weight were also registered at birth, 1, 6, 12, and 18 months. Z-scores of anthropometric measures were electronically computed using WHO Anthro software (www.who.int/ childgrowth/software/en/). The WHO Child Growth Standards provide child growth measures standardized by age and sex using z-score.

Fecal DNA Extraction

Total DNA was extracted from the fecal material (approx. 50-100 mg) using the Master-Pure DNA extraction Kit (Epicentre, Madison, WI, US) following the manufacturer's instructions with the following modifications: samples were treated with lysozyme (20 mg/mL) and mutanolysin (5U/mL) for 60 min at 37ºC and a preliminary step of cell disruption with 3-μm diameter glass beads during 1 min at 6 m/s by a bead beater FastPrep 24-5G Homogenizer (MP Biomedicals). Purification of the DNA was performed using DNA Purification Kit (Macherey-Nagel, Duren, Germany) according to manufacturer's instructions. DNA concentration was measured using Qubit® 2.0 Fluorometer (Life Technology, Carlsbad, CA, US) for further analysis.

Sequencing and Bioinformatics Analysis

DNA libraries were performed with the amplification of the V3-V4 variable region of the 16S rRNA gene following the 16S rDNA gene Metagenomic Sequencing Library Preparation Illumina protocol (Cod. 15044223 Rev. A). The primers were selected from [495]. A multiplexing step was conducted by the NextEra XT Index Kit (FC-131-2001) (Illumina, San Diego, CA, United States) and DNA quality of the library PCR product was measured by a Bioanalyzer DNA 1000 chip (Agilent Technologies, Santa Clara, CA, United States) to verify the size, the expected size on a Bioanalyzer trace is ~550 bp. The libraries were sequenced using was a 2 x 300 bp paired-end run (MiSeq Reagent kit v3) on a MiSeq-Illumina platform (FISABIO sequencing service, Valencia, Spain) according to manufacturer instructions. Obtained reads were searched for residual adaptors using the program Trimmomatic [496].

Quality-trimmed and filtering was assessed using DADA2 pipeline [389]. After quality examination, reads were trimmed at the 270th and 210th nucleotide in forward and reverse position, respectively. Additionally, adapters were also removed in the filtering process and a maximum of 2 expected errors was considered. The following denoising and merging steps were performed, and chimeras were also removed.

Taxonomic assignment was conducted using the Silva v132 database with the addition of the specie level classification by the same database.

Taxa occurring <3 reads in at least 10% of the total samples number and those representing less than 0.01% of the reads across all the samples were filtered. Furthermore, the *decontam* package [408] in R environment [445,497] was used to determine the presence of potential contaminants-related sequence. Samples with less than 1000 reads were also removed from the final analysis (n=6). One of those samples was from a vaginally born infant with samples only at delivery time and this infant data was eliminated in further analysis. The 16S rRNA gene sequence data generated is available through NCBI Sequence Read Archive Database under project accession number BioProject ID PRJNA614975.

Predictive inferred functional analysis was performed using PICRUST pipeline [639] and the linear discriminant analysis effect sized (LEfSe) analysis was performed for the biomarker discovery using a size-effect cut-off of 3.0 on the logarithmic LDA score [640].

Bacterial Quantification by quantitative PCR Analysis

A small subset of samples (n= 248) according to DNA availability (delivery n=78; 7d n=100; and 31d n=86) were used for the specific bacterial count determination by the qPCR. Total bacterial and *Bifidobacterium* genus counts were measured by quantitative system based on the amplification of specific 16S rRNA gene region by use of Light Cycler 480 Real-Time PCR System (Roche, Basilea, Switzerland). The (Details in **Supplementary file V-3**). Reaction mixture consisted in SYBR Green I master mix (Roche, Basilea, Switzerland), 0.25 µM of each specific primer set and 1 ul of DNA. The amplification process consists of one cycle at 95ºC for 5 min, followed by 40 cycles at 95ºC for 20 s, annealing temperature (**Supplementary file V-3**) for 10 s and 72ºC for 10 s. Melting curves were also assessed to test the specificity of the reaction. Standard curves for the specific targeted bacterial group were generated using *Ct* values and the calculated gene copies numbers were determined based on the fragment amplification length.

Cell culture

All the reagents for cell culture were purchased from Sigma-Aldrich, Spain, otherwise stated.

NF-κB-SEAP HT29 reporter cells. The HT-29-transfected cell line was previously established in the Laboratory of Lactic Acid Bacteria and Probiotics of IATA-CSIC, by stable transfection of HT-29 cells with a NF-κB- secreted alkaline phosphatase (SEAP) plasmid (pNiFty2-SEAP; Invitrogen, Carlsbad, CA, US) as a reporter. Cell maintenance was performed in 75 cm^2 flask with Dulbecco's Modified Eagle Medium (DMEM) High glucose medium supplemented with 1% (v/v) L-Glutamine 200 mM, 1% (v/v) Na-Pyruvate, 1% (v/v) penicillin/streptomycin and 10% (v/v) of inactivated Fetal Bovine Serum (Biowest). Zeocin (200 µg/ml) (InvivoGen) was added to the medium for the clone selection in each passage. All cultures were used between the passage 15 and 20.

THP-1 cells. The THP-1 cells were obtained from the European Collection of Authenticated Cell Cultures (THP-1 ECACC 88081201, Public Health England, UK). Cell maintenance was carried out as described in Boudish et al. [641]. All cultures were used between the passage 40 and 50.

Caco-2 and LS17T cells. The Caco-2 (ECACC 86010202, Public Health England, UK) and LS174T (ECACC 87060401, Public Health England, UK) cells were obtained from the European Collection of Authenticated Cell Cultures. Caco-2 cells were maintained as described in [642]. LS174T cells were maintained in 25 cm^2 flasks with Eagle's Minimum Essential Medium (EMEM), supplemented with 1% (v/v) GlutaMax, 1% (v/v) Non Essential Amino Acids (NEAA), 10% inactivated Fetal Bovine Serum (FBS) and 1% (v/v) penicillin/streptomycin. Medium was refreshed every two days and cells were subcultured when they reached 80% confluence, as described in [642]. All cultures were used between the passage 40 and 50. Cell morphology was analyzed and checked by phase- contrast microscopy (Olympus CKX41, Olympus Corporation, Tokyo, Japan).

Stimulation of HT-29 and THP-1 cell line

To investigate the role of microbial shifts on NF-κB activation and innate immune response, NF-κB-HT-29-reporter cells and macrophage-like cell line (THP-1) were exposed to filtered fecal supernatant obtained from a subset of samples of each group (n=4, total n = 12) at 1 month. Samples were randomly selected among the samples with fecal material availability.

Fecal supernatants prepared as described above (n=4 individuals of each group, total n = 12) were filter-sterilized (0.22 µm PES; Sarstedt SA, Barcelona, Spain) and

exposed to the cells as described below. pH adjustment to 7.0-7.2 was performed when required with 0.5M filter-sterilized NaOH (Panreac, Barcelona, Spain) and buffered with HEPES (1% v/v).

HT-29 cells were seeded at 7 x 10^4 cells/well in 96-wells plates and incubated for 24h in DMEM High Glucose medium without FBS supplementation. Cells were exposed to filtered fecal supernatant obtained from the three studied groups diluted in 1:10 v/v in DMEM with FBS. Supernatants were collected after 24h of stimulation and SEAP activity was measured using p-nitrophenyl phosphate according to manufacturer's instructions (Thermo Fisher Scientist, Waltham, US). The signal was quantified using a Spectrostar Nano microplate reader (BMG Labtech, Ortenberg, Germany) at 405 nm.

THP-1 cells were seeded at 5 x 10^4 in 96 wells plates in RPMI 1640, supplemented with 100 ng/mL of phorbol 12-myristate 13-acetate (PMA). After 48 h, cells were refreshed with RPMI without PMA and incubated for 4-5 days, to allow macrophage-like differentiation of THP-1 cells. Thereafter, the cells were exposed fecal supernatants diluted 1:20 (v/v) in RPMI and incubated for 5h. Next, cell culture supernatants were collected, and cells were washed twice with PBS. Cells and supernatants were stored at -80ºC for gene expression determination and cytokine quantification, respectively.

Triple co-culture in Transwell plates: Long-term stimulation

A subset of samples from each group at 1 month of life (n per group=3, total n = 9) were selected to investigate in a more physiologically model the long-term effect of the differences observed in microbiota composition of the infants on the gut barrier and innate immunological state.

Cell differentiation and the posterior tests were carried out in double chamber wells (Corning® Transwell®-6 well, pore size 0.4 µm; Costar, NY) equipped with separate apical and basolateral compartments and a porous support on which the Caco-2 and LS174T cells grow into a monolayer. The cells were seeded at a density of 7.5 x 10^4 cells/cm², in a proportion of 90/10 Caco-2/LS174T in supplemented DMEM. After 24 hours, apical media was refreshed, basal media was removed, and THP-1 cells were seeded in the bottom of the 6 well plate at a density of 1 ·10^5 cells/cm^2 in RPMI 1640 media containing 100 ng/mL of phorbol 12-myristate 13-acetate (PMA).

After 48 hours of incubation, both apical and basolateral compartments were refreshed with DMEM or RPMI 1640 without PMA and without antibiotics, respectively. Thereafter, the triple co-culture was maintained for 4 days more and refreshments of the apical and basal media were done every 2 days.

Filter-sterilized fecal supernatants selected from each studied group (n=3 individuals, total n = 9) were diluted 1:20 in cell culture media without antibiotic/antifungals and added to the apical compartment of the triple co-culture model (1.5 mL). Treatments were maintained for 7 days, with daily refreshments of the apical and basolateral compartments, with fecal supernatants or RPMI 1640, respectively. Aliquots of the culture supernatant from apical and basolateral media were stored at -80°C for cytokine measurements.

Epithelial barrier function: Trans-epithelial electrical resistance (TEER) and apparent permeability.

The monolayer integrity was assessed by measuring the trans-epithelial electrical resistance (TEER) and the apparent permeability (Papp) of the paracellular transport marker Lucifer yellow (LY).

A Millicel-ERS (Millipore Corporation, Spain) was used for the TEER measurements. Measurements of the TEER were performed every day at the beginning of the exposure to sterilized fecal supernatant (7 days post-seeding) and every day until the end of the assay (15 days post-seeding). TEER values are reported as delta (Δ) of the initial time point before of the exposure and final time point (7 days). TEER values (ohms/cm^2) are presented in **Supplementary file V-11**.

Papp of LY was measured by adding the marker (100 µM) to the apical compartment of the wells. After 30, 60 and 120 min, 100 µL of medium was removed from the basolateral compartment and replaced with an equal volume of fresh medium (supplemented RPMI-1460 without antibiotics). LY fluorescence was measured at an excitation/emission wavelength of 485/520 nm in 96 black plates (Greiner), using a microplate fluorescence reader CLARIOstar Plus (BMG Labtech, Ortenberg, Germany). A calibration curve (0, 5, 10, 25, 50 and 100 µM) for LY quantification was run in duplicate in each reading. The Papp coefficients were calculated as previously described in [643].

Mucus production and Intestinal alkaline phosphatase (IAP) determination

At day 15 post-seeding (7 days of fecal supernatant exposure), the transwell inserts containing Caco-2/LS174T cells were incubated with 1 mL of 10 mM of N-acetyl cysteine (Sigma) for 1h at 37°C, 95% humidity in DMEM, adjusted at pH 7-7.2, and the mucus produced by the cells was collected recovering the media and washing once with 0.5 mL of DMEM. The solution containing mucus was concentrated using a Vacuum Concentrator (Eppendorf, Hamburg, Germany) until dryness and re-suspended overnight in 100 µL of PBS at 4ºC. The amount of mucus was measured by a Bradford protein quantification assay, following the manufacturer instructions. Blanks containing cell culture media were subtracted and a standard curve of bovine serum albumin (BSA) was used for the calibration curve (0-5 mg/ml).

Intestinal alkaline phosphatase (IAP) activity was assessed in the apical and basal compartment supernatants by enzymatic assay following manufacturer's instructions (Sigma-Aldrich, Missouri, US) scaling the reaction to 100 µl using 4 µl of cell supernatant. Results were read in a SpectroStar Nano (BMG Labtech, Ortenberg, Germany) at 405 nm.

Cytokine quantification in cell supernatant

IL6, IL8 and TNF-α released by the cells after acute and long-term exposures to fecal supernatants were quantified by Enzyme-Linked ImmunoSorbent Assay (ELISA), following manufacturer instructions. Human IL8, TNF-α or IL6 Uncoated ELISA kit (Invitrogen, Carlsbad, CA, US) were used for the cytokine determination. In the long-term exposure, cytokine released values were expressed as percentage of variation comparing each treatment to control in order to avoid time-dependent effect. Samples were diluted in assay buffer to adjust the concentration to the linear range of the standard curve.

Gene expression by real time RT-qPCR

HT-29 and THP-1 cells from the acute exposure as well as those from tripe co-culture system were collected by scraping at the end of the treatment. RNeasy mini kit (Qiagen, Hilden, Germany) was used to RNA extraction following the manufacturer's instructions. Total RNA was converted to cDNA by Transcriptor First Strand cDNA Synthesis Kit (Roche, Basilea Switzerland) adjusting to the same amount of RNA for each cell type. RT-qPCR analysis was performed using 1 µl of resulted cDNA reaction

and 0.25 µM of the specific primers using Lightcycler 480 SYBR Green I master mix (Roche, Basilea, Switzerland). Plates were read in the LightCycler 480 (Roche, Basilea, Switzerland) at annealing temperature of 58ºC. Sequences of the primers used in the study are listed in **Supplementary file 3**. Genes related with microbes sensing as TLRs and related transcription factors were analyzed and Actin (ACTB) gene was used as housekeeping gene expression, except for the studies in THP1 cell line in the triple co-culture system where hypoxanthine phosphoribosyl transferase (HPRT) gene was used as reference gene for showing higher stability between samples than Actin gene.

Statistical analysis

Chi-Squared test was used to assess differences in the categorical variables of the studied population and ANOVA or Kruskall-Wallis, followed by a Dunn's post-hoc test, was used for the continuous variables according to the distribution of the data. Normality distribution was tested by Shapiro-Wilk test.

Sequencing data were transformed to relative abundance before further analysis. Pyloseq [500] and vegan [505] packages were used for the analysis conducted in the sequencing data, including alpha diversity estimation. For alpha diversity analysis, samples were rarefied with a 90% of the minimum sample depth. Kruskall-Wallis followed by Dunn's post-hoc tests were performed to detect significant differences in the gut microbial alpha-diversity according to categorical variables studies. Spearman correlations were used to find associations between alpha-diversity measures and continuous variables of the population, including gestational age or maternal body mass index, among other, and between microbial species.

For beta-diversity analysis, Permutational multivariate analysis of variance (PERMANOVA) was conducted to assess the effect of the studied factors on the neonatal gut microbial composition (Bray-Curtis distance) and its functionality. For each factor, differences in the dispersion were also tested. In groups with different dispersions, ANOSIM test was also addressed. Calypso web platform (v. 8.56) [443] was used for visualizing the multivariate analysis. The clustering of the samples according to the different studied variables, including mode and place of delivery was performed by discriminant analysis of the principal components (DAPC).

Kruskal Wallis test followed by a Dunn's post-hoc test with the FDR method for multiple comparisons correction was applied to find significant differences in gut

microbial composition or functionality (KEGG categories) between studied groups at each time point.

For RT-qPCR analysis, LC480 Conversion and LinRegPCR software [644,645] were used for efficiency calculation and gene expression data were analyzed by REST2009 [646]. Statistical analysis of data from triple co-culture system was performed by Graphpad software (v. 5.04) (GraphPad Software, La Jolla CA, US). Unpaired t-test was used for statistical analysis of mucus production, IAP, TEER and apparent permeability. Kruskall-Wallis and Mann-Whitney test was used for ELISA measurements, considered as non-parametric data. A $p<.05$ was considered as a threshold to accept a statistically significant difference. All multiple comparisons were adjusted by false discovery rate (FDR) adjustment method. Each cell culture experiment was performed in triplicate.

RESULTS

Study population

A total of 180 mother–neonate pairs from hospitalized births, both VAG (n=91) and elective CS (n=66), and vaginal HB deliveries (n=23) were included in this study. All the babies were born at full term. The CS-born neonates were born before both groups of vaginally delivered neonates (39 and 40 weeks of pregnancy for the VAG and HB neonates, respectively) ($p=0.004$). No differences were observed in the neonatal weight between groups, which showed a median of 3250 g (range 2973–3573 g). Other maternal clinical parameters are presented in the supplementary material (**Supplementary file V-2**).

The HB neonates showed higher length measurements than the hospital-born infants for both delivery modes ($p=0.003$). Additionally, the HB infants had higher ratios of exclusive breastfeeding than the hospital-delivered infants at both seven and 31 days of life ($p<0.001$).

Perinatal factors related to the place and mode of delivery shape the neonatal microbiome composition at birth

At birth, the place (hospital versus home) was the main contributor of neonatal microbiota composition ($p=0.001$), followed by the mode of birth ($p=0.025$) (**Fig. V-1, A**). Other perinatal factors did not significantly influence the neonatal microbiota during delivery.

The place and mode of birth shaped the microbial richness and diversity (**Supplementary file V-4**). The hospital-born neonates showed a bacterial community with greater richness ($p=0.002$) and diversity ($p=0.072$) at birth than the HB neonates. The CS-born neonates harbored higher index of observed species ($p=0.023$), diversity ($p=0.001$) and richness ($p=0.031$) than the HB infants.

The neonatal fecal microbiota was dominated by the Proteobacteria phylum, followed by Firmicutes and Actinobacteria (**Supplementary file V-5**). The hospital-born infants showed higher relative abundances of the *Finegoldia* ($p<0.001$), *Clostridioides* ($p<0.001$), *Klebsiella* ($p=0.025$) and *Peptoniphilus* ($p=0.006$) genera, including *Clostridioides difficile* ($p<0.001$) and *Clostridium neonatale* ($p=0.006$), while the HB infants showed higher relative abundances of the *Staphylococcus* ($p<0.007$) and *Enterococcus* ($p<0.001$) genera.

As for the mode of birth, the VAG infants showed higher relative abundances of the Bacteroidetes ($p=0.035$) and Firmicutes ($p=0.035$) phyla, including the *Bacteroides* and *Escherichia/Shigella* genera. A linear discriminant analysis of effect size (LEfSe) was used to confirm which genera were responsible for the clustering of the rectal swab microbial population (**Supplementary file V-6**). The microbiota of the HB neonates was enriched by species from the *Enterococcus* and *Staphylococcus* genera, while the hospital-born infants were enriched with species from the *Klebsiella*, *Veillonella* and *Clostridioides* genera. We also observed a microbial core at each time point and noted some unique genera not shared between the groups (**Supplementary file V-7**). For instance, we observed that the *Akkermansia* genus was only present in the HB.

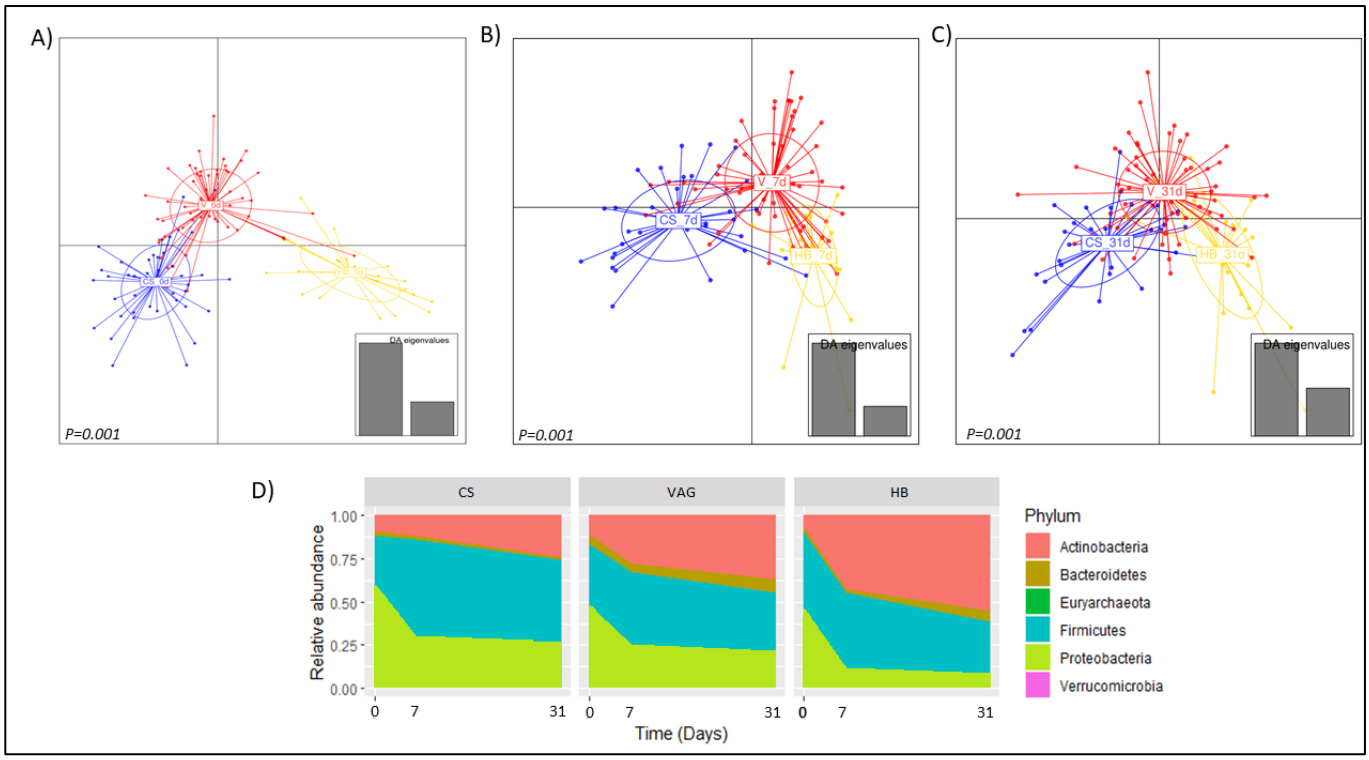


Figure V-1. Factors affecting meconium and neonatal microbiota during the first month of life. A-C) Discriminant analysis of principal components (DAPC) of the neonatal (**A**) and infant fecal microbiota at 7d (**B**) and 31d (**C**). Each point represented microbiota from a neonate. Adonis analysis was used to stablish the significance of studied variables. **D**) Colonization patterns during the first moth of life. Neonatal microbiota composition at phylum level at birth (0d), 7 days (7d) and 1 month (31d). C-section (CS), vaginal delivery at hospital (VAG) and homebirth (HB).

Perinatal factors related to the place and mode of delivery shape neonatal microbiota development

Mode of birth (*p=0.001*) and time (*p=0.001*) were the main contributors to the overall microbiota structure during the first month of life. Generally, neonatal microbiota at birth showed significantly higher microbial richness (Chao1 index) and diversity (Shannon index) than the microbiota at 7 and 31 days of life (**Supplementary file V-4**). No differences in alpha diversity (Chao 1, Shannon index) were found according to the place or mode of delivery at the 7- or 31-day time points.

Distinct colonization patterns were identified between the hospital born (VAG and CS) and HB neonates (**Fig. V-1, D**). The mode of birth shaped the microbiota colonization process at 7 (Adonis *p<0.001*) and 31 days (Adonis *p<0.005*).

The infant microbiota showed differing development in the VAG and CS-delivered neonates at the phylum and family levels (**Fig. V-1, D**). The VAG deliveries exhibited a colonization pattern somewhere between the patterns of the CS-born and HB deliveries. The HB and CS-born infants showed significant differences in the Firmicutes phylum at delivery (*p=0.035*) and in the Proteobacteria at seven days (*p=0.019*), although they did not show differences when compared to the hospital-based VAG infants. The relative abundance of the Actinobacteria phylum increased during neonatal life in the both groups of vaginally (VAG and HB) born infants but not in the CS-born neonates (**Supplementary file V-5**). In the HB neonates, the Actinobacteria increase occurred from delivery to 31 days, while in the hospital-based VAG neonates, the increase was delayed from seven to 31 days of life. The Bacteroidetes phylum abundance was slightly higher in the vaginal births (VAG and HB) at seven (*p=0.014*) and 31 days (*p=0.01*) than in the CS births.

At the phylum level, the vaginal births (VAG and HB) showed higher relative abundances of the Actinobacteria (*p=0.001*) and Bacteroidetes (*p=0.018*) phyla, especially *Bifidobacterium* (p=0.003 at seven days), when compared with the CS-born infants at both times (7and 31 days). The CS-born infants were enriched with the Firmicutes phylum (*p=0.020*), including *Enterococcus* (*p=0.005* at seven days) and *Clostridium* (*p=0.039*).

The HB neonates harbored a higher relative abundance of Actinobacteria (*p=0.004* and *p=0.006* at 7 and 31 days, respectively) and a lower relative abundance of Proteobacteria (*p=0.026* and *p=0.008* at seven and 31 days, respectively) when compared with the hospital-born infants (VAG and CS) at both times. Hospital-born infants had higher relative abundances of the *Klebsiella* species and lower relative abundances of *Bifidobacterium* genus, including *B. bifidum* (*p=0.013*), and *Collinsella* genus, including *Collinsella aerofaciens* (*p=0.028* at 31 days), compared with the HB neonates.

In the vaginal births (both, VAG and HB), the relative abundances of the *Collinsella* and *Bacteroides* genera increased during the first month of life. An opposite trend was observed for the *Escherichia* and *Enterococcus* genera (**Fig. V-2**). In the CS-born infants, *Escherichia*, *Enterococcus* and *Klebsiella* genera increased from birth to seven and 31 days (**Fig. V-2**). The relative abundance of *Bifidobacterium* genus was higher in the vaginal births, especially the HB infants at 7 (*p<0.001*) and 31 days (*p=0.004*), when compared with the CS births. Moreover, CS-neonates harbored a

higher relative abundance of the *Clostridium sensu stricto* genus than the HB infants at 7 days (*p=0.007*), although no difference was observed with the hospital-based VAG infants (*p=0.250*), indicating intermediate colonization patterns in neonates born in hospital via vaginal route. At both 7 and 31 days, the *Bacteroides* genus was present between the vaginal-delivered neonates but not the CS-delivered infants, who showed *Enterobacter* as a unique genus at 31 days.

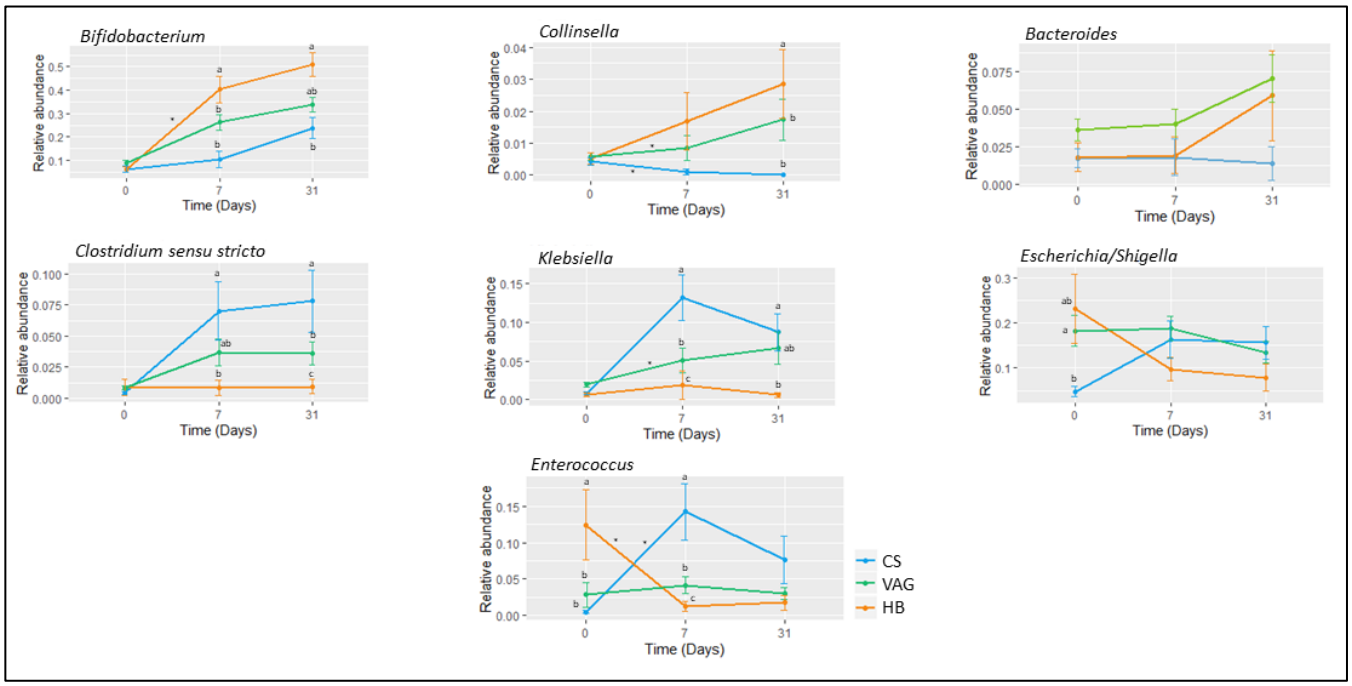


Figure V-2. Differences in relative abundance of most important and variable genera in fecal microbiota among the first month of life. Each point represented the mean and SEM of relative abundance of each genus in that point from the fecal samples of babies born by cesarean section (blue), vaginal delivery at hospital (green) and at home (orange). Kruskall-Wallis test with a Dunn's post-hoc test was performed to compare the different groups. Data not sharing the same letter in each point were significantly different (*p<0.05*). Significant variations within the same group at different time points were marked by an asterisk (*). C-section (CS), Hospitalized vaginal delivery (VAG), homebirth (HB).

In a subset of samples, total bacterial counts by qPCR were significantly lower at birth than the counts obtained at seven days in all three groups (**Supplementary file V-8**). We also found that the CS-born fecal samples showed significantly lower bacterial counts than the VAG (*p=0.043*) and HB (*p=0.008*) samples at delivery. The HB infants showed higher total bacterial counts than the VAG (*p=0.008*) and CS-born (*p=0.043*) infants at seven days. Similarly, in the case of the *Bifidobacterium* genus counts in the vaginal-delivered groups (HB [*p<0.001*] and VAG [*p=0.003*]), the counts increased

during the first month. Furthermore, the HB infants had higher *Bifidobacterium* counts than the VAG and CS-born infants at seven ($p=<0.001$ for CS, $p=0.004$ for VAG) and 31 days ($p=0.027$ for CS, $p=<0.001$ for VAG).

Impact of the perinatal environment on the infant weight status at 18 months

Higher BMI and W/L z-scores were observed in the CS-born infants than in the HB ($p=<0.001$ for BMI, $p<0.001$ for W/L at 12 months) and hospital-born vaginal births ($p=<0.001$ for BMI and $p=0.003$ for W/L at 12 months) (**Fig. V-3, A-B**). This Indeed, at 18 months of life, CS infants exhibit also higher BMI z-scores than HB ($p<0.001$) and VAG ($p=0.016$) children. Additionally, a multivariate linear analysis (adjusted by breastfeeding duration, antibiotic intake during the first year of life, maternal pre-gestational BMI, and BMI and W/L z-scores at delivery) showed the CS-born neonates to exhibit significantly higher BMI and W/L z-scores across the first 18 months of life.

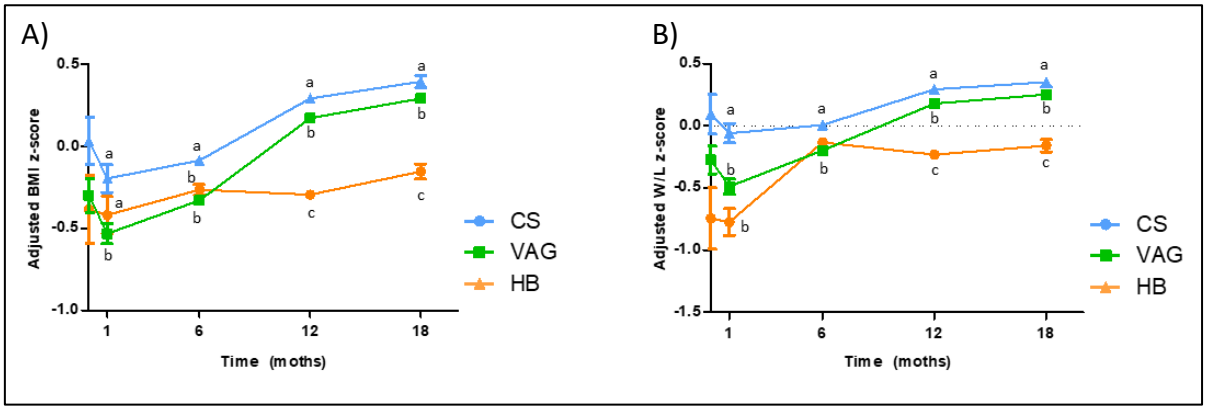


Figure V-3. Place and Mode of birth impact the infant growth. BMI z-scores (**A**) and Weight for Length (**B**) z-scores curves from delivery to 18 months of life according to mode of birth and place adjusted by covariates, breastfeeding duration, antibiotic intake during the first year of life, maternal pre-gestational BMI and infant BMI and Weight for length (W/L) z-scores at birth. General Linear Model Multivariate test adjusted by covariates was done and $p<0.05$ was considered significant. Kruskal-Wallis was performed on the adjusted values (different letters indicate significant differences between three studied groups). C-section (CS), Hospitalized vaginal delivery (VAG), homebirth (HB).

Microbiota functionality during the first month of life is influenced by the birth mode and place

The inferred microbial functionality at birth was mainly affected by the mode of birth ($p<0.001$), but also by the birthplace ($p=0.049$). In addition, mode of birth also influenced the microbiota functionality at 7 ($p=0.001$) and 31 days ($p=0.02$); however, the birthplace was only significant at 7 days ($p=0.001$) (**Fig. V-4, A-B**).

Regarding concrete functions, the microbiota of the vaginal-delivered infants (VAG and HB) were enriched in functional routes related to the synthesis of secondary metabolites ($p<0.001$), amino acids ($p=0.002$), lipids ($p=0.034$) and carbohydrate metabolism ($p=0.046$) when compared with the CS-born neonates at delivery (**Supplementary file V-9**). At seven and 31 days, the vaginal-delivered infants showed microbiota enriched in lysine, phenylalanine, tyrosine, tryptophan, valine, leucine and isoleucine biosynthesis pathways. At one month of life, the CS-born infants expressed higher energy metabolism pathways, including carbohydrates, lipids, and propanoate biosynthesis.

The immune-system-related paths (e.g. antigen processing and presentation [$p<0.001$] and NOD-like receptors [$p<0.001$]) were overrepresented in the CS-born infants at birth. Contrarily, the lipopolysaccharide (LPS) biosynthesis routes were enriched in the vaginal-delivered infants at all time points (**Fig. V-4, C-D**), being also influenced by the birthplace. However, the CS-delivered infants showed increasing representation of pathways related to bacterial toxins at seven ($p=0.011$) and 31 days ($p=0.004$) when compared with the HB neonates.

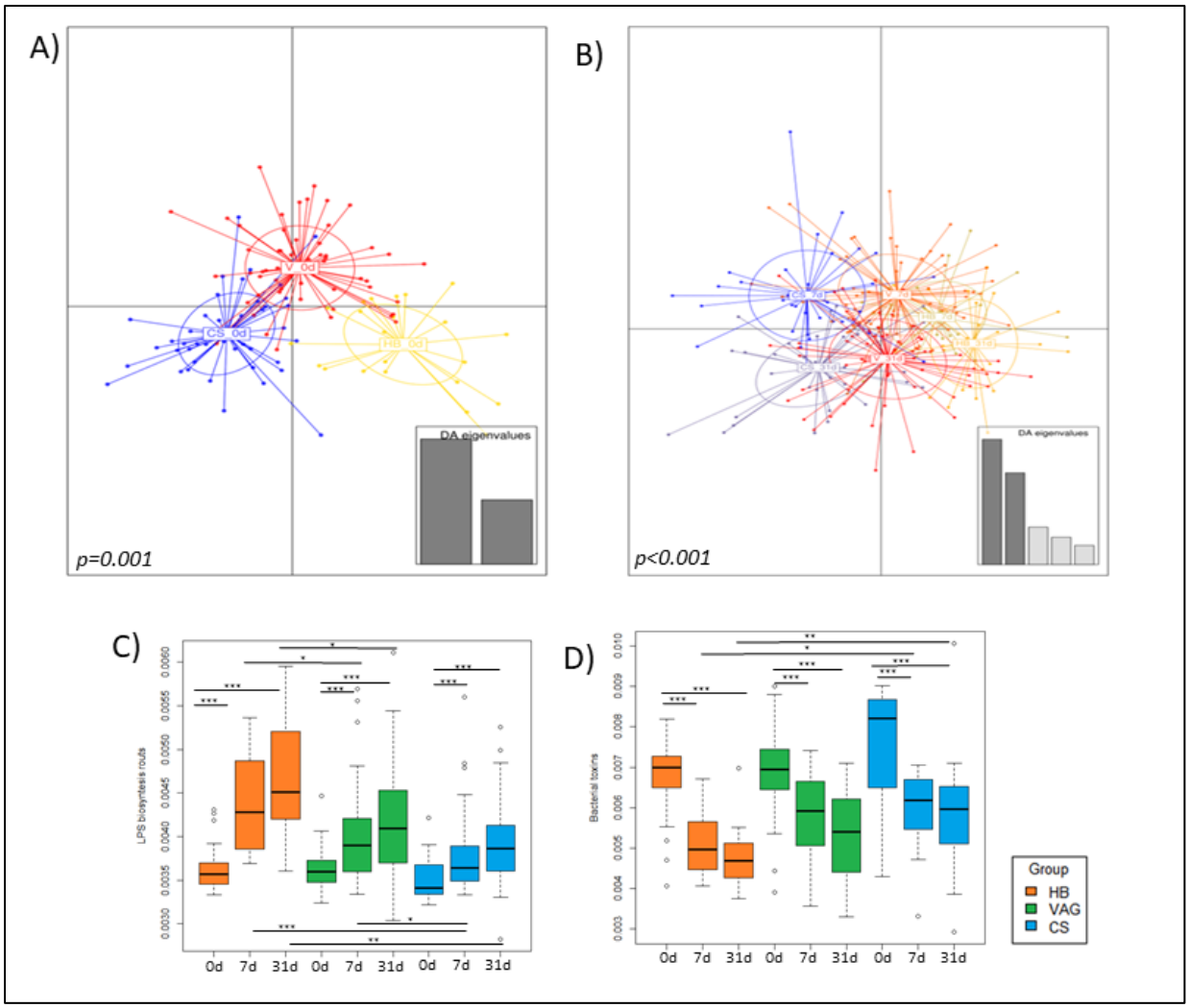


Figure V-4. Microbial functions computationally predicted present in neonatal microbiota along the first month of life. A-B) Discriminant analysis of principal components (DAPC) of the neonatal (**A**) and infant fecal microbiota at 7d and 31d (**B**) Adonis analysis was used to stablish the significance of studied variables. **C-D**) Computational analysis of lipopolysaccharide (LPS) biosynthesis (**C**) bacterial toxins (**D**) routs presents in the fecal microbiota of newborns along the first month of life. Results were expressed as percentage of total functional routs for each sample. *$p<0.05$, **$p<0.01$, ***$p<0.001$. C-section (CS), Hospitalized vaginal delivery (VAG), homebirth (HB).

Gut microbiota shifts impact on the in vitro host response

Due the differences observed *in silico* microbiota functions related to the innate immune system related to the bacterial pathways, especially those involving the toll-like receptor (TLR) signal, we designed *in vitro* experiments to assess the effects of microbiota composition on the innate immune host response.

Fecal supernatants from all groups induced a pro-inflammatory response in the *in vitro* models, with differences noted between different cell types (enterocyte-like versus macrophage-like cells).

Fecal supernatants induce the mRNA expression of TLR4 and IRAK4 in intestinal epithelial cells

The HT-29 reporter cells showed increased NF-kB activation independently of the place and mode of delivery in all the groups (data not shown). All fecal supernatants induced IL8 and TNF-alpha production in the HT-29 cells when compared with control cells exposed to cell culture media (**Fig. V-5, A**). No differences were found between the groups in relation to any of the cytokines.

HB fecal supernatants up-regulated the *TLR4* mRNA in a 1.405-fold change (*p=0.024*) and the *IRAK4* mRNA in a 2.654-fold change (*p<0.001*) when compared with the control samples. This increase was not observed in the cells exposed to hospital-born (VAG and CS) neonatal samples (**Supplementary file V-10**). Generally, the fecal supernatants all reduced the mRNA expression of the tight-junction proteins, including zonulin-1 (*HP*) (0.202- and 0.265-fold change in the HB and CS samples, respectively), e-cadherin (*CDH1*) (0.226- and 0.326-fold change in the HB and CS, respectively) and occludin (*OCLN*) (0.192- and 0.253-fold change in the HB and CS). However, no differences were found between the different studied groups.

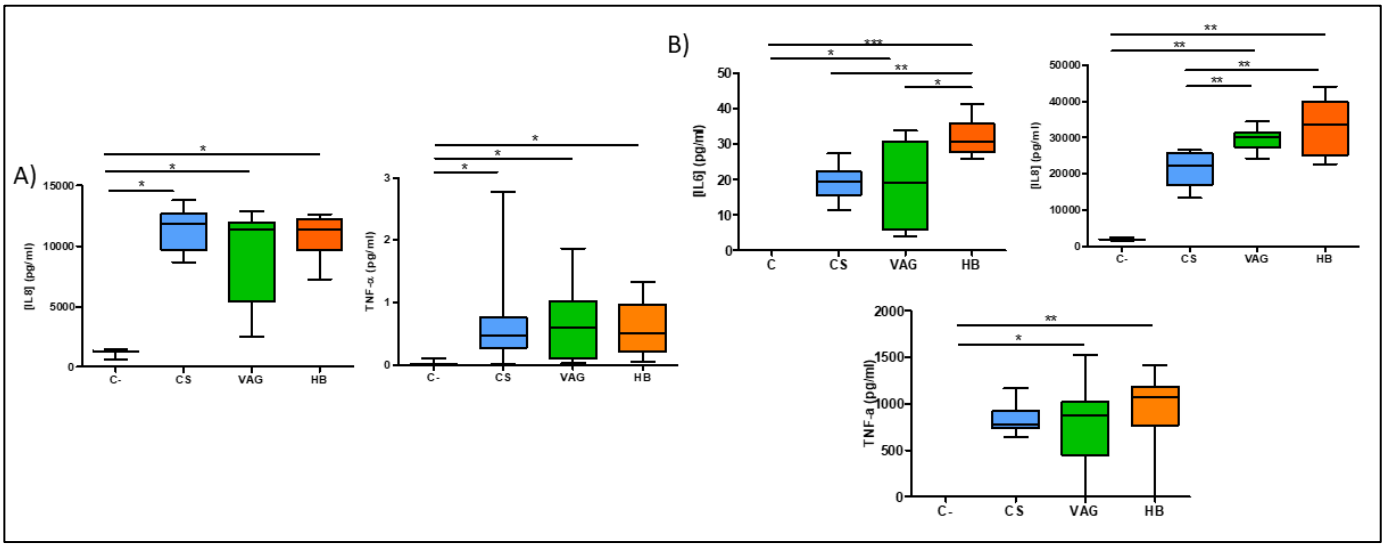


Figure V-5. Effect of 1-month infant fecal water exposure in epithelial (A) and macrophages-like (B) cell lines after 24h. **A**) Cytokine production by HT-29 cells after exposure to fecal water from neonates born by C-Section (CS), vaginal delivery at hospital (VAG) and Homebirth (HB). IL6 production in HT-29 cell line was below detection limit. **B**) Cytokine production of THP1 cells after 24h exposure to faecal water of each group. Data was presented as median and whiskers represented the 5-95 percentile. Kruskal-Wallis and Dunn's post hoc (FDR adjustment) test was used to test the significance of the differences in cytokine response between the groups. *$p<0.05$, **$p<0.01$, ***$p<0.001$. C-section (CS), Hospitalized vaginal delivery (VAG), homebirth (HB).

HB fecal supernatants trigger higher immune response in macrophage-like cells than CS fecal supernatant.

The mode of birth significantly affected the production of cytokines in the PMA-differentiated THP-1 cells (**Fig. V-5, B**). The HB and VAG fecal supernatants had a higher pro-inflammatory capacity than the CS samples. Higher levels of IL6 and IL8 were detected after HB ($p<0.001$), followed by the VAG ($p=0.043$) samples, when compared with the CS fecal supernatant. The VAG and HB samples triggered a significantly higher response than the control condition for IL6, IL8 and TNF-α, which was not observed in cells exposed to CS samples.

The CS fecal supernatants down-regulated the *TLR4* (0.509-fold change, $p=0.006$) and *FOS* mRNA expression (0.238-fold change, $p=0.038$) (**Supplementary file V-10**). The expression levels were not quantifiable for the interferon gamma (*IFN-γ*) and *IL10* genes in either the HT-29 or THP-1 cell lines following faecal supernatant exposure.

Triple co-culture system for host–microbiome interaction

Place and mode of birth impacts on intestinal barrier function and maturation

With regard to the integrity of the cell monolayer (**Supplementary file V-11**), HB promoted a higher increase in the transepithelial electrical resistance (TEER) values after seven days of exposure (**Fig. V-6, A-B**) when compared with hospital birth (VAG [$p<0.001$] and CS [$p<0.001$]). These results were confirmed via the measurement of lucifer yellow dye (LY) transport through the epithelial layer. Despite all the fecal supernatants triggered an increase in LY transport when compared to control condition ($p<0.05$), hospital-based birth, both VAG ($2.1\cdot10^{-6}\pm5.7\cdot10^{-7}$ cm/s, $p=0.018$) and CS ($2\cdot10^{-6}\pm3.5\cdot10^{-7}$ cm/s, $p=0.033$), led to higher LY transport than HB ($1.5\cdot10^{-6}\pm1.7\cdot10^{-7}$ cm/s).

The intestinal alkaline phosphatase (IAP) activity was also measured in the supernatant of cell cultures from both; the apical and basal compartments (**Fig. V-6, D**). Indicative of functional cell polarization, the IAP activity was significantly higher (p=0.006) in the apical compartment at 7 days of treatment, while all the fecal supernatants enhanced the IAP activity when compared with the control. Similar to the TEER and LY results, HB samples induced higher IAP activity than the ones observed in VAG ($p=0.043$) and CS samples ($p=0.049$). Furthermore, higher mucus production

was observed in cells exposed to hospital fecal samples (0.92±0.2 mg/ml) when compared with the HB samples (0.69±0.09 mg/ml) (*p=0.006*).

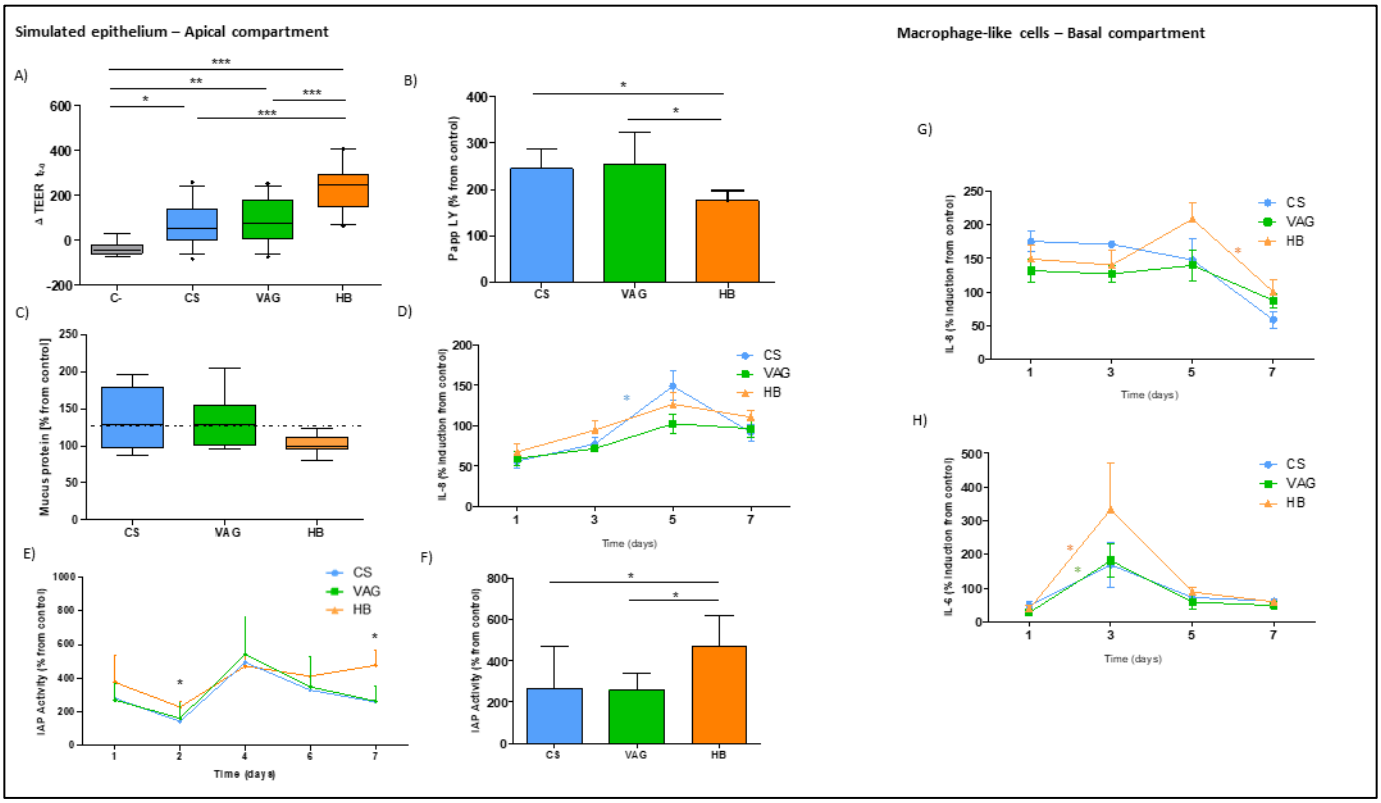


Figure V-6. Effect of fecal water long-term exposure (7d) on the triple co-culture system. A-B) Epithelial barrier function measured as trans-epithelial electric resistance (TEER) (A) and Lucifer Yellow transport (LY) (B). C) Mucus production by LSTH17 cells after the long-term exposure on the triple co-culture system measured by Bradford assay. D) Interleukin (IL) 8 production by cells on the apical compartment (CacO-2 and LSTH17) measured by ELISA and expressed as increment respect to control condition. E-F) Intestinal cells functional maturation degree measured as intestinal alkaline phosphatase activity (IAP) on apical compartment during the treatment (E) and at final time point (F). G-H) Cytokine production in the basal compartment by THP-1 cells. IL- 8 (G) and IL-6 (H) production after fecal supernatant long-term exposure expressed as increment respect to control condition.

The treatments were fecal water from infants born by C-section (CS), vaginal delivery at hospital (VAG) and homebirth (HB). Non-normal data was presented as median and whiskers represented the 5-95 percentile while normal data was showed as mean and SD. Kruskal-Wallis/Anova and Dunn's/Tukey's post hoc (FDR adjustment) test was used to test the significance of the normal/non normal distributed variables between the groups. In the cytokine analysis, the symbol (*) represented variations between time within the same studied group according to the colour. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

Immune system response

The immune response to the fecal supernatants was generally higher in the basal than in the apical compartment. In the simulated intestinal epithelium, the exposure to

CS-born fecal supernatants down-regulated (0.43-fold change) the *IRAK4* mRNA expression when compared with the control-no stimulus (*p=0.01*) and HB samples (0.45-fold change, *p=0.001*). Contrarily, the CS-born fecal supernatants up-regulated the toll-interacting protein (*TOLLIP)* mRNA (*p=0.007*) expression in the HB samples (1.54-fold change (**Table V-1**).

Table V-1. Gene expression of cells in the apical and basal compartment in the triple co-culture system after 7 days of exposure

	HB		CS	
	Fold expression	**p-value**	**Fold expression**	**p-value**
Apical compartment (Caco-2, LSTH-17 cells)				
HP	1.139 (0.767 - 1.627)	*0.495*	1.097 (0.957- 1.214)	*0.106*
CDH1	1.054 (0.462 - 1.675)	*0.841*	0.916 (0.736-1.237)	*0.399*
OCLN	1.163 (0.673 - 1.534)	*0.531*	1.321 (0.867-1.702)	*0.083*
IRAK4 (#)	0.94 (0.610 - 1.168)	*0.837*	0.425 (0.273-0.595)	***0.010**** ↓
TLR2	1.492 (0.837 - 2.142)	*0.181*	1.302 (0.960-1.710)	*0.061*
TLR4	0.89 (0.722 - 1.074)	*0.201*	0.826 (0.482-1.276)	*0.475*
TOLLIP	0 (0.000 - 1.381)	*0.509*	1.231 (0.658-2.076)	*0.474*
Basal compartment (THP-1 cells)				
TLR4	3.566 (1.103 - 13.629)	*0.094*	1.117 (0.235 - 7.013)	*0.896*
TLR3	1.346 (0.939 - 1.853)	*0.378*	3.546 (1.712 - 8.099)	*0.094*
TOLLIP	5.182 (3.667 - 9.351)	***<0 .001**↑*	7.186 (4.388 - 11.727)	***0.010**↑*
IL10	7.888 (3.496 - 31.769)	***< 0.001**↑*	1.737 (0.912 - 5.478)	*0.061*

Total RNA was extracted from cells treated with homebirth (HB) samples and C-section (CS) fecal supernatant. For each condition, n=6 in triplicates, data was presented as fold change expression (95% C.I). Values expressed relative expression fold-change of each condition compared to control. *P<0.05* (*) and blond letters marked significant differences between treatment and control, up (↑) or down (↓) regulation was represented by the arrows. Symbol # represented genes expression that was different between HB and CS.

IL8 was detectable at all time points in the apical compartment (**Fig. V-6, E**). Generally, the IL8 exhibited a gradual increase until the fifth day of exposure (126±39%

average increase for the three groups). Thereafter, the IL8 levels decreased until the end of treatment (7 days, 99±24% average of three groups). The IL6 concentration was only above the detection limit at the final time point (7days), and there were no differences between the groups.

In the basal compartment, similar to the epithelium-like layer, we observed a 7.2-fold change in the downregulation of the *TOLLIP* mRNA expression after exposure to CS-born samples ($p<0.001$) (Table 1).

IL8 release was observed at 24 hours (151.3±42.7% increase from the control), with the maximum being seen after five days of treatment (164±65% increase). At day seven, the IL8 levels reached similar values to those seen on the first day of treatment (82±67% increase) (**Fig. V-6, F-J**).

The IL6 production by the THP-1 cells showed a late response to faecal supernatant exposure, lasting from day one of treatment until day three (218±223% increase from control); however, the increase was only significant for the HB ($p=0.003$) and VAG ($p=0.017$) samples. At the subsequent time points, the IL6 release was stabilised to concentrations similar to those seen on day one (61.45±11.69% for CS, 48.88±11.14% and 59.97±13.4% for HB). No significant differences were observed between the groups in terms of the cytokine production patterns over the seven days.

DISCUSSION

In this study, we evaluated gut microbiota evolution during the first month of life in CS-delivered and both HB and hospital-based VAG infants. Our results highlight the relevance of perinatal factors to the gut microbial colonization pattern, which affects the innate defensive mechanisms and functional maturation at the intestinal level. We observed how distinct microbial colonization patterns were associated with the mode of delivery and how HB altered the host gut barrier and innate immune response in an *in vitro* model.

CS procedures are associated with specific conditions, including the use of antibiotics, longer hospitalization, low neonatal–maternal contact and the delayed initiation of breastfeeding [227,534], which may influence microbial colonization patterns [634]. A higher percentage of bottle or mixed feeding is commonly observed in CS-born infants [227], including the studied cohort. In our cohort, the HB infants

showed a higher rate of exclusive breastfeeding than the hospital-born infants (95% in HB verses 67% and 61% in CS and VAG at hospital, respectively). Furthermore, the practices associated with hospital delivery, as antibiotic use, vaginal cleansing, controlled maternal food intake and mobility, oxytocin administration or anesthesia, may affect the maternal microbiota and, consequently, mother–infant microbial transmission. Researchers have described the importance of maternal–neonatal microbial transmission, especially certain specific strains from the maternal gut microbiota, at the onset of infant colonization [153]. These birth-related factors may affect the microbial establishment process, and these cannot be studied independently. Therefore, our results sum up the consequences of all the concomitant factors affecting the different groups of study.

Thus, the hospital environment, especially in the case of CSs, could be considered an intervention-heavy condition characterized by the high pressure of antibacterial therapy and instrumentalization. HB rates have increased in Europe over recent decades, ranging from 0.1% in Sweden to almost 20% in the Netherlands [230]. However, limited information concerning the possible benefits of HB is currently available due to the lack of adequate randomized clinical trials meaning that it is not possible to determine the risk of neonatal–maternal mortality and morbidity during HB [647]. Further, little is known about the impact of a non-hospital environment on infant gut colonization.

The mode and place (hospital versus home) of birth shaped the neonatal microbiota. The microbiota of hospital-delivered infants was enriched with gram-positive anaerobic cocci, including the *Peptoniphilus* and *Finegoldia* genera, while those of HB infants exhibit higher relative abundances of species from the *Enterococcus* and *Bifidobacterium* genera. In all three groups, time was the main factor affecting the composition of the infant microbiota, with a significantly different pattern being observed at birth than at the subsequent time points. Primary events concerning gut microbial colonisation may impact the assembly and acquisition of the early microbiome, and they may have long-lasting consequences for gut ecology [68].

At delivery, we observed higher diversity indices in the neonatal microbiota than at the subsequent time points; however, the quantitative total bacteria count was lower at delivery. This is likely caused by bias in the sequencing data obtained from low biomass samples and by higher contact with environmental contaminants. The HB infants had lower microbial diversity but a higher total number of bacteria than the

hospital-born infants, likely due to the latter having increased contact with bacterial environmental contaminants [217].

We found clearly identifiable differences in the global microbiota structure during time between the three groups, even at the phylum level, which is in agreement with previous studies [217,232,281]. Interestingly, the differences in the colonization patterns become more pronounced over time, at least during the first month of life. These results indicate the importance of birth-related events to neonatal colonization processes, which may affect the microbiome composition in later developmental stages of infants. Similar to prior studies, we found that CS-born infants showed higher relative abundances of *Clostridium* and *Klebsiella* and lower species from the *Bifidobacterium* genus [217]. We observed that both VAG and HB infants had higher quantitative *Bifidobacterium* species than CS-born neonates. The depletion of the *Bifidobacterium* species in the gut environment has been described in relation to immune-related diseases [648,649], and members of this genus are commonly used as probiotics due to their capacity to modulate microbiota–immune system homeostasis [650].

Could these shifts in the microbiota composition alter the functional profile of the neonatal microbiota?

Descriptive and observational studies are relevant to understanding microbiome evolution during the neonatal period; however, mechanistic studies of the host–microbiome interplay during early life are still required. Thus, microbiota functional analysis has been proposed as a tool for clarifying the host–microbiome interactions [651]. Our results showed functional differences between the place and mode of birth in the infant microbiota at delivery, 7 and 31 days. Several amino acid (AA) biosynthesis routes were over-represented in the vaginal-delivered microbiota when compared with the CS-born infants, including tryptophan-related paths. It is known that AAs serve as regulators of several metabolic pathways in the host [652]. Specifically, tryptophan interacts with the immune system through microbial serotonin production [653], regulation by TLRs or interactions within the aryl hydrocarbon receptor–microbiota–immune system relationship [654,655].

We also found that vaginal-delivered infants, especially HB babies, had a microbiota enriched with LPS biosynthesis-related functions. Similarly, Wampach et al. found differences in the earliest functional profile according to the delivery mode,

including LPS biosynthesis routes being enriched in vaginal deliveries when compared with CS-born neonates [358], which may influence immune system maturation and neonatal health.

Do these differences in the microbiota influence the host immune system response?

Despite studies in animal models highlighting the possible effects of microbial colonization patterns on the host gut epithelium maturation and immune system response, little evidence is available from human studies. To the best of our knowledge, this is among the first studies to address this important issue. The intestinal epithelium is the gateway through which gut microbiome–host crosstalk effects intestinal functionality in the form of enterocytes maturation, mucus production or epithelial barrier development, which means it is a key anatomical location for host–microbiome interplay.

The samples from HB infants exhibited a higher immune stimulatory capacity than those from hospital-born infants (both VAG and CS), with an increased ability to induce the expression of immune system-related genes (*TLR4* and *IRAK* mRNA) and cytokine responses, including IL6 and IL8, in the HT-29 and THP-1 models. In concordance with our results, Wampach et al. identified the higher immunostimulatory potential of the microbiota of vaginal-delivered infants when compared with CS-born infants, although they used LPS purified from infant fecal samples and primary human macrophages differentiated into dendritic cells [358]. Combellick et al. noted the higher expression of *TLR4* and *IL8* by the HT-29 cell line following exposure to sterile fecal supernatant from HB infants when compared with hospital-born infants. However, they also found higher levels of *TGF-β,* which is mainly an anti-inflammatory cytokine, in hospital-born neonates [232]. We observed that the THP-1 cell line response was more affected by the mode and place of delivery than the HT-29 cell line, which highlighted the importance of the epithelial integrity and the innate immune system on the *in vitro* assessment of the host-microbiome interplay.

Most prior studies with similar objectives involved acute exposure on unique cell lines. We hypothesized that acute exposure to microbial products could not accurately reflect the biological effect of microbial metabolites and so proposed long-term (7-days) *in vitro* exposure assays, including the crosstalk between different cell types, which enabled us to obtain personalized results for each participant, thereby translating the individual signatures to the *in vitro* system.

In our model, the HB fecal supernatants induced higher gut barrier integrity (**Fig. 5**) and functionality (IAP; **Fig. 5**) following a time-dependent response that highlighted the relevance of the dynamics of host–microbiome interplay. Interestingly, the impaired closure of gut mucosal membranes has been shown, alongside higher intestinal permeability, in preterm infants who received formula feeding rather than breastfeeding [656]. This increased permeability could be related to allergic diseases in non-breastfed children [657] and to other health disorders [658]. In addition, the HB samples induced the expression of anti-inflammatory molecules (e.g. *IL10*, *TOLLIP*) to a higher extent than the CS samples, indicating negative feedback on inflammatory signaling in the gut. Specifically, IL10 down-regulate the microbiota-activated mucosal inflammatory cytokines and reinforce the gut epithelium barrier and control gut permeability, which are both essential to maintaining intestinal homeostasis [659].

Higher mucus production was observed after cell-exposure to fecal supernatants obtained from hospital-born infants when compared with HB infants. Both, microbiota [335] and TLR expression [660] are involved in the regulation of mucus production. Skoczek et al. found increased mucus production to be mediated by TLR signaling following the microbial invasion of the epithelial layer [661]. Despite lower mucus production in the gut being associated with disease phenotypes (e.g. inflammatory bowel disease, higher susceptibility to bacterial infections) [662] in adults, little is known about the role of mucins in neonatal colonization processes. We hypothesize that a penetrable mucus layer in newborns would allow for microbial colonization and interaction with the epithelium during the immune-priming window.

Many researchers have discussed the possible relationship between CS and altered immune system development [634]. Generally, CS delivery is associated with the poor stimulation of the immune system [222,663,664]. Urbanization is also associated with an impaired immune system response [665]. Some researchers have proposed non-diverse environments in early life, including delivery, to play a role in this effect. Furthermore, recent evidence has shown that early exposure to rural areas or farm environments could affect microbial composition and diversity [126]. This may be reflected in the observed reduction in the atopy risk in adults [666]. Kirjavainen et al. recently described how a farm-like indoor microbiota could also decrease the asthma risk in a non-farm environment [125].

However, most of these results were derived from observational studies, as very few mechanistic analyses have been conducted to date. Our results suggest a possible

link between CS and the delayed maturation of intestinal function and the innate immune system. Such a link could play a significant role in the diseases associated with intervention-based deliveries, including autoimmunity, allergy and other immune- and metabolic-related disorders.

In our study, CS-born infants showed higher BMI and W/L z-scores during the first 18 months of life. Other researchers have reported similar results, associating CS with the risk of overweight in children [581,667]. This could indicate the relevance of priority events, including those that alter microbial colonization, in infant health, thereby supporting the early programming hypothesis [668,669]. Researchers have described how antibiotic therapy during early life modulates weight gain in different ways depending on the antibiotic dose in both animal [121] and human epidemiological studies [581]. It has been suggested that high-dose antibiotics can cause important reductions in the microbiota population, which may be related to the weight loss observed in some studies [254]. However, lower antibiotic doses would cause microbiota composition shifts, more than population size variation, and trigger the weight gain shown in the above-mentioned studies. Thus, it remains to be discovered whether the proposed mechanism could be extended to other perinatal factors that also disrupt the microbiota composition and transmission.

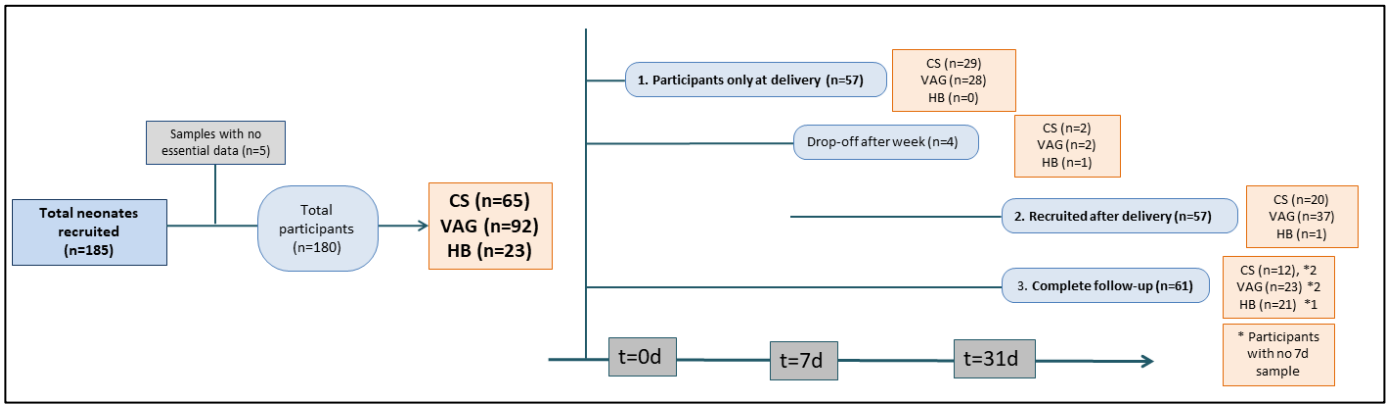
The limitations of this study include the low number of participants and the possible confounding factors not included in the analysis (e.g. maternal diet, lifestyle or number of siblings, pets among others). Our microbiota analysis was based on the taxonomic profile obtained via 16S rRNA gene sequencing, which offers less resolution than complete shotgun metagenome sequencing. As we used sterile fecal supernatant, we observed the effects of soluble bacterial metabolites and, of non-bacteria-related products, including growth factors or eukaryotic extracellular vesicles, which may have influenced the observed results. Yet, fecal supernatants contain a complex array of molecules representative of the *in vivo* condition, which retain inter-individual differences and features. The use of cell lines may hamper the translational results, although it offers a reproducible and economically viable strategy for further testing on more physiologically relevant models. Among the strengths of the study are the inclusion of three groups and the comparison of CS and vaginal delivery at both hospital and home, including 18 months of follow up. We performed the cellular exposure assays in cellular models with different degrees of complexity and different

exposure times, including the epithelial barrier function and maturation as relevant targets, together with the innate immune response.

CONCLUSION

Our results may provide a mechanistic linkage between studies associating CS and immune-related diseases with colonization pattern alterations, although we cannot rule out other possible factors that might participate in the process. The study has shed light on the effects of hospitalization and HB on neonatal microbial colonization and on the possible effect on innate immune system development, specifically at the intestinal level. The results highlight both the importance of host–microbial contact during the first month of life and the dynamism of the process. However, further research is needed to determine the impact of these observations in neonatal *in vivo* clinical conditions. Such knowledge would facilitate the design of strategies for adjusting medical practices with the aim of reducing intervention during the birth process and ensuring the correct initiation of bacterial colonization and, consequently, the immune system response during early life.

CHAPTER V - SUPPLEMENTARY DATA



Supplementary file V-1. Flowchart detailing the number of participants in each time point according to study group. The three main participation mode of MAMI cohort [420] is shown in bold letters. Mode 1 (participants only at delivery), Mode 2 (Participants recruited in health care centers after delivery), Mode 3 (Participants recruited at hospital before delivery and follow in the study during the first month of life). Additionally, it is also shown the drop-off of some participants according to study group (n=4) and number of participants with missing values at 7d (n=6).

Supplementary file V-2. Characteristics of studied population according to place and mode of delivery.

		C-section (n=65)	Hospital Vaginal birth (n=92)	Home birth (n=23)	p-value
MATERNAL DATA					
Maternal age (years)		35.5 [33-38]	34 [31-37]	34 [32-36]	*0.056*
Pre-gestational BMI (Kg/cm^2)		23.19 [21.5-25.9][a]	21.69 [20.37-23.85][b]	22.77 [19.75-24.77][ab]	***0.027****
Weight gain over pregnancy (Kg)		13.42 ± 4.91	12.16 ± 4.01	13 ± 4.33	*0.315*
Antibiotics during pregnancy		27 (41.5%)[a]	31 (34%)[a]	1 (4.34%)[b]	***<0.001****
Antibiotics during delivery		65 (100%)[a]	10 (10.9%)[b]	0 (0%)[c]	***<0.001****
NEONATAL DATA					
Delivery	Gestational age (weeks)	39 [38-40][a]	40 [39-40][a]	40 [39-41][b]	***0.004****
	Neonatal weight (g)	3305 [2925-3710]	3200 [2965-3440]	3360 [3145-3630]	*0.140*
	Infant length (cm)	50 [48-51][b]	49.5 [48.5-51][b]	52 [50.25-53][a]	***0.003****
	Sex				
	Female	22 (33.8%)	52 (57.2%)	12 (52.17%)	
	Male	43 (66.15%)	39 (42.8%)	11 (47.83%)	
	Siblings	28 (43.08%)	34 (37%)	10 (43.4%)	*0.588*
	BMI z-score	0.22 [-0.52-0.86] [a]	-0.29 [-0.88-0.2] [b]	-0.37 [-1.03-0.13] [ab]	***0.015****
	W/L z-score	0.26 [-0.55-0.96] [a]	-0.19 [-0.87-0.31] [b]	-0.51 [-1.52-0.16] [b]	***0.002****
7 days	Infant weight (g)	3275 [2845-3585][2]	3200 [2975-3530][4]	3480 [3290-3620]	*0.126*
	Infant length (cm)	50.75 [49-52][2b]	51 [49-51.5][4b]	52 [51-53][a]	***0.028****
	Infants antibiotics	0 (0%)[2a]	7(7.7%)[3b]	0 (0%)[a]	***<0.001****
	Exclusive breastfeeding	73.5%[a]	61.29%[a]	95.5%[b]	***<0.001****
31 days	Infant weight (g)	4150 [3650-4728]	4100 [3685-4438] [1]	4540 [3935-4810]	*0.054*
	Infant length (cm)	53.75 [52-55.5][b]	53.5 [52-55.5] [1b]	55.5 [54.25-57.5][a]	***0.013****
	Infants antibiotics	3 (4.6%)[2]	5 (5.5%)[4]	1 (4.34%)	*0.922*
	BMI z-score	-0.45 [-1.24-0.65]	-0.65 [-1.27-0.16][1]	-0.52 [-1.035-0.175]	*0.552*
	W/L z-score	-0.19 [-0.89-0.55]	-0.54 [-0.54-0.34]	[-0.85- -1.275]	*0.104*
	Exclusive breastfeeding	67.6%[a]	61.29%[a]	95.5%[b]	***<0.001****
18 moths	Infant weight (Kg)	11 [10.04-11.85]	10.85 [10.85-11.54]	10.85 [9.75-11.54]	*0.158*
	Infant length (cm)	82 [79.5-83]	81.5 [81.5-83.5]	81.5 [79-83.5]	*0.818*
	BMI z-score	0.44 [0.22-0.58][7 a]	0.31 [0.13-0.41][7 b]	-0.13 [-0.36—0.05] [c]	***<0.001****
	W/L z-scores	0.39 [0.15-0.56][7 a]	0.26 [0.05-0.38][7 b]	-0.17 [-0.34- -0.02] [c]	***<0.001****

Normally distributed data was presented as mean ± SD and non-normal data as median [IQR]. Categorical variables were expressed as positive cases (percentage). Data no sharing letters was significantly different between studied groups. Number of missing values for each variable was represented as a superscript. Z-scores of anthropometric measures were electronically computed using WHO Anthro software (www.who.int/ childgrowth/software/en/). Z-scores values were adjusted by breastfeeding duration, antibiotic intake during the first year of life, maternal pre-gestational BMI and infant BMI and Weight for length (W/L) z-scores at birth.

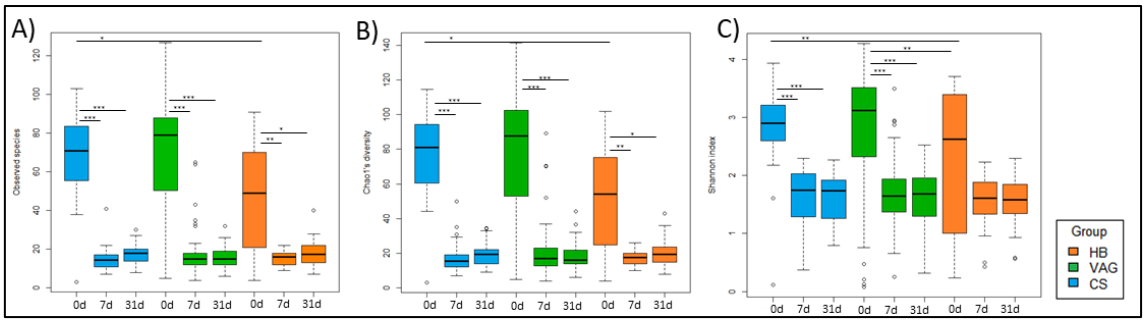
Supplementary file V-3. Primers of 16s rRNA gene of prokaryotic targets and human genes tested by RT-PCR.

Target	Sequence (5'-3')		Amplicon size (bp)	Reference
PROCARIOTIC PRIMERS		**Annealing (Cº)**		
Total Bacteria	CGTGCCAGCAGCCGCGG TGGACTACCAGGGTATCTAATCCTG	62	274	[670,671]
Bifidobacterium	GATTCTGGCTCAGGATGAACGC CTGATAGGACGCGACCCCAT	60	232	[672]
HUMAN GENES PRIMERS		**Efficiency at 58ºC**		
ACTB	ATGCTATCACCTCCCCTGTGTG TTGTTACAGGAAGTCCCTTGCC	1.79	101	[673]
TLR2	CTGGTAGTTGTGGGTTGAAGCA GATTGGAGGATTCTTCCTTGGA	1.71	102	[674]
TLR3	ACCTCAACTGGGATCTCGTCA ACAACTTAGCACGGCTCTGGA	1.82	124	[675]
TLR4	GGGTTCAGGGACAGGTCTAAAGA AATCTAGAGCACTTGGACCTTTCC	1.81	116	[675]
TOLLIP	AAGTGGTACAGCCTGAGCGG GATGGGCACATAGCCAACGC	1.75	159	This work
IRAK4	CGCCCGGGCAGGAATAGAA TTCAGAAGTGGGACTTTTTCCAGT	1.79	227	This work
FOS	CAGACTACGAGGCGTCATCC CGTGGGAATGAAGTTGGCAC	1.67	166	[676]
IP10 (CXCL10)	GTGGCATTCAAGGAGTACCTC GCCTTCGATTCTGGATTCAGACA	1.77	193	[677]
IL12	TTGTGGCTACCCTGGTCCT AGAGTTTGTCTGGCCTTCTGG	1.75	151	[678]
IL10	GGCGCTGTCATCGATTTCTT TGGAGCTTATTAAAGGCATTCTTCA	1.77	79	[679]
ZO-1 (HP)	TTAAGC-CAGCCTCTCAACAGAAA GGTTGATGATGCTGGGTTTGT	1.75	83	[680]
OCLN	TATAAATCCACGCCGGTTCCT ACGAGGCTGCCTGAAGTCAT	1.78	77	[680]
ECAD (CDH1)	ACAGCCCCGCCTTATGATT TCGGAACCGCTTCCTTCA	1.79	60	[681]
HPRT1	TGACACTGGCAAAACAATGCA GGTCCTTTTCACCAGCAAGCT	1.80	93	[682]

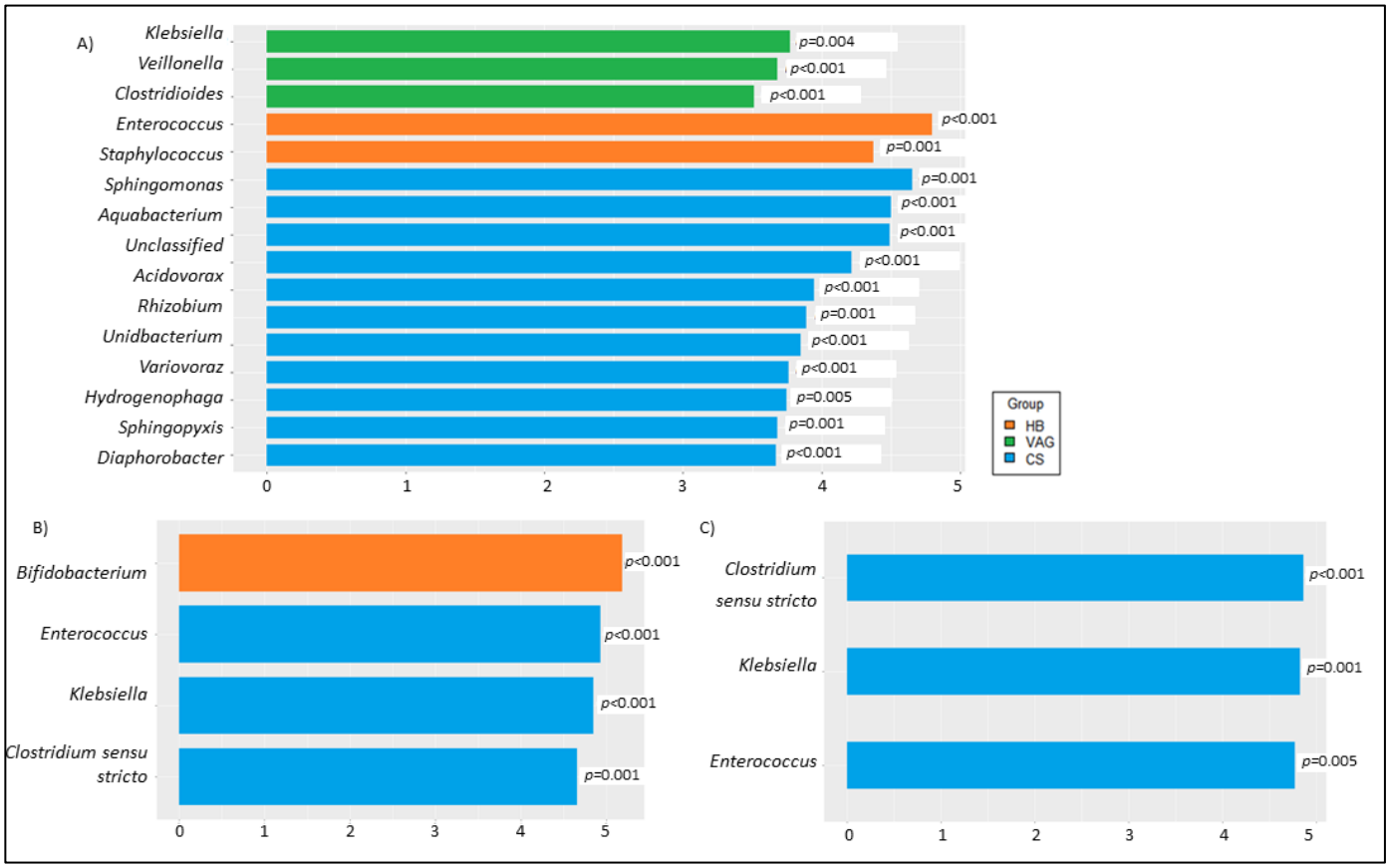
Supplementary file V-4

	Homebirth			Hospital Vaginal birth			C-section		
	Delivery	7d	31d	Delivery	7d	31d	Delivery	7d	31d
Proteobacteria	40.3a [22.8-70.2]	6.73b* [1.67-16.8]	3.06b* [1.13-12.59]	43.5a [29.2-64.75]	24.15b [8.11-39.95]	11.9b [3.95-29.63]	66.2a [35.8-77.9]	28.2b* [14.25-43.6]	24.1b* [6.19-40.4]
Firmicutes	43.8* [20.3-64.15]	40.6 [24.8-47.3]	26.55 [14.55-43.58]	32.8 [17.55-46.05]	36.15 [19.8-61.45]	32.2 [16.38-51.68]	19.7a* [9.27-41.1]	56.5b [38.63-76.78]	43.9b [24.4-64.1]
Bacteroidetes	0.25a [0.057-1.53]	0.048b [0-1.72]	0.28b [0-4.02]	2.49a [0.58-7.16]	0.1b* [0-5.13]	0.021b* [0-12.28]	0.21a [0.08-0.92]	0b* [0-0.03]	0b* [0 -0.021]
Actinobacteria	6.04a [1.35-11.05]	45.9b*# [27.8-53.4]	54.2b [37.73-77.3]	9.62a [3.65-15.15]	26.15a*# [0.22-48.10]	39.5b [11.87-55.43]	5.83 [2.6-8.92]	0.50 * [0.04-19.35]	14 [0.68-40]

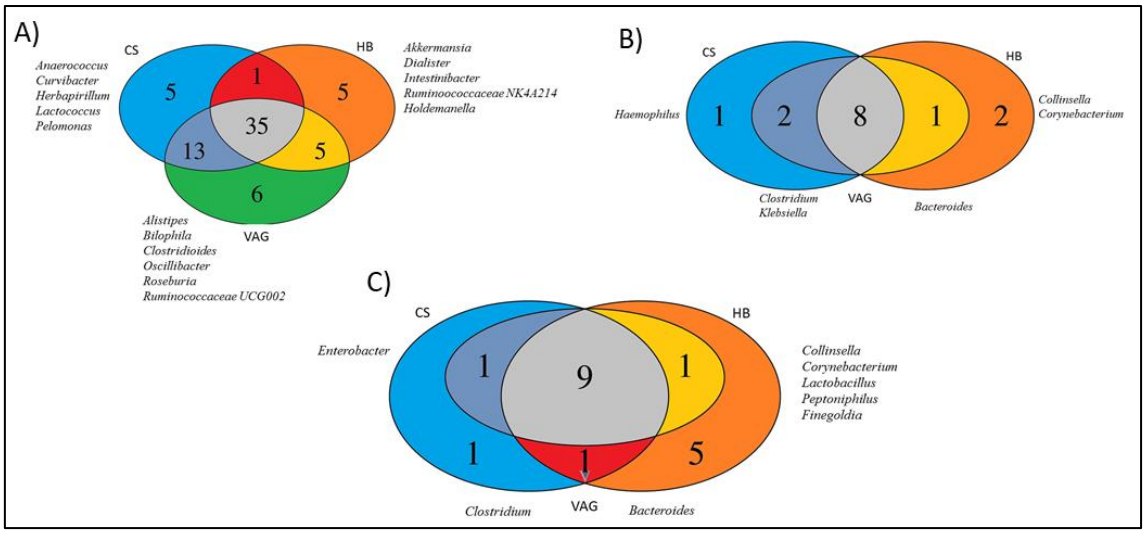
Data was presented as median [IQR]. Data no sharing letters represent significantly different composition between times within the same delivery group. Symbols (*, #) marked differences in the microbial composition between the three studied groups at the same time.



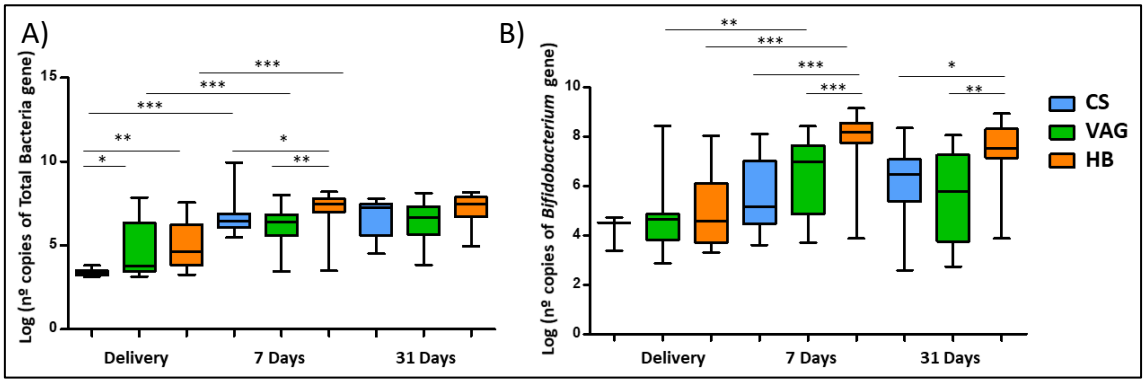
Supplementary file V-5. Neonatal fecal microbiota diversity and richness of meconium and infant fecal samples at 7 and 31 days. Observed species (**A**), Chao1 (**B**) and Shannon (**C**) index were used to measure richness and diversity respectively. Samples were rarefied at 90% of the minimum depth sequencing to assess diversity. *$p<0.05$, **$p<0.01$, ***$p<0.001$. C-section (CS), Hospitalized vaginal delivery (VAG), homebirth (HB).



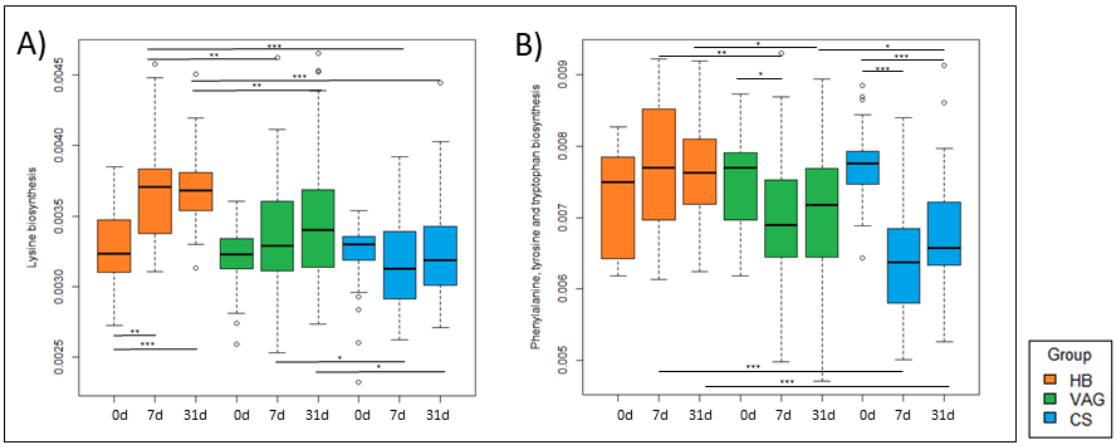
Supplementary file V-6. Taxonomic biomarkers of microbiota composition of each group depending on place and mode of delivery. Linear discriminant analysis effect size (LEfSe) was performed with a threshold of LDA score >4 for faecal swabs microbiota at genus levels at delivery time (**A**), seven (**B**) and 31 (**C**) days of life.



Supplementary file V-7. Core group of neonatal microbiota composition at genus level over the first moth of life. Venn diagram was conducted at delivery (**A**), seven (**B**) and 31 days (**C**) of life.



Supplementary file V-8. Quantitative analysis of intestinal microbiota from infants born at hospital (vaginal and C-section delivery) and at home across the first month of life. Total bacterial (**A**) and Bifidobacterium genus counts (**B**) expressed as log (nº copies of 16S rRNA gene of each group). A subset of total population was selected according to sample availability. Percentage of samples measured according to time and groups was as follow, Delivery: VAG (47.3%), CS (69.8%), HB (95.7%); 7d: VAG (88.3%), CS (82.4%), HB (90.5%); 31d: VAG (67.2%), CS (75.8%), HB (90.9%). Data was presented as median and whiskers represented the 5-95 percentile. Kruskal-Wallis and Dunn's post hoc (FDR adjustment) test was used to test the significance of the differences in cytokine response between the groups. *p<*0.05*, **p<*0.01*, ***p<*0.001*. C-section (CS), Hospitalized vaginal delivery (V), homebirth (HB).



Supplementary file V-9. Microbial functions related to amino acids metabolism computationally predicted present in neonatal microbiota along the first month of life. Computational analysis of lysine (**A**) and Phenylalanine, tyrosine and tryptophan biosynthesis (**B**) routs presented in the fecal microbiota of newborns along the first month of life. Results were expressed as percentage of total functional routs for each sample. *p<*0.05*, **p<*0.01*, ***p<*0.001*. C-section (CS), Hospitalized vaginal delivery (VAG), homebirth (HB).

Supplementary file V-10. Gene Expression of HT-29 and THP-1 cells after 24 h of fecal supernatant exposure

	HB		CS	
Gene	**Fold-expression**	**p-value**	**Fold expression**	**p-value**
HT-29 cells				
HP	0.202 (0.088 - 0.478)	***< 0.001**** ⬇	0.265 (0.098 - 0.726)	***0.006**** ⬇
CDH1	0.226 (0.065 - 0.704)	***0.005**** ⬇	0.326 (0.102 - 1.129)	***0.035**** ⬇
OCLN	0.192 (0.080 - 0.485)	***<0 .001**** ⬇	0.253 (0.088 - 0.739)	***0.012**** ⬇
TLR4	1.405 (1.052 - 1.735)	***0.024**** ⬆	1.482 (1.115 - 2.052)	*0.100*
IRAK4	2.654 (1.216 - 4.330)	***<0 .001**** ⬆	1.965 (0.952 - 4.218)	*0.174*
IL12	0.609 (0.311 - 1.036)	*0.118*	0.725 (0.435 - 1.310)	*0.210*
CXCL10	989.712 (654.81 - 1637.46)	***< 0.001**** ⬆	677.505 (214.19 - 1584.86)	***0.033**** ⬆
FOS	1.029 (0.796 - 1.244)	*0.814*	1.333 (0.847 - 2.046)	*0.210*
THP-1 cells				
TLR4	0.917 (0.342 - 12.306)	*1*	0.509 (0.345 - 0.626)	***0.006**** ⬇
IRAK4	1.345 (0.399 - 45.200)	*0.969*	0.538 (0.209 - 0.959)	*0.113*
IL12	0.418 (0.110 - 16.175)	*0.599*	8.437 (0.831 - 107,927.33)	*0.379*
CXCL10	1751.59 (104.706 - 197,216.41)	***< 0.001**** ⬆	893.79 (652.843 - 1295.368)	***<0.001**** ⬆
FOS	0 (0.000 - 0.233)	*0.271*	0.238 (0.097 - 0.628	***0.038****

Total RNA was extracted from cells treated with homebirth (HB) samples and C-section (CS) fecal supernatant. Values expressed relative expression fold-change of each condition compared to control (95% C.I). *P<.05* (*) and blond letters marked significant differences between treatment and control, up (UP) or down (DOWN) regulation was represented by the arrows.

Supplementary file V-11. Epithelial barrier function and maturation of simulated intestinal epithelium of the triple co-culture during the long-term exposure

		HB	VAG	CS	p-value
Trans-epithelial electrical resistance (TEER) - Ohms/cm^2					
Day	**1**	666.02 ± 12.06	652.41 ± 29.97	643.10 ± 6.66	*0.226*
	2	735.09 ± 40.76	831.83 ± 62.11	782.08 ± 43.62	*0.211*
	3	763.20 ± 111.65	848.98 ± 105.23	838.91 ± 76.81	*0.624*
	4	555.72 ± 63.47	732.92 ± 220.33	689.30 ± 246.44	*0.198*
	5	556.10 ± 17.99	638.29 ± 77.09	642.19 ± 209.07	*0.350*
	6	682.83 ± 65.45^a	865.44 ± 86.64^b	822.35 ± 47.32 ^{b}	***0.001****
	7	903.66 ± 72.439^a	727.81 ± 101.87^b	732.35 ± 56.60^b	***0.004****
Apparent permeability – cm/s					
		$1.45{\cdot}10^{-6}$ ± $1.72{\cdot}10^{-7a}$	$2.09{\cdot}\ 10^{-6}$ ± $5.68{\cdot}10^{-7b}$	$2.01{\cdot}10^{-6}$ ± $3.49{\cdot}10^{-7b}$	***0.013****
Intestinal alkaline phosphatase – mg/ml					
Day	**1**	0.56 ± 0.2	0.40 ± 0.13	0.42 ± 0.12	*0.106*
	2	0.75 ± 0.11^a	0.48 ± 0.21^b	0.47 ± 0.13^b	***0.023****
	4	1.02 ± 0.46	1.02 ± 0.38	1.01 ± 0.34	*0.997*
	6	0.85 ± 0.28	0.68 ± 0.32	0.69 ± 0.27	*0.494*
	7	0.75 ± 0.24^a	0.42 ± 0.24^b	0.43 ± 0.32^b	***0.027****
Mucus production – mg/ml					
		0.691 ± 0.09	0.928 ± 0.25	0.920 ± 0.28	*0.107*

Variations in the Transepithelial electrical resistance (TEER) and intestinal alkaline phosphatase were presented along the seven days of treatment. Apparent permeability and mucus production were measured at final time point. Letters indicated groups that showed significant differences between them within a measurable variable. *P<0.05* (*) and blond

letters marked significant differences between three studied groups.

GENERAL DISCUSSION

GENERAL DISCUSSION

In this thesis, maternal and neonatal samples were longitudinally collected with the aim of investigating the network of associations between maternal and neonatal microbiota, perinatal factors that could modify this interaction and the possible outcomes in the maternal and/or neonatal health. We described the relationship of maternal microbiota with the maternal amniotic fluid immunological state (**Chapter I**) at birth time as well as several maternal factors, including those related to delivery (**Chapter II**) and also maternal diet during gestation and its impact on infant health (**Chapter III–IV**). Thus, we have observed that Firmicutes spp. in maternal microbiota were related to higher intakes of SFA and MUFA, which also influenced the maternal intestinal permeability. These observations in maternal microbiota were also seen in neonatal microbiota. Furthermore, a group of taxa, including *Peptoniphilus*, *Finegoldia*, *Anaerococcus*, *Porphyromonas* and *Campylobacter* were over-represented in the mothers who had undergone a C-section, and were negatively associated with salivary cortisol concentration and with amniotic fluid concentrations of IL-2, IL-5, IL-17 and TNF-alpha.

We have also shown the importance of these factors in the neonatal microbiota at delivery and in the microbial colonization process during the first month of life (**Chapter III–V**) and beyond, describing the possible long-lasting effects of the alterations associated with these factors (**Chapter III–V**). Furthermore, we propose a molecular mechanism that mediates in the observed outcomes in the neonate (**Chapter V**), where the exposure of samples from C-section-born neonates on a simulated gut epithelium model triggered less immunostimulation and membrane integrity compared to those induced by samples from vaginal-delivered neonates, especially those born at home. Our data shed light on the field of maternal–neonatal microbiota and open the door to conducting further mechanistic analyses that allow, in the future, to design strategies to modulate the possible alterations derived from microbial-disrupter practices.

Maternal microbiota and gestation

Maternal microbiota has been highlighted as one of the main factors that contribute to the neonatal microbial colonization [153]. However, most of the studies

have been focused on the neonate at birth [130] or during the breastfeeding period [277]. Studies on the maternal microbiota during pregnancy are still scarce and most are only descriptive of the microbiota composition, with very few of them noticing the interaction with other body systems. We have described that several factors were associated with maternal microbiota at delivery. The bacterial community in the maternal gut was influenced by delivery mode and associations between bacterial taxa and salivary cortisol concentrations and several cytokines in amniotic fluid, as well as placental expression of some metabolic genes, such as GLUT3 and GPX. Indeed, we have shown that maternal diet during gestation was related to both maternal and neonatal microbiota as well as being associated with neonatal BMI at 18 months of life.

Recently, there has been growing concern about the *in utero* microbiota [160,165,668], but also a focus on the possible significance of this fact on the neonatal development. Several studies have been developed on the meconium microbiome [163,185,568,683]; however, most of them are based mainly on sequencing results and very few studies have isolated viable bacteria from meconium [172,684]. In order to assess the question of the possible *in utero* colonization using meconium microbiota, we consider it essential to test the influence of labour in the meconium microbial composition. Recently, a study was performed on the possible initial microbial colonization using meconium collected before birth [684]. They found bacterial DNA in 40 samples of 50 tested individuals. However, only 15 samples showed viable bacteria and a clearly differential microbiome profile compared to controls. Interestingly, the authors found that these foetal bacterial profiles were associated with distinct foetal epithelial transcriptome with differences in patterns of T cell composition, which could imply that the presence of bacterial DNA rather than viable bacteria could be enough to affect the *in utero* immune imprinting. Indeed, the exposure of the isolated bacteria to primary human foetal intestinal cells *in vitro* reproduced the mentioned *in vivo* transcriptome profile.

In this regard, in our study, the neonatal faecal samples were collected in the delivery room by use of a rectal swab immediately after birth (**Chapter I-V**). Thus, we preferred to denominate our samples as first-pass faeces since they were very likely to have been influenced by faecal maternal contact at delivery and we could not ensure that meconium had the same bacterial DNA profile.

Although this is an important and relevant field, there could be other routes by which maternal microbiota could affect the foetal development with the same degree as

direct contact with bacteria. The capability of bacteria to interact with all maternal systems, including digestive [685], hormonal [60], metabolic [592], immune [21] and even neurologic [686], make the study of microbiota and derived products – including exosomes, bacterial DNA, and metabolic products, and the relations between them and the host – one of the most suitable approaches to compose an overview of the gestation process in a holistic mode. Furthermore, not only could a direct effect be studied, but also the possible alterations that microbial dysbiosis could cause in the other mentioned systems [540,687], thus impacting on the normal development of gestation. With regard to this, several authors have described that microbial shifts occurring in periodontal disease are associated with bad pregnancy outcomes, including preterm delivery [506,688,689] with other conditions involved, such as a low-grade pro-inflammatory state [689].

Indeed, it has been suggested that the possible changes that the maternal microbiome undergoes during the last period of gestation could play a role in the foetal and even neonatal development, mainly contributing to host adiposity through a microbial profile with an increased capacity for energy extraction from diet, or by an alteration of metabolism due to the promotion of low-grade inflammation through an imbalance in the host–microbial relationship [132]. Thus, it is possible to hypothesize that factors that alter these microbial adaptations during pregnancy would also impact on foetal development.

A greater number of human studies, especially from gestational cohorts, are needed to clarify the actual extent of the maternal microbiota's impact on foetal development and adult health. Plasma levels of SCFAs have been shown to be higher in offspring from a high fibre diet and also to have a higher frequency of thymic Treg [154]. In humans, similar results were found by Vuillermin et al., who described that the carrying of *Prevotella* species by mothers during pregnancy was associated with a protective factor against allergic disease later in life [690]. They observed that children from those mothers with a high intake of both fat and fibre had a lower risk of allergic disease. *Prevotella* genus is characterized by the capacity of these dietary components to produce SCFAs and succinate, which both stimulate the immune system cell development and function [691]. Indeed, *Prevotella* species express some endotoxins, including LPS [692], which have been observed to play a role in the maturation of the neonatal immune system by TLR4-dependent routes [693].

This growing body of evidence highlights the importance of the complex link between diet, gut bacteria and bacteria metabolites, and the gestational process [156]. Despite this, the effect of maternal diet on the women's microbiota during pregnancy has been understudied in the field. A lot of knowledge is available on the effect of some specific nutrients on gut microbiota in the adult population [579], especially for high fat (HFD) [694] or fibre diets [695]. Indeed, the possible systemic effects of these diets mediated by bacterial metabolism or response to those nutrients have been described. The link between HFD and systemic circulating LPS has been observed, which could be responsible for the low-grade inflammation associated with this diet. However, pregnancy as discussed in this thesis, is a unique state in human life that may not be regulated by the same rules, and analysis in the pregnant population – especially in the third trimester – would be essential to classify these differences.

We have described that maternal dietary intake was associated with both maternal and neonatal microbiota at delivery (**Chapters III–IV**). In general, we have observed that the Firmicutes phylum was positively associated with mothers that had a higher fat intake, but only SFA and MUFA, while the relative abundance of Proteobacteria phylum showed a negative relationship with this nutrient. Indeed, we have shown that this could be related to the effect of fat-associated components in intestinal permeability, since those followed a positive correlation with excreted zonulin levels, which have been proposed as a measure of gut permeability (**Chapter III**). Importantly, these patterns of associations were also observed in neonatal microbiota, especially in vaginal-delivered infants.

Limited number of studies have explored the effect of diet on the pregnant population [194–196,198,489] in humans, despite the evidence available in animals [154,155,193,536,696]. However, Mandal et al. focused on maternal microbiota four days post-delivery, and in accordance with our results they found that fat products and cholesterol affect the maternal microbiota at delivery [196]. Indeed, they described that saturated fat was associated with a decrease in Proteobacteria relative abundance. In contrast, they found a difference in the association patterns between saturated and unsaturated fat, which was related with an increment in this phylum. In agreement with our results, Chu et al. observed in a primate model that a high-fat diet showed a depletion of the *Campylobacter* genus from the Proteobacteria phylum in the offspring, even 6 months after delivery. However, these same authors found in their human study a slight depletion of the *Bacteroides* genus in the microbiota of the offspring of mothers

that followed a high fat diet[193]. Although we found a lower relative abundance of Bacteroidetes phylum in mothers with higher consumption of SFA (**Chapter III**), we did not confirm that observation in *Bacteroides* genus. However, our population followed an intake of all nutrients that is in the range of a healthy diet and very few mothers could be classified as HFD consumers, given that this diet is characteristic of at least 60% of daily total energy contribution that comes from lipids intake [697].

In this regard, although a study in populations with extreme diets could be a suitable initial approach for microbiota study or even to confirm observations, the analysis of the associations in a healthy pregnant population with a non-intervention or predesigned diet may be a more real situation, which should provide a more accurate overview of the maternal diet effect on normal gestational development. However, this approach could have some disadvantages, including the difficulty in discriminating between the intrinsic inter-individual variability derived from other factors and those associations related to the maternal diet. Thus, big cohorts as homogeneous as possible in terms of other factors, including genetic background, environmental conditions or physical activity, are necessary to overcome these issues [698].

Furthermore, we have observed differences in the way by which maternal diet affects neonatal microbiota depending on the delivery mode (**Chapter III**), similarly to what was observed by Lundgren et al. [195]. Thus, it is possible that the physiological processes that cause labour could modify, in some unknown way, the interaction between diet and gut microbiota. Besides this, with the delivery mode and associated conditions being a great altering factor of maternal microbiota, as we have shown in this thesis, the study of the effect of dietary intake on maternal gut microbiota during throughout the pregnancy without the possible impact of labour processes provided relevant information on the maternal health during pregnancy. Indeed, the analysis of how maternal diet may be modifying the normal physiological variations in gut microbiota in a healthy pregnancy would be especially interesting.

To evaluate in greater measure the effect of fibre, we decided to study a population with more differences among the participants in terms of dietary fibre intake and we included a deeper analysis of dietary profile. In this population we confirmed the association between maternal fat intake and Firmicutes phylum and their negative relation with Proteobacteria, which was also observed in neonatal microbiota. Indeed, we found (**Chapter IV**) two clusters of mothers according to their microbiota composition with differences in terms of dietary fibre and of omega-3 fatty acids

(mainly of docosahexaenoic acid [DHA] and docosapentaenoic acid [DPA], as well as polyphenol intake. In this group of mothers, fibre intake significantly affected the maternal and neonatal microbiota at delivery and that had an effect on infant health at 18 months post-partum. Fibre and PUFA have been previously described as modifiers of gut microbiota [613,691,699] and even of the health condition of offspring [155] and microbiota [700]. In our study (**Chapter IV**), offspring from mothers with a higher fibre and PUFA intake, which were associated with a specific microbiota profile, showed lower z-scores at 18 months post-partum, especially in those children born by C-section.

Although some results are convergent among different studies, such as the relation between Firmicutes and Proteobacteria with SFA intake, the possible implication of this observation in maternal and neonatal health is still unclear. In animals, HFD has also been observed to increase the Firmicutes phylum, but similar results were found for *Proteobacteria* spp. [694]. The molecular mechanisms beyond this result and their relevance to pregnancy development are totally unknown. In a non-pregnant population it has been suggested that Firmicutes spp. would be more efficient in energy extraction with the subsequent increase in body mass index [701]. Thus, the analysis of the Firmicutes to Bacteroidetes ratio was proposed as a measure of the 'obesogenic' grade of the microbiota [237]. That hypothesis was supported by some studies that found higher relative abundance of Firmicutes associated with obese condition [237,702–704]. Some studies have examined the relevance of this idea, suggesting that the influence of microbiota could be more complex, and this ratio could not explain this relationship [705]. Thus, several questions remain concerning the mechanisms beyond the relation between the Firmicutes phylum and increased weight or adiposity.

Interestingly, some studies have described that the obese phenotype related to HFD could be transferred to mice through a microbiota transplantation [64,706]. Thus, it is reasonable to expect that the possible effect of SFA intake in systemic health, which could be mediated by maternal microbiota, could also be transferred to the neonate during delivery in the initial bacterial seeding of the neonate, with possible long-lasting consequences.

Moreover, our study about factors affecting maternal microbiota during gestation has been focused on diet; however, we consider it essential to highlight the importance of the research in this period, not only into diet but also other factors, including medication, BMI, weight gain during pregnancy and physical activity, to

improve the health of both infant and mothers beyond the duration of pregnancy. Other studies have found the effect of some of these factors, especially BMI [152] or weight gain [707] on neonatal microbiota, despite the fact that we did not observe the same results. Regarding this, our maternal and neonatal samples were collected at delivery time and they were possibly influenced by all of those physiological processes involved in labour, and only those factors that deeply affect the maternal microbiota, such as maternal diet, were detectable in the microbiota of faecal samples at delivery. Thus, as we discussed, more studies focused on the gestation are needed in order to find the relevance of these other factors for foetal development.

Maternal and neonatal microbiota: the birth challenge

The triggering of labour is an extremely well-orchestrated process in which all the body systems are involved [708]. It is reasonable to speculate that maternal microbiota will also be affected by these physiological alterations; however, the nature of these alterations in maternal microbiota and whether they could contribute in some way to the labour process and / or the following neonatal requirements for the colonization are totally unknown.

Our study (**Chapter II**) is one of the few that describe the variations of maternal microbiota according to delivery mode and other related factors since most of the research is focused on neonatal microbiota [37,162,163]. Although we have also found an effect that clustered the samples according to their microbiota, this unidentified factor interacted in some way with delivery mode, since one of the clusters was enriched by mothers who had undergone a C-section. As we described in **Chapters II** and **III** of this work, a possible effect of maternal diet could be involved in this clustering, since some of the bacterial groups associated with fat or fibre were also associated with the clusters.

In this regard, resilience as a bacterial community property, which consists of resistance to changes in terms of composition and/or functional activity caused by possible exogenous factors has been suggested [36] – in this case, the delivery mode and concomitant alterations, including antibiotic intake [709] or reduced maternal contact. Thus, maternal diet could alter the manner by which the delivery mode affects maternal microbiota, and some mothers could be more 'sensitive' to those microbial disrupters.

In any case, the fact is that, traditionally, most of the research has been focused on the delivery mode effect but our results suggest that changing the paradigm to an all-inclusive analysis that notes the interaction between the main drivers for maternal microbiota would be essential to obtain more accurate results. Otherwise, there could be other physiological variables, including metabolic [449,710] and hormonal [432] processes, that may be altering the observations that we obtained from the microbiota analysis. Regarding this, we have described that a hormonal signal associated with vaginal delivery, such as saliva cortisol concentration [523], was negatively associated with taxa related to C-section, independently of the delivery mode (**Chapter II**). Thus, hormonal signals that accompany the labour could be altering the maternal microbiota [60,711] and be related to the differences that we observed in terms of bacterial composition according to delivery mode. In this regard, the association between progesterone concentration and the relative abundance of *Bifidobacterium* in maternal gut microbiota during pregnancy has been reported [451] . Furthermore**,** variations in adiponectin concentrations according to delivery mode have also been shown [535]**,** which could also impact maternal microbiota [712].

As this physiological process could be initiated even a few days before delivery [713], it is possible that the differences in these metabolic and hormonal signals could also be contributing to the fact that some of the mothers who undergone a vaginal delivery were grouped with those that had a C-section. However, information regarding the exact molecular signals that could be affecting the maternal microbiota as a consequence of the labour is still scarce. Some mechanisms have been proposed, such as alterations in bile acid and cholesterol regulation or motility and permeability [525].

Not only hormonal alterations, but also several pro-inflammatory cascades are crucial for the triggering of labour and, as in the case of the other body systems, their activity could be initiated before the clinical definition of delivery [713]. The role of cyclooxygenase [714], NF-kB [449] and prostaglandins [524,715], among others, in the parturition is well known and evidence that labour is an inflammatory reaction is available [716]. The differences in concentrations of different cytokines implicated in the Th cell regulation during labour and the post-labour period have been described [459]. Also, differences in the cytokine concentrations during the perinatal period according to delivery mode have been reported, which could have implications for neonatal immunity [460].

We have described associations between amniotic fluid's cytokine profile and maternal microbiota at delivery, suggesting a possible relation between these signals and shifts in gut microbiota from both mother and neonate (**Chapter I**). As we have presented in the literature background section, the immune system and the gut microbiota have the ability to affect each other reciprocally [257,335,350], and the delivery time may not be an exception for this observation. We have described that several cytokines in amniotic fluid were associated with different bacterial patterns in maternal microbiota (**Chapter I**). Interestingly, even ignoring the effect of delivery mode, the taxa that we have observed to be less related to C-section delivery in the other chapters were negatively associated with the cytokine profile dominated by the IL-2 cytokine concentration, which is known to have a role in T-cell amplification and function regulation [463,464]. The taxa related to this profile, including *Roseburia*, *Faecalibacterium*, *Lachnospira* or *Bacteroides* genera, are known to be SCFA producers [40,50] and/or with immunomodulatory properties [325,348]. Interestingly, some of these genera and also the cytokine profile were related to serum cholesterol and triglycerides, but taking into account the mothers had non-pathological values.

The other group of taxa, a C-section-associated bacterial pattern, composed of *Finegoldia*, *Peptoniphilus*, *Campylobacter* or *Porphyromonas* genera, was associated with a cytokine profile characterized by IL-4 and IL-10 concentration (**Chapter I**). These cytokines are more related to a less pro-inflammatory and activated immune response [717,718]. Indeed, both IL-2 and IL-4 cytokine concentrations were observed as being elevated in the umbilical cord compared to maternal serum, suggesting a possible role in the development of the immune system or in its regulation [460]. Our results suggest that there exists a tight relationship between the *in utero* immune environment, blood clinical parameters and maternal microbiota near to the time of birth, independently of the delivery mode. Despite the fact that we conducted the analysis in a small population, which could limit the significance of the conclusions, this is one of the first studies to explore the possible effect of pro-inflammatory signals that occurred at the end of the gestation on maternal microbiota. As in the case of cortisol levels, our results open the door to the hypothesis that these concomitant physiological processes were, in part, associated with the observed differences in terms of gut microbiota according to delivery mode.

Furthermore, important questions appear as a result of the present study. Could these results be more than a simple consequence of the labour-associated process? Or

could the alterations in microbiota be contributing to the initiation of labour? Could those physiological processes play a role in modifying the microbiota composition to make it suitable for starting the colonization process? All these questions remain unclear and they compose a new field of study in the maternal–neonatal health research.

However, the studies focused on preterm birth (PTB) have provided valuable information that could shed light on the possible answers to those questions. Shifts in maternal microbiota, especially in vaginal [719] and oral [720] niches, have been related to the initiation of labour in early gestational age and several studies have associated the periodontal diseases with PTB [721,722]. Besides this, a study with approximately 12,000 samples described the existence of preterm-birth-associated taxa in vaginal microbiota that correlated with pro-inflammatory cytokines in amniotic fluid [723]. Hocevar et al. found that women who had undergone a PTB showed a vaginal microbiome with higher richness and diversity and a decreased relative abundance of *Lactobacillus* genera [724]. Thus, these results could be a signal for what could also be occurring in a non-pathological situation, suggesting that maternal microbiota could be participating along with the other body systems in the labour process.

Clarifying these aforementioned questions could impact not only on maternal health but also on infant growth through an influence on the colonization process. As we presented in the background literature section, maternal microbiota is the main bacterial source for the neonatal colonization initiation [37,49,153]. Several years ago, it was reported that meconium microbiota from neonates born by vaginal delivery showed a microbiota that resembled the maternal vagina, with the presence of *Lactobacillus*, *Prevotella* or *Sneathia* genera. However, those born by C-section showed a microbiota dominated by *Staphylococcus*, *Corynebacterium* and *Propionibacterium* genera, which are commonly found in maternal skin [130]. Since this work, other authors have reported differences in meconium microbiota according to delivery mode [163]. However, recent studies found that the effect of delivery mode on meconium microbiota could be limited [725]. It has been found that the meconium microbiota, foetal membranes and placenta bacterial profiles were very similar among them and not affected by delivery mode, suggesting that meconium microbiota could be more related to the *in utero* environment than the delivery period [725]. Regarding this, we found a slight effect of delivery mode on the first-pass neonatal microbiota, the place of delivery and associated factors being more relevant at birth, which suggested that the first-pass bacterial profile was influenced by more variables than only birth mode. As we

discussed in **Chapter V**, mothers who decided to have a home birth usually showed other associated aspects, such as rural environment, higher consumption of vegetables, less medicalized gestations, among others, which could be influencing our observations. In any case, we could not discard an effect of environmental microbiota in the initial neonatal seeding that we described in **Chapter V**, which reflected the differences between a hospitalized vs home delivery.

Despite the huge amount of knowledge about the effect of delivery mode in neonatal colonization, the influence of other environmental factors in this process are poorly understood. Our study is one of the first analyses focused on the influence of place of delivery on neonatal microbiota. Indeed, we described that at delivery time, the place of birth had even more influence than the mode of birth.

At the time of birth, hospital-born infants showed higher relative abundance of *Finegoldia*, *Clostridioides* and *Klebsiella* genera, while those born at home had a first-pass microbiota enriched in *Staphylococcus* and *Enterococcus* genera. Previously, intestinal microorganisms were found to be involved in the differences in the risk of asthma observed in vaginal home deliveries and C-section [231]. However, other authors found no influence of birthplace at 6 days of life [219]. Domínguez-Bello et al. showed that hospital-born infants had lower relative abundance of *Bacteroides*, *Streptococcus* and *Lactobacillus* genera while they showed higher relative abundance of *Clostridium* and the Enterobacteriaceae family [232].

In this regard, due to the associational studies that have related high-intervention deliveries with some immune-based diseases, as we presented above in this dissertation, public health organizations are making efforts to design hospital environments with fewer medical interventions that could disturb the maternal–infant contact [726]. However, the benefits that confer these practices are still under debate and our results could contribute to the body of evidence that show the possible influence of less intervened birth environments on the neonatal microbiota and health.

Molecular mechanisms beyond microbiota-host interactions in early life

Although the delivery mode effect on microbiota is commonly accepted by the research community, the results regarding the duration of this influence are much more contradictory [217]. In this context, while some studies reported differences in infant microbiota composition at 6 [71,219,222] and 12 months of life [223], other authors found no significant effects of delivery mode at 1 month post-delivery [37,218].

Whether the impact of delivery mode on neonatal microbiota was observable only during the first month or beyond, these microbial shifts occur in an extremely important period of infant development since they overlap in time with immune system maturation [668].

In **Chapter V** we described that in agreement with the mentioned studies, delivery mode significantly impacts the neonatal microbiota composition during the first month of life. Despite the great variability among the studies, some observations have been consistently found in the analysis of different cohorts, including the one studied in the present thesis [217]. Specifically, C-section-born neonates have a delayed Bacteroidetes colonization and an enrichment in *Klebisella* and *Clostridium* genera during the first month of life. In contrast, vaginal-delivered infants showed higher relative abundance of *Bacteroides* and *Bifidobacterium* genera [225]. Other authors have found similar results in other cohorts, which were reviewed by Rutayisire et al. [217]. Recently, our results have been confirmed in a large cohort with almost 600 children enrolled [225]. They also found higher relative abundance of *Klebsiella* and *Clostridium* genera in C-section-born infants.

The differences in these taxa are especially relevant since the immunomodulatory properties from both *Bifidobacterium* and *Bacteroides* genera have been widely reported [348,648]. Shifts in the colonization patterns of these genera would have consequences for immune system development [223]. Indeed, taxa from *Klebsiella* and *Clostridium* genera have been considered as opportunist pathogens more commonly observed in C-section-born infants. The authors conclude that the hospital environment was the main source of these microbes in infant colonization since those neonates showed a similar bacterial profile as those presented in operating rooms [727] and neonatal intensive care units [728]. Thus, the interruption of *Bacteroides* spp. transmission from mother to infant has been hypothesized to predispose the newborn to be colonized by hospitality taxa, such as the mentioned *Clostridium* and *Klebsiella* spp.

Furthermore, our results suggested that the observed differences in neonatal microbiota could be related to the immune system development. Faecal samples for children born by vaginal delivery, especially those from the home birth group, which was related with higher Bacteroidetes and Actinobacteria phyla, showed higher immunomodulatory capacity with higher capacity to activate monocyte-like cell culture. In our simulated gut epithelium system, the exposure to samples from home birth

neonates also caused higher epithelial membrane integrity measured through transepithelial electrical resistance (TEER) and Lucifer yellow transport.

Although the delivery mode effect on neonates has been widely described, the possible mechanisms related with this observation, which could also explain the associations of C-section with immune-based disease, are very scarce. Thus, our study is one of the first to propose a possible mechanism that links the C-section, the neonatal microbiome shifts and the described outcomes (**Fig. 5**).

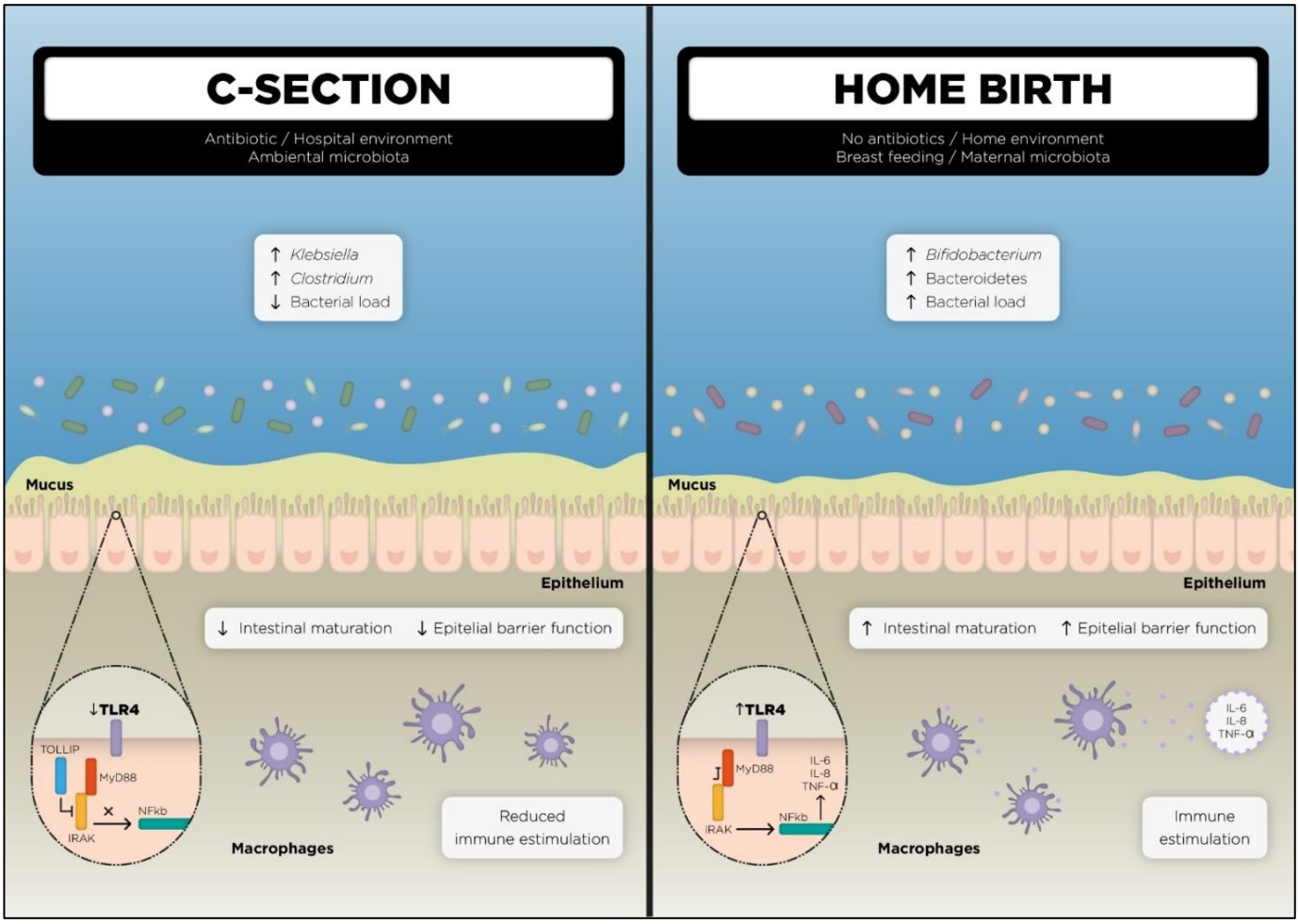


Figure 6. Proposed mechanism for the observed cellular response in the simulated gut epithelium system after exposure of neonatal faecal samples. The differences in microbiota composition and bacterial load according to mode and place of delivery would be related to shifts in intestinal epithelial maturation as well as immune system response during the first month of life. The exposure of faecal samples from homebirth infants in a simulated gut epithelium cellular model resulted in higher integrity of the epithelial membrane and higher immunostimulatory activity than those that were exposed to samples from C-section infants (own drawing).

In agreement with our results, similar results have been described in a study comparing vaginally delivered infants and those born by C-section. They found an over-representation of routes involved in LPS biosynthesis in microbiota from children born by vaginal delivery at 3 days after birth. Indeed, the LPS isolated from faecal samples from these children also showed higher immune activation capacity than those obtained from C-section-born infants' samples [358]. A higher expression of *TLR4* and *IL8* by the HT-29 cell line following exposure to sterile faecal supernatant from home birth infants when compared with hospital-born infants has been noted. However, they also found higher levels of *TGF-β*, which is mainly an anti-inflammatory cytokine, in hospital-born neonates [232].

Our observations along with Wampach results suggest that the differences in microbiota acquisition and maturation according to delivery mode could affect the the development of the immune response in early life, which could have consequences in adult health Although further similar studies are needed, our results would suggest that the immune activation could be essential for the adequate maturation of the immune system, which could be mediated by the early recognition of the microbial components during the microbiota acquisition process. As we mentioned in the literature background section, some authors suggest this lack of this recognition is the cause of the association between the colonization disrupters in early life and some diseases, constituting a reformulation of the hygiene hypothesis.

Indeed, the crucial role of antigen recognition by the innate immune system in the activation of the adaptative immunity is well known [729]. The inflammatory response increases the flow of lymphocytes to lymphoid tissues and antigen signals can activate them [730]. Furthermore, macrophages and dendritic cells also activate naive T-cell response, contributing to the immune memory [729]. Regarding this, lower memory T-cells and Tregs in adult mice born by C-section [627] have been found, which would support the hypothesis of the importance of this initial recognition in early life.

In this regard, an elegant study performed on three populations from Finland, Russia and Estonia described how the discrepancies in the LPS content of these three groups could be related to the differences in the incidence of allergy [361]. Children from Finland and Estonia showed higher relative abundance of *Bacteroides* genus and higher incidence of allergic diseases compared to the Russian population, which showed higher a proportion of *E. coli*-derived LPS. Furthermore, they described differences in

the immunogenicity of the LPS according to their bacterial origin, *E. coli* or from *Bacteroides* spp. mediated by their structural configurations. Thus, *Bacteroides* spp. failed in the activation of the immune system and the authors conclude that the early colonization by immunological silencing microbiota may be a key aspect of immune education and the cause of the differences in autoimmune diseases observed between the three populations.

The ability of *Bacteroides* spp.-derived LPS to silence the immune response through a signalling dependent on the TLR4 route have been confirmed by other studies [350]. However, these authors suggested that the attenuation of the immunogenicity of *Bacteroides* spp. LPS could be a mechanism to support host–microbiome tolerance and in some way facilitate the colonization process [350]. Also, it has been described that the polysaccharide A (PSA) from *Bacteroides fragilis* could induce the regulatory T cells to secrete anti-inflammatory cytokines, mainly IL-10, through an action by TLR2 in plasmacytoid dendritic cells [731]. Similarly, other authors have found that PSA could promote Treg expansion [732] and function [733]. Interestingly, in addition to the discussed higher immunostimulatory capacity of the home birth infants' faecal supernatants, they also showed a higher induction of the anti-inflammatory molecules expression (e.g. *IL10, TOLLIP*) to a higher extent than the CS samples, indicating a possible regulation of negative feedback on inflammatory signalling in the gut. Thus, while a complex equilibrium may exist in the early neonatal gut between the activation of the immune system to recognize the 'new' bacterial challenge and the establishment of a 'tolerant' environment, the mechanisms that rule this balance still need to be elucidated.

Our study revealed that the integrity of the gut epithelium could also be an aspect that participates in this process. While we have observed a higher immune response after exposure to the samples from home birth infants, we have also found a better maturation of our simulated gut epithelium compared to C-section samples. Indeed, we described the relevance of the epithelium-like cells in the protection of the system to an over-immune response in the simulated epithelium. In this sense, other authors have observed that one of the benefits of breastmilk feeding is the enhancement of the gut membrane closure in the neonates [656]. Furthermore, increased permeability has also been related to allergic diseases in non-breastfed children [657] and to other health disorders [658]. The newborn's digestive system has been described as an immature organ with some physiological mechanisms, such as mucus [734] and bile

acid production [735], pH and permeability regulation [736] are not fully developed. Microbial colonization could participate in the maturation of these processes. Thus, factors that could alter this maturation, such as place and delivery mode, could also affect them.

Although evidences in animal studies are available [35], the confirmation of some of these results in human studies needs to be further addressed. Moreover, studies based on human samples that propose molecular mechanisms are also essential to generate a body of evidence that could have clinically relevant data. In our study related in **Chapter V**, we designed a simulated gut epithelium system with the presence of an intestinal epithelium cell line, a mucus producer cell line and macrophage-like cells subjected to the same stimulus. Indeed, we performed a long-term exposure instead of a traditional acute exposure in order to better mimic the intestinal environment in the neonatal gut. We hypothesized that all these modifications would allow us to obtain more accurate results than those found using traditional approaches. Improvement in the design and standardization of protocols needed for these new tools for molecular mechanistic studies will provide a great advantage in the knowledge about the host–microbiome relationship.

Microbiota as an essential node in the infant development network during the first 1000 days

As we have shown in the chapters of the present thesis, microbial components of the human body, from both mothers and infants, could play a role in the regular development of the gestation, the delivery and the first month of life, with implications for maternal and neonatal health, including weight status and immune system response (**Fig. 6**).

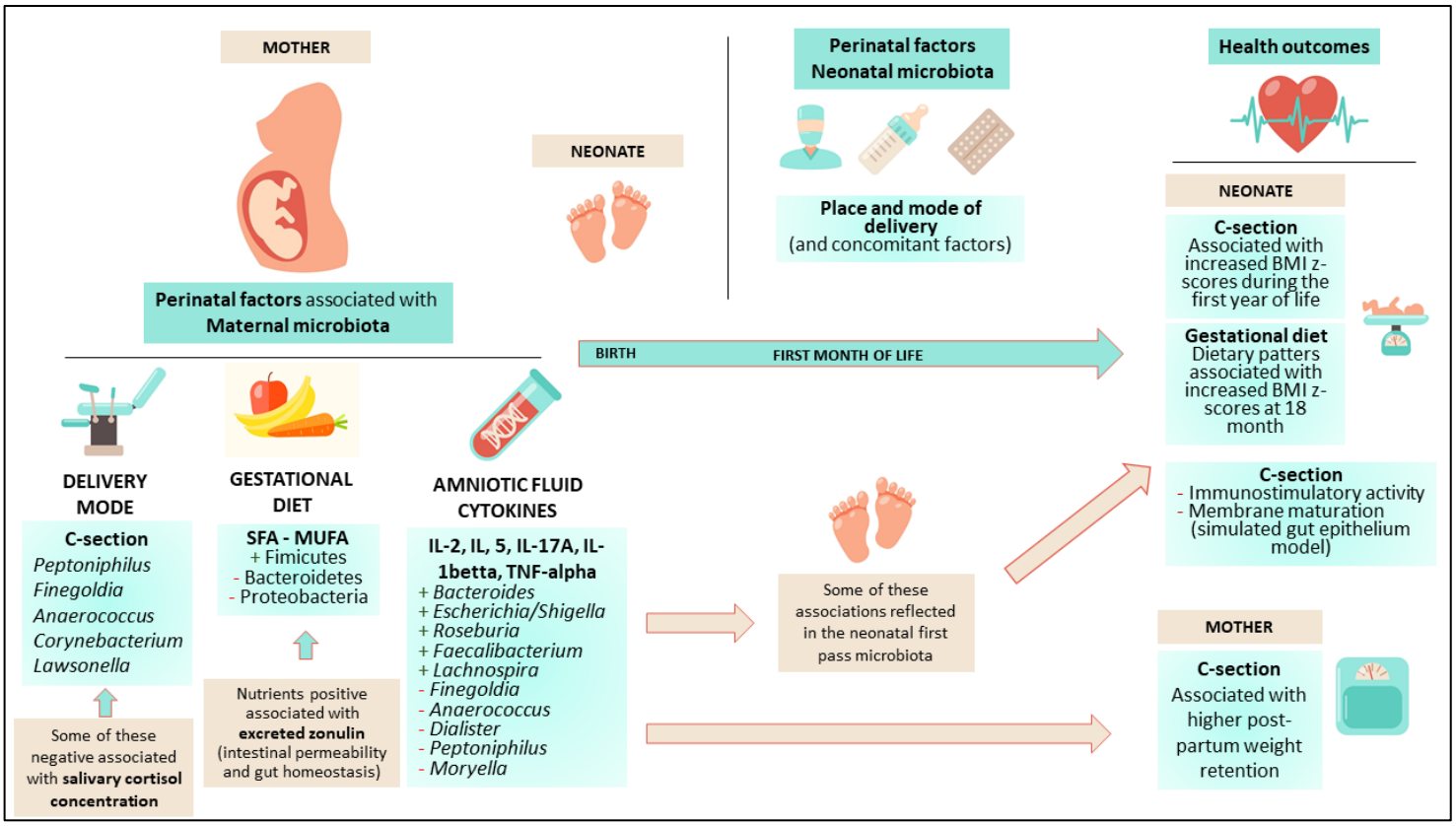


Figure 7. Summary of the main perinatal factors that affected maternal-neonatal microbiota during the first month of life and related health outcomes found in the present thesis (pictograms designed by macrovector / Freepik) (own drawing).

However, the influence of the microbiota on the maternal–neonatal field could be crucial even before the gestation period. Over the last years, interest in the possible impact of the preconception microbial environment has increased. Some studies have linked vaginal [737] and reproductive tract microbiota [738] with fertility problems and pregnancy complications [739–742]. Similarly, semen-microbiome studies have focused on the issue of infertility, highlighting differences in the microbial profiles composition and diversity [743–745]. A recent study reported different seminal microbiomes in patients with obstructive or non-obstructive azoospermia, thereby showing an increase in the Bacteroidetes and Firmicutes as well as a decrease in the Proteobacteria and Actinobacteria when compared to a healthy man [746].

During gestation, maternal microbiota could also be affecting the foetal immune system development. In animal studies, it has been shown that pups born to mothers treated with antibiotics during the gestation and post-partum period showed a reduced IFN-γ production by CD8+ T cells compared to controls [244]. Maternal TLR signalling during pregnancy has been reported to be required for prenatal asthma protection by

non-pathogenic microbes, showing the possible role of maternal microbiota in the immune system education during the foetal stage [693].

We have reported associations between maternal microbiota and the immune environment in the amniotic cavity, which could influence foetal development. Our study highlighted the importance of the maternal microbiota changes as a variable in the study of labour among hormonal, metabolic and immune adaptations necessary for this process. Indeed, we considered it crucial to perform analyses concerning the interconnection between these systems, the maternal microbiota, and infant development outcomes in order to ascertain their influence in maternal–neonatal health.

At the time of birth, we found that mothers who had undergone a vaginal delivery had differences in gut microbiota compared with those that had a planned C-section which showed higher post-partum weight retention than the VAG mothers. We hypothesized that the changes observed in microbiota according to delivery mode would be involved in the recovery of pre-gestational weight status during the post-partum period. With regard to this, it has been suggested that there is a differential effect of antibiotic treatment in infants, depending on the administered dose. Thus, high-dose antibiotics could cause important reductions in the microbiota population, which may be related to the weight loss observed in some studies [254]. However, lower antibiotic doses would cause microbiota composition shifts with low population size variation, triggering the weight gain shown in previously mentioned studies [70,83,99,100]. Thus, it remains to be discovered whether the proposed mechanism could be extended to other perinatal factors that also disrupt the microbiota composition and transmission.

The developing infant: microbial influence beyond birth

Similarly, it is unknown whether these differences would affect the infant's development. In this regard some studies have reported differences in the breastmilk composition according to maternal BMI [747]. Other authors have shown that for each 1 Kg/m^2 increase in pre-pregnancy BMI, there was a 4.5% increase in risk of obesity compared to offspring from normal BMI mothers [748]. Furthermore, maternal obesity in early gestation is associated with lower rates among women intending exclusive breastfeeding [749,750]. Thus, we hypothesized that maternal microbiota and its effect on maternal health during the post-partum period would be an essential aspect to explore in the study of infant development during early life.

Nyangahu et al. reviewed the possible mechanisms by which maternal microbiota during pregnancy would affect the infant immunity, including the influence on foetal microbiota, the direct effect on the immune system maturation, the bacterial metabolites and the epigenetic programming or through maternal IgG action [751].

Our results suggest that early interaction with commensal microbes could be crucial for healthy immune development and metabolic programming, and aberrant microbial neonatal colonization could be related to long-term effects on host metabolism or impaired immune development. In the study described in **Chapter V**, we reported that children from the HB group, whose microbiota was enriched in *Bifidobacterium* spp., showed higher BMI z-scores index at 18 months of life, suggesting that alterations in microbiota related to perinatal factors could affect weight development during childhood. Indeed, we discussed that this observation could be related not only to the birthplace, but also co-dominant variables, such as maternal contact, no hospital environment, and no antibiotic treatment. Similarly to the results found in mothers, it is reasonable to discuss the hypothesis about the effect of low doses of antibiotics on the weight gain in those children born by C-section. Indeed, we observed that maternal diet could also be associated with the infant's BMI development over the first 18 months of life. However, further studies are needed in order to confirm the results obtained in our population and propose molecular mechanisms related to these observations.

As we have discussed throughout the present thesis, our results would support the hypothesis that the microbial acquisition and colonization during the first month of life participate in the developmental origin of the health and disease hypothesis [72] through an influence in the immune system's development.

During the studies in the present thesis, we have shown that blood cholesterol and triglyceride levels (in non-pathological ranges), lower concentrations of anti-inflammatory signals and higher salivary maternal cortisol were related to a bacterial profile less associated with C-section delivery. Indeed, higher immunostimulatory responses were found after exposure of samples from vaginal-delivered infants, especially those born at home and with a specific bacterial colonization profile. Thus, in accordance with other authors [358,693], we highlight that the relation between maternal–neonatal microbiota and the immune stimulation during labour and the first weeks of life may be essential in the correct neonatal development and immune system maturation. Further studies that explore this hypothesis in more depth are needed in

order to clarify the relationship between these two systems and possible mechanisms involved in this relation.

Strategies of microbiota restoration after a disrupting event

Our study and similar works that we have discussed, open the door to the design of strategies that could invert the possible shifts caused by perinatal factors during pregnancy and early life. Traditionally, the majority of approaches in this field have used pro and prebiotics. Thus, the administration of probiotics has been proposed during gestation to reduce the risk of preterm birth [752–754], the pathogenic gestational cavity [755,756] and urogenital infection [757–759]. They have also been studied to reduce the incidence of some diseases, such as preeclampsia or gestational diabetes mellitus (GDM), or as concomitant measure along with the treatment of these diseases [753,760–763]

On the part of infant health, the main targets of probiotics intake have been the prevention of diarrhoea and infection [764–766], allergy [767–769], necrotizing enterocolitis (NEC) and late-onset sepsis (LOS) in preterm neonates [770,771] as well as infant colic [772,773]. All of these studies have focused on the prevention or treatment of diseases; however, as we have shown in the present study, there are perinatal factors that could also be related to alterations in microbial composition from both maternal and neonatal microbiota. It could be possible to design strategies to restore microbial shifts related to these perinatal factors, including diet, place or mode of birth or antibiotic use during pregnancy. The greatest difficulty in addressing this question would be to find the indicators that show the microbiota have been restored and to identify those signals that point to actual and measurable benefits for maternal and infant health.

Our study reported that mothers who had undergone a vaginal delivery showed a lower rate of post-partum weight retention (PPWR) than those that had had a C-section, with a possible influence of the differences observed in maternal gut microbiota at delivery (**Chapter II**). Although further studies are needed in order to confirm our results, it is reasonable to think that the design of strategies for mimicking the vaginal delivery-associated bacterial profile in those mothers who undergo a C-section could contribute to maternal post-partum weight status, with possible consequences in maternal and even in infant health.

Thus, dietary counselling may be especially relevant in those mothers who need a planned C-section due to clinical conditions [774,775]. Although probiotic use could provide an extra benefit, diet is one of the main drivers of the microbiota composition and dietary counselling could contribute to a greater extent to the microbial restoration after the birth [776]. However, it remains to be established which foods, food components, nutrients and other dietary compounds affect the microbiota during the perinatal period [575]. Not only nutrients but also the effects of differences in modes of food processing have been studied in terms of gut microbiota variations and also host energy status [777]. Thermal processing of foods reduced gut microbiota diversity and triggered the adaptation of the bacterial community to the thermal processed food [778]. Similarly, the impact on human microbiota of eating behaviours and time of food intake have recently been receiving more attention [779,780]. Our results on the effect of perinatal factors on maternal microbiota along with these kinds of studies could contribute to develop personalized strategies to modulate maternal and neonatal microbiota, which could lead to personalized medicine in the future.

Taken together, these observations explain the increasing research interest in perinatal dietary interventions, as well as in the use of probiotics, prebiotics and symbiotics during pregnancy in order to modulate the microbiota and promote a 'favourable microbial vertical transmission'.

Beyond the probiotic approach, other methodologies have been recently proposed to overcome the effect of delivery mode, mainly with regard to infant health. Domínguez-Bello et al. reported a partial restoration of the microbiota from children born by C-section. In their study, the neonates were exposed to their maternal vaginal fluids at birth by swabbing the neonate within 2 minutes post-delivery with a sterile gauze that had been previously incubated in the vagina of the mothers [215]. The results revealed that in the exposed infant's microbiota, the proportion of maternal skin taxa were reduced and those from the maternal vagina were increased compared to non-exposed C-section born infants [215]. However, this was a pilot study and the small sample size (n=4 exposed neonates) does not enable the confirmation of the hypothesis of restoration. Indeed, the possible benefit of this methodology has not been confirmed since analyses made in larger cohorts and with the study of health outcomes are still not available.

Although more studies focused on this methodology are needed, this could be one of the first integrative strategies for restoring neonatal microbiota after a C-section.

However, as we showed (**Chapter I–II**), the differences in neonatal microbiota according to delivery mode could also be influenced by those differences in maternal microbiota even before delivery, probably associated with the hormonal [522], metabolic [781] and immune [459] processes necessary for labour in the spontaneous vaginal delivery. Thus, approaches targeting maternal microbiota would also be essential to produce a complete restoration of neonatal microbiota. In agreement with that, it has been suggested that the lack of vaginal exposure would not be the unique factor responsible of the alterations in C-section-born infants' microbiota. Other variables such as the intrapartum antibiotic intake, the absence of labour signal or even the differences in breastfeeding behaviours usually observed in those children need to be considered in order to produce a possible real benefit for infant and human health [782].

Future perspectives

Although the last decades have seen an explosion of knowledge concerning the importance of microbiota for maternal and infant health, a lot of questions remain unclear and some aspects need to be further addressed.

Regarding this, there is still room for improvement concerning technical and analytical protocols in the human microbiome data acquisition and bioinformatical handling [783]. Despite some consistent results in the field, most of the microbiome studies showed a huge amount of variability between them and, sometimes, contradictory results [784]. Indeed, some authors have shown that differences in sample collection and storage protocols [785], DNA extraction methods and commercial kits [786,787], and sequencing methods [788], which could be partially responsible for introducing bias into the results. Some authors have found that the inclusion of a bead-beating step was linked to a higher DNA yield and more efficient DNA extraction from Gram-positive bacteria [789].

Furthermore, the rapid development of the NGS technologies has prompted the study of human microbiome, and their diminishing costs have made them available to a wide range of laboratories worldwide. However, a standardized pipeline for data generation and analyses is required to reduce the variability among studies. Multiple bioinformatical tools for microbiome analysis are available, including different 16S database [790], OTU or ASV clustering methods [387,389], functional profiles

predictors [639] and tools for metagenome assembly [791] and all the possible combinations among them. Although shotgun sequencing has been proposed as an approach to improve the quality of the results obtained by other methodologies [792], the increment in the cost as well as the large amount of data generated requires specialized analysis pipelines to efficiently detect the relevant information. The combination of all these different approaches for microbiome analysis would contribute to the variability between the studies and the lack of consistent data that has led to relevant clinical recommendations (**Fig. 7**). Thus, standardized procedures and guidelines are necessary for reproducibility across microbiome studies [784].

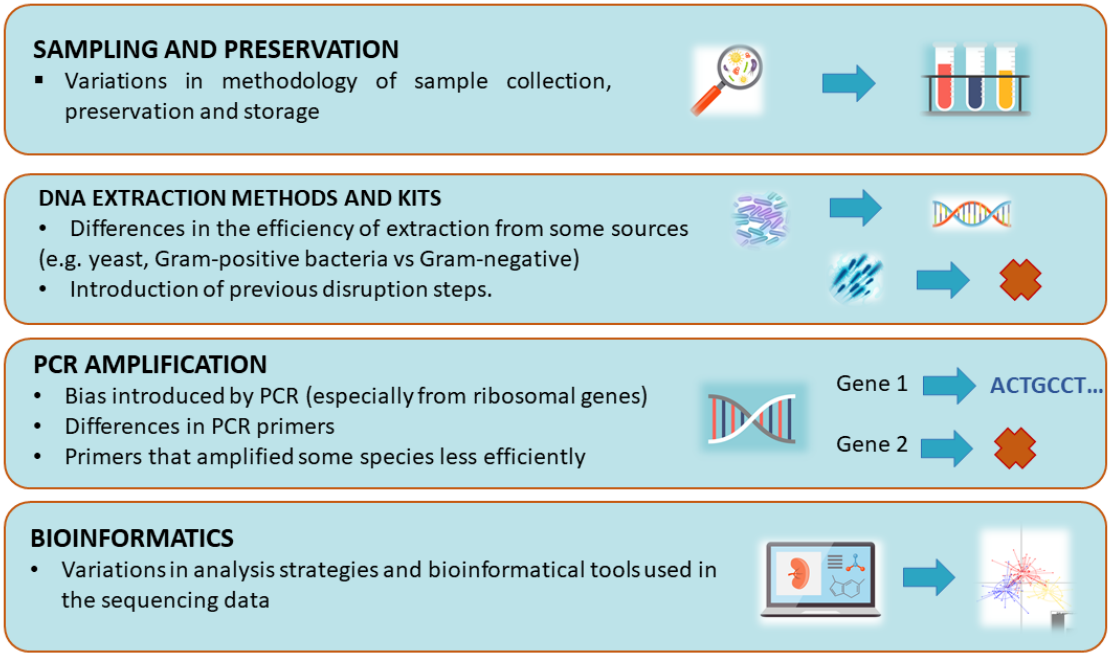


Figure 8. Sources of variability among microbiome studies through differences in technical and analytical procedures (own drawing with data from [793] with permission, pictograms designed by macrovector/ Freepik).

The improvement and development of these bioinformatical tools that lead to a deeper analysis of the microbial taxa relations in the community and with the host are essential for progress in microbiota study. This would be extremely important in the maternal–neonatal microbiome field since some of the targeting niches showed a low-biomass environment [794] and the discrimination between contamination and genetic material from the goal sample is essential to finding significant conclusions. In this regard, one of the most discussed questions in the microbiome field is the existence of a placenta and/or an amniotic cavity microbiome, which would suggest an *in utero*

colonization [568]. While some authors have found evidences of its existence [160], others have not observed differences between the microbial content of these niches and sequencing controls [166,795]. Thus, the handling of extraction and sequencing controls are crucial in these kinds of studies. Recently, the inclusion of a mock community control composed of predetermined ratios of DNA from a mixture of bacterial species has been proposed [796], which would allow the quantification of sequencing error and the bias introduced during sample processing [797]. In 2019, Hornung et al. reviewed the issues and current standards of control management in microbiome analysis [178].

In recent years, bioinformatical tools have been developed to manage this issue [408]; however, as in the case of data generation, a well-defined technical protocol and bioinformatical pipeline to contaminant handling is still not available. In this regard, the available literature has been reviewed and general recommendations suggested for 16S rRNA gene studies, from sample preparation to data analysis [797]. An exhaustive literature review and experimental planification are needed before a microbiome study in order to avoid some of these possible issues.

In addition to the technical aspects, the research community is demanding a change of paradigm from correlation to causation in microbiome studies. In recent years, plenty of works have described the associations between microbial shifts, environmental factors, and an increased risk of some diseases [90–94]. However, available data about the molecular mechanisms behind these observations are still scarce. Intense effort has been made to develop methodologies that provide more information in this field, such as the germ-free mice experiments that enable the transfer of the whole gut microbiome or specific components to study the cause–effect relation [65,180,335,341]. Thus, germ-free animal studies have provided valuable information in the field. The presence of specific microbial populations in the intestine has been correlated to the differentiation of the Th17 cells also influencing the T regs repertory in the mice [798], which was also confirmed by others [799]. Host–commensal interaction has been shown to be critical for the generation of inflammatory response since microbiota promoted the TLR expression, which was essential for TLR-responsive monocytes [455].

In recent years, the development of new methodologies for *in vitro* mechanistic analysis has offered new opportunities in the field of host–microbiota interactions, including the gut-on-a-chip system [800], the human enteroids [801] and the co-culturing in Transwell systems [802]. Contrary to the traditional *in vitro* experiments,

these new approaches permit the reproduction of more realistic situations, with the inclusion of different cell lines, such as epithelial or immune cells, in the same experiment [803]. Indeed, they allow researchers to move from animal studies to molecular mechanistic analysis in human cell lines. Besides the independent mechanistic or the observational analysis of each individual factor that has arisen during this last decade, we consider it essential to move the research to a more holistic molecular perspective, based on the interconnection of the data compared to the traditional approaches. The omics era has provided the opportunity to generate a high amount of data from several aspects of a system, including genome [804], microbiome [805], metabolome [404], transcriptome [806], epigenome [807] and proteome [406] from both animal models and human studies.

However, all these methodologies showed inherent data differences, and integrating multiple omics platforms remains a challenge for most researchers [808]. In this regard, systems biology has been proposed as the approach that could better contribute to the advance in microbiota–host interaction knowledge [809]. Systems biology is an interdisciplinary research field that has the aim of unravelling the complexity of a biological system through an integration of multiple types of quantitative molecular measurements [810,811]. This discipline could help to design a complete overview of several host–microbiome interactions and facilitate the advance in the understanding of the alterations in those relations that could be triggered in the development of disease [809] or even during the immune system–microbiome maturation period. Mechanistic and phenomenological models that could provide a predictive understanding of the microbiome's dynamics are an especially promising route [812].

Likewise, for that purpose, the collaboration between researchers of those different techniques will be crucial for the development of a truly useful system biology-based microbiome network, which will open the door to new methodological and technical approaches that offer a complete view of the microbiome maturation impact on the human health.

CONCLUSIONS

CONCLUSIONS

Based on the findings of this thesis, the following conclusions can be drawn.

1. The maternal microbiota was associated with both the immune status of the amniotic cavity and placental metabolic gene expression, with possible consequences in relation to neonatal microbial colonisation at the time of delivery.IL-2, TNF-alpha, IL-1beta, IL-5 and IL-17A cytokines in amniotic fluid were positive associated with *Faecalibacterium*, *Roseburia* and *Lachnospira* genera in maternal microbiota at delivery time and negatively related to *Peptoniphilus*, *Corynebacterium*, *Porphyromonas*, *Prevotella* or *Finegoldia* genera. The placental mRNA expression of the glucose transporter GLUT3 and the glutathione peroxidase (GPX) enzyme was positively correlated with several Ruminococcaceae groups as well as with the *Murdochiella* and *Ezakiella* genera.
2. Both the mode of delivery and the codominant hormonal changes (salivary cortisol) could influence the maternal microbiota at the time of delivery and also possibly impact maternal weight recovery during the post-partum period.
3. The maternal microbial clusters modulated the effect of the mode of delivery on the maternal microbiota. Moreover, the salivary cortisol concentration was significantly higher in those mothers who underwent a VAG delivery, and it was negatively associated with those genera related to a C-section birth found in the maternal microbiota.
4. The maternal diet during pregnancy, mainly the fat intake (SFA and MUFA), was related to intestinal homeostasis, meaning that it likely altered the maternal microbiota and transmission to the neonate. Higher fat-related nutrient intakes were associated with excreted intestinal zonulin and Firmicutes spp. and with a lower relative abundance of Proteobacteria spp. in the maternal microbiota.
5. Several associations were identified between the composition and diversity of the neonatal microbiota and maternal diet during gestation. SFA and proteins derived from animal sources were positively correlated with the richness of the neonatal microbiota, as measured using the Chao index. It was observed that the fat-related nutrient intake of the mother, including the total lipids, SFA and MUFA, but not PUFA, resulted in the enrichment of the Firmicutes phylum

genera as well as the depletion of the Proteobacteria phylum genera in the offspring's microbiota. This effect of the maternal diet was observed to differ according to the delivery mode.

6. The maternal microbiota could be grouped into two distinct microbiota clusters characterised by either the *Prevotella* (Cluster I) or the *Ruminococcus* (Cluster II) genera. Higher intakes of total dietary fibre, omega-3 fatty acids and polyphenols were observed in Cluster II when compared with Cluster I. Higher intakes of plant-derived components were associated with a greater presence of the Christensellaceae family, *Dehalobacterium* and *Eubacterium* as well as with lower amounts of the *Dialister* and *Campylobacter* species.
7. The longitudinal BMI and WL z-score trajectories from birth to 18 months of life were shaped by the maternal microbial cluster, maternal diet and mode of birth. The neonates born via C-section from Cluster I showed the highest BMI z-scores at the age of 18 months, along with a higher risk of being overweight.
8. The mode and place of birth influenced the neonatal gut microbiota, likely shaping its interplay with the host through the maturation of the intestinal epithelium, the regulation of the intestinal epithelial barrier and the control of the innate immune system during early life, which can all affect the phenotypic responses linked to metabolic processes in infants.
9. The Bacteroidetes spp. and *Bifidobacterium* genus were found to be decreased in the infants born via C-section, who showed higher BMI and WL z-scores during the first 18 months of life. In fact, stronger epithelial barrier function and intestinal maturation, in addition to a higher immunological response (both TLR4 route activation and pro-inflammatory cytokine release), were noted in those intestinal simulated epithelium which were exposed to home-birth faecal supernatants when compared with infants born via C-section.

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