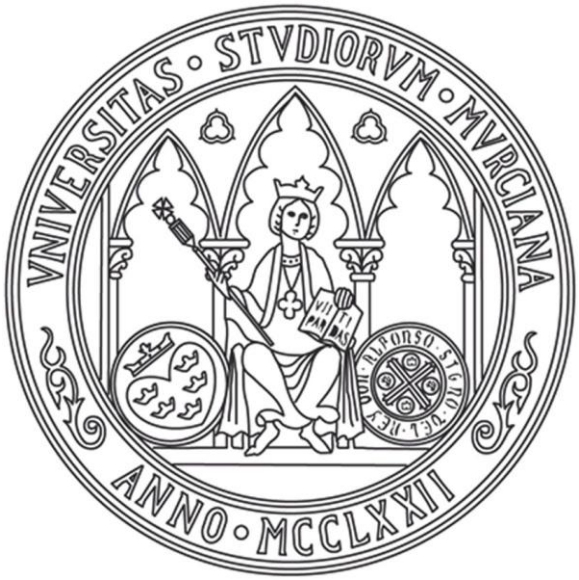

UNIVERSIDAD DE MURCIA

ESCUELA INTERNACIONAL DE DOCTORADO

Adherence to the Mediterranean diet in pregnant women from the NELA cohort (Nutrition in Early Life and Asthma). Influence on the microbiota of infants and the early onset of possible asthma precursor symptoms.

Adherencia a la dieta Mediterránea en gestantes de la cohorte NELA (Nutrition in Early Life and Asthma). Influencia en la microbiota de los lactantes y en la aparición de posibles síntomas precursores de asma.

Dña. Clara Suárez Martínez

2021

Adherence to the Mediterranean diet in pregnant women from the NELA cohort (Nutrition in Early Life and Asthma). Influence on the microbiota of infants and the early onset of possible asthma precursor symptoms.

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2021

This research is inserted into the study of the cohort “Nutrition in Early Life and Asthma (NELA)” and has been funded by different projects and fellowships:

- The excellence project named “Unravelling in utero determinants predicting lung function in infants: a step for prenatal prevention of Asthma” (PIE15/00051) funded by the Instituto de Salud Carlos III and with Dr. Luis García-Marcos as principal investigator.

- The project ”Mediterranean diet during pregnancy, antioxidant defence, lung function, asthma and allergies in pregnant women and their offspring: a cohort study” (CP14 / 00046) founded as well by the Instituto de Salud Carlos III and with Dr. Eva Morales Bartolomé as principal investigator.

- Two Miguel Servet Fellowships (MS14/00046 and CPII19/00019) awarded by the Instituto de Salud Carlos III (ISCIII), Spanish Ministry of Science, Innovation and Universities, and Fondos FEDER.

- The Project “Impacto de la exposición in útero a contaminación atmosférica sobre la función inmune al nacimiento: identificación de mecanismos epigenéticos” (PI16/00422) founded by the Instituto de Salud Carlos III and with Dr. Eva Morales Bartolomé as principal investigator.

- The project “Effect of anemia during pregnancy and breast feeding on microbiota of mother-chIld binomial in NELA cohort” (PID2019-106693RB-100/AEI/10.13039/501100011033)) founded by the Ministry of Science, Innovation and University of the Government of Spain and Dr. Carmen Martínez Graciá as principal investigator.

I would like to acknowledge the economic opportunity of the PhD grant (FPU MECD 15/05809) given by Ministry of Education, Culture and Sport of the Government of Spain.

I would like to thank to ERASMUS PLUS and EIDUM (2018/2019) for the economic support which made possible my internship in a foreign research centre (Food Research Centre, TEAGASC MOOREPARK, Fermoy, Cork, Ireland).

Finally, I want to particularly acknowledge the patients and the BioBank “Biobanco en Red de la Region de Murcia” (PT17/0015/0038) integrated in the Spanish National Biobanks Network (B.000859) for its collaboration.

List of contents

Extended Summary

Asthma is the most common chronic disease in children, causing great morbidity (www.Globalasthmareport.org). At least one in three infants has asthma-like symptoms 3 or more times during the first year of life. This condition is a source of costs to the health care system estimated at € 20 million per year from emergency room visits alone. Although many infants present wheezes early and are able to outgrow this condition by the age of 6 or 7 years, the prevalence of asthma is 10 % among school-aged children and adolescents in Spain. Although it is not a major cause of death, neither in childhood nor in adulthood, asthma is the leading cause of disability during the 5-9 years period and the second leading cause during early adolescence (10-14 years) in developed countries.

In humans, lung development takes place during the 2nd and 3rd trimester of pregnancy and continues until at least the age of 7 years, when alveolarization is complete. During the early stages of growth and development in utero, the lungs can be vulnerable to any environmental aggression, which can cause permanent changes throughout the course of a person's life. Although a long list of risk and protective factors for asthma in children have been described, few have been studied about their ability to interact with the foetal environment, and in particular how they may affect lung development in utero.

If the early programming hypothesis extends to asthma, it seems reasonable to think that the interaction of the foetus with the environment in utero and after birth with the outdoor environment during the first weeks of life could be crucial in the development of the lungs and programming towards those respiratory conditions. Knowledge of the changes brought about by the interaction of the mother (and therefore the foetus) with the environment is probably the best way to understand the mechanisms involved in early programming. Similarly, the interaction of the infant with the environment in the early stages of life is of enormous importance.

Pregnant women can influence the intrauterine environment through interaction with their own physical environment, primarily through diet. Some food intake patterns such as a pattern of low adherence to the Mediterranean diet by

the mother during pregnancy have been associated with asthma in her children in school years. But other studies have also been found in which this effect has not been observed. That is why one of the main objectives of this thesis is to characterise the adherence to the Mediterranean diet of mothers belonging to the NELA cohort and to study whether low adherence to this dietary pattern is associated with early onset of asthma precursor symptoms in offspring, such as wheezing, atopic skin, dry cough, bronchitis and/or bronchiolitis, at 3 months of age.

On the other hand, the so-called "hygiene hypothesis" indicates that allergic diseases are probably associated with a cleaner environment and that the lack of contact of the immune system with bacteria tends to shift the immune system to the Th2 side. Antibiotic use in pregnancy or delivery by caesarean section or type of breastfeeding appear to be risk factors for asthma in offspring and it is hypothesised that this effect is mediated by disruption of proper colonisation of the new-born’s microbiota. The gastrointestinal tract is the major source of exposure to microbes. Several studies in recent years have demonstrated differences in the composition of the gut microbiota in infants exposed to different conditions before, during and shortly after birth. A number of diverse maternal factors are responsible for the creation and colonisation of the infant's microbiota. Differences in microbial diversity have been associated with the development of allergies and/or asthma over time. Therefore, another aim of the thesis is to characterise the intestinal microbiota of infants and their fatty acid profile at 3 months of age and to determine the influence of prenatal and neonatal factors (microbiota and short-chain fatty acids) on the early onset of asthma precursor symptoms in 3-month-old infants.

Firstly, **the state of the art** of this doctoral thesis has been described, which can be subdivided into 3 sections. The first one reports how the whole process of transmission, colonisation and microbial evolution takes place during the first months of life of the neonate. Regarding the transmission process, there are currently different theories among the scientific community, on the one hand there is the theory that the intrauterine environment is a sterile environment and that the first colonisation occurs during the birth process and on the other hand, several authors have studied the microbiota of the meconium, finding

microorganisms and therefore considering that the intrauterine environment is not sterile and that during pregnancy there must be a transmission of bacteria from the mother to the newborn through the placenta or the amniotic fluid. The second part of this literature review summarises the different prenatal, perinatal and postnatal factors involved in the correct colonisation and development of the intestinal microbiota of infants during the first months of life. Finally, the third section aims to provide the reader with an overview of asthma as a disease and the possible relationship between the early onset of asthma and the gut microbiota of children. It also considers other factors that may be involved in this relationship, such as the type of birth, antibiotic use, type of breastfeeding, etc. The main conclusion of this review is that, although there is no consensus on whether there is prenatal colonisation through microbial transmission between mother and child, there is a certain consensus on the main factors that may influence the transmission and subsequent colonisation of the infant's intestinal microbiota, with the type of birth, the use of antibiotics and the type of feeding during the first months of life being the 3 main modulating factors. Another conclusion is that childhood asthma is a disease with a prevalence that has been increasing in recent decades and its diagnosis is difficult due to the wide range of associated symptoms and the variability in the age at which it is diagnosed. As a consequence, there is no single criterion within the scientific community when it comes to establishing the definition of an asthmatic child in the different studies carried out to date, which may be the cause of the great discrepancy in the results obtained in relation to whether there is any association between the profile of the intestinal microbiota of the infant and asthma in infancy.

Next part is a **general material and methods** section describing in detail the recruitment of the NELA cohort from which this thesis is derived and which is the basis for the information and samples of the studies carried out. Specifically, a description of its design, stages, objectives, the study population, the inclusion criteria and the collection of data and samples are described. On the other hand, a first indication is made of the selection of a sub-sample of 200 mother-child pairs that was carried out for a case-control study based on whether the infants presented episodes of wheezing, eczema, dermatitis, bronchitis and/or bronchitis

at 3 months of age. Both populations will be used in the different studies that make up this doctoral thesis.

The rest of the methodologies and materials used during the different studies carried out in the doctoral thesis are described in each of the 2 chapters that make up the section on the **research studies derived from the doctoral thesis**, which are described below.

Specifically, in **chapter 1**, the main objective was to establish whether a healthy maternal diet during pregnancy, such as adherence to a Mediterranean dietary pattern, is associated with a lower occurrence of asthma precursor symptoms in infants at 3 months of age. In order to achieve this objective, this chapter has been subdivided into 3 studies.

Study I characterised adherence to the Mediterranean diet and to a healthy dietary pattern by mothers belonging to the NELA cohort, using 3 dietary indices and comparing them with each other, as well as a study of the possible socio-demographic and lifestyle factors that influence the degree of adherence to this dietary pattern. The main conclusion of this study was that pregnant women who are younger, have fewer previous births, have a low level of education and practice unhealthy lifestyles are more likely to have a low adherence to the Mediterranean diet and a healthy dietary pattern.

Study II deepens the degree of adherence to the Mediterranean diet in pregnant women and its association with the early onset of asthma precursor symptoms in infants at 3 months of age. The conclusion drawn from this study is that no differences were observed in the dietary patterns of mothers whose children were classified as cases or controls, nor was an association observed between a low degree of adherence to the Mediterranean diet and an increased risk of the appearance of precursor symptoms of asthma in offspring. As a final conclusion, we believe it is necessary to establish or create a single index of adherence to the Mediterranean diet specific to pregnant women, thus reducing the heterogeneity of future research.

After carrying out this second study, the need arose to further research the diet of pregnant women and its relationship with the symptoms indicated above, this time focusing on food groups or nutrients that make up the diet such as

vegetables, fruits, legumes, pastries, etc. **Study III** was carried out for this purpose. We can conclude that a higher consumption of pastries and sweets by pregnant women is associated with a higher risk of their offspring developing precursor symptoms of asthma at an early age. However, a high consumption of coffee, tea and infusions during pregnancy, probably due to their antioxidant nature, is associated with a lower risk of early onset of asthma precursor symptoms in the offspring.

Having done all of the above with the aim of studying the prenatal, perinatal and postnatal factors that may influence the infant microbiota and its metabolites, as well as their association with the early onset of asthma precursor symptoms at 3 months of age, **chapter 2** was conducted, This chapter comprises a single pilot case-control study with the 200 mother-child pairs selected on the basis of whether or not the infants had presented symptoms such as wheezing, eczema, dermatitis, dry cough, bronchitis and/or bronchiolitis at 3 months of age. In this work, on the one hand, a characterisation of certain bacterial species and groups present in the faeces of infants at 3 months of age, most of them classified by the scientific community as beneficial bacteria for health, was carried out using quantitative PCR. On the other hand, short-chain fatty acids present in the faeces of infants at 3 months of age were analysed and quantified by gas chromatography. Once the data were obtained, a comparison was made between the control group and the case group. Subsequently, several bivariate and multivariate statistical analyses were performed to observe the influence of different prenatal, perinatal and postnatal factors on infant gut microbiota and its metabolites. Finally, using logistic regression models, the possible relationship between infants' gut microbiota and their fatty acid profile, and the occurrence of asthma precursor symptoms in infants was studied. We found differences in the gut microbiota of infants between the control group and the case group, as well as in the molar proportions of the main major short-chain fatty acids. Specifically, the gut microbiota of infants belonging to the case group was characterised by lower counts of *Atopobium, Bacteroides, B. breve, B. longum, Clostridium cluster XIVa* and *cluster IVa* and *Clostridium difficile*, and higher counts of *Akkermansia muciniphila* compared to controls. It could also be concluded that the faeces of infants classified as cases had higher molar proportions of acetic acid and lower

proportions of butyric and propionic acid, both also related to beneficial health effects. Another conclusion was that higher counts of *Akkermansia muciniphila* and *B. breve*, and lower counts of *Bacteroides-Prevotella, Clostridium cluster IVa* and *cluster XIVa*, and *B. longum*, were associated with an increased likelihood of developing asthma precursor symptoms at 3 months of age. In relation to the major short-chain fatty acids and their possible association with early onset of asthma precursor symptoms in infants, a lower concentration of butyric acid in faeces at 3 months of age was associated with an increased likelihood of onset of these symptoms.

Resumen extendido

El desarrollo y función pulmonar en el ser humano se ha relacionado con la exposición a diferentes factores tanto prenatales, en el medio ambiente del útero materno, como en las primeras semanas de vida, siendo éstos fundamentales en la aparición de determinadas enfermedades como el asma.

El asma es la enfermedad crónica más frecuente en niños, causando una gran morbilidad (www.Globalasthmareport.org). Al menos uno de cada tres lactantes tiene durante el primer año de vida tres o más episodios de síntomas respiratorios similares al asma. Esta condición es una importante fuente de gastos para el Sistema de Salud estimada, tan sólo contabilizando las visitas a los Servicios de Urgencias, en 20 M € al año. Aunque muchos lactantes padecen sibilancias tempranas que desaparecen a la edad de 6 o 7 años, la prevalencia de asma es del 10 % entre los niños en edad escolar y adolescentes en España. A pesar de que no es una causa importante de muerte, ni en la infancia ni en la edad adulta, el asma es la principal causa de discapacidad durante el periodo comprendido entre los 5 y 9 años y la segunda durante la adolescencia temprana (10- 14 años) en países desarrollados.

En los seres humanos, el desarrollo pulmonar tiene lugar durante el 2º y 3º trimestre del embarazo y continúa al menos hasta la edad de 7 años, cuando la alveolarización es completa. Durante las primeras etapas de crecimiento y desarrollo en el útero, los pulmones pueden ser vulnerables a cualquier agresión del ambiente, lo que puede causar cambios permanentes a lo largo del curso de la vida de la persona. Aunque se han descrito una larga lista de factores de riesgo y de protección para el desarrollo del asma en niños, pocos han sido estudiados acerca de su capacidad para interactuar con el medio ambiente fetal, y en particular la forma en que pueden afectar el desarrollo pulmonar en el útero.

Si la hipótesis de la programación temprana se extiende para el asma, parece razonable pensar que la interacción del feto con el medio ambiente en el útero y después del parto con el medio ambiente al aire libre durante las primeras semanas de vida, podría ser crucial en el desarrollo de los pulmones y de la programación hacia aquellas afecciones respiratorias. El conocimiento de los cambios producidos por la interacción de la madre (y por consiguiente del feto)

con el medio ambiente es probablemente la mejor forma de comprender los mecanismos que intervienen en la programación temprana. Del mismo modo, la interacción del bebé con el medio ambiente en las primeras etapas de la vida es de enorme importancia.

Las mujeres embarazadas pueden influir en el medio ambiente intrauterino a través de la interacción con su propio medio físico, principalmente a través de la respiración y de su dieta. Algunos patrones de ingesta de alimentos, como un patrón de baja adherencia a la dieta Mediterránea por parte de la madre durante el embarazo se han asociado con el asma en sus hijos en años escolares. Pero también se han encontrado otros estudios en los que este efecto no ha sido observado. Por ello uno de los principales objetivos de este proyecto es caracterizar la adherencia a la dieta Mediterránea de las madres pertenecientes a la cohorte NELA y estudiar si una baja adherencia a este patrón dietético está asociada con la aparición temprana de síntomas precursores de asma en la descendencia, como pueden ser las sibilancias, piel atópica, tos seca, bronquitis y/ o bronquiolitis, a los 3 meses de edad.

Por otro lado, la llamada "hipótesis de la higiene" indica que las enfermedades alérgicas están probablemente asociadas con un medio ambiente excesivamente limpio y que la falta de contacto del sistema inmunológico con bacterias tiende a desplazar el sistema inmunológico hacia el lado Th2. El uso de antibióticos en el embarazo, parto mediante cesárea o el tipo de lactancia parecen ser factores de riesgo para el desarrollo de asma en la descendencia y se formula la hipótesis de que este efecto está mediado por la interrupción de una correcta colonización de la microbiota del recién nacido. El tracto gastrointestinal es la mayor fuente de exposición a microbios. Varios estudios realizados en los últimos años han demostrado diferencias en la composición de la microbiota intestinal en los niños que están expuestos a diferentes condiciones antes, durante y poco después de nacer. Un número diverso de factores maternos son responsables de la creación y la colonización de la microbiota del lactante. Las diferencias en la diversidad microbiana se han asociado con el desarrollo de alergias y/o asma a lo largo del paso del tiempo. Es por ello que otro de los objetivos del proyecto es caracterizar la microbiota intestinal de los lactantes y su perfil de ácidos grasos en las heces de los lactantes a los 3 meses

de edad y determinar la influencia de factores prenatales y del neonato (microbiota y ácidos grasos de cadena corta) en la aparición temprana de síntomas precursores de asma de los lactantes de 3 meses de edad.

Primeramente, se ha descrito el **estado del arte de esta tesis doctoral**, dividido en tres apartados. En el primero de ellos se describe el proceso de transmisión, colonización y evolución microbiana durante los primeros meses de vida del neonato. Respecto al proceso de transmisión, actualmente existen diferentes teorías entre la comunidad científica. Por un lado, está la teoría que considera al ambiente intrauterino como un ambiente estéril y que la primera colonización por microorganismos se produce durante el proceso de parto. Por otro, estudiando la microbiota del meconio, diversos autores han encontrado microorganismos presentes y, por ende, consideran que el ambiente intrauterino no es estéril y determinan que durante el embarazo debe existir una transmisión de bacterias de la madre al neonato a través de la placenta o del líquido amniótico. La segunda parte de esta revisión bibliográfica resume los diferentes factores prenatales, perinatales y postnatales que intervienen en una correcta colonización y desarrollo de la microbiota intestinal de los lactantes durante los primeros meses de vida. Por último, en el tercer apartado se intenta establecer la posible relación entre la aparición temprana de asma y la microbiota intestinal de los niños, contemplando otros factores que pueden intervenir en esta relación como el tipo de parto, el uso de antibióticos, el tipo de lactancia, etc. La principal conclusión de esta revisión es que, aunque no hay consenso sobre la existencia de colonización prenatal por transmisión microbiana entre la madre y el niño, si existe cierto consenso acerca de los principales factores que pueden influir en la transmisión y posterior colonización de la microbiota intestinal del lactante, siendo el tipo de parto, el uso de antibióticos por parte de la madre y el tipo de alimentación durante los primeros meses de vida los 3 principales factores moduladores. Otra de las conclusiones es que el asma infantil es una enfermedad con una prevalencia que va en aumento en las últimas décadas de difícil diagnóstico debido a la amplitud de síntomas asociados a ella y a la variabilidad en la edad a la que se diagnostica. Como consecuencia, no existe un criterio único dentro de la comunidad científica en la definición de niño asmático lo que puede ser causa de la gran discrepancia que existe en los

resultados obtenidos en diferentes estudios a la hora de establecer la relación entre el perfil de la microbiota intestinal del lactante y el asma en la infancia.

Tras la revisión bibliográfica, en el apartado de **material y métodos general** se describe en detalle el diseño experimental de la cohorte NELA del cual se deriva esta tesis y que es la base de la información y las muestras utilizadas en los diferentes estudios llevados a cabo. En esta sección se realiza una amplia descripción del diseño de la cohorte, etapas, objetivos, población objeto de estudio y criterios de inclusión y la recogida de datos y muestras. Por otro lado, se detallan los criterios para la selección de una submuestra de 200 parejas madre-hijo utilizadas en la realización de un estudio Casos-Control definidos en función de si los lactantes presentaron episodios de síntomas relacionados con asma como sibilancias, eczema, dermatitis, bronquitis y /o bronquitis a los 3 meses de edad. Ambas poblaciones serán utilizadas en los diferentes estudios que conforman esta tesis doctoral.

El resto de métodos y materiales utilizados se describen dentro de cada uno de los **dos capítulos** que componen el apartado de **estudios de investigación.**

En el **capítulo 1** el principal objetivo fue establecer si una dieta materna saludable durante el embarazo, como es la adherencia a un patrón de dieta Mediterránea, se asocia con una menor aparición de síntomas precursores de asma en los lactantes a los 3 meses de edad. Para la consecución de este objetivo este capítulo se ha subdividido en 3 estudios.

En el **estudio I** se realizó la caracterización de la adherencia a la dieta Mediterránea y a un patrón de dieta saludable por parte de las madres pertenecientes a la cohorte NELA, utilizando 3 índices dietéticos y comparándolos entre sí, así como el estudio de los posibles factores sociodemográficos y de estilo de vida que influyen en el grado de adherencia a dicho patrón dietético. La principal conclusión de este estudio fue que las mujeres embarazadas de menor edad, menor número de partos previos, bajo nivel educativo y que practican estilos de vida poco saludables tienen mayores probabilidades de presentar una baja adherencia a la dieta mediterránea y un patrón dietético saludable.

En el **estudio II** se intentó determinar la asociación entre el grado de adherencia a la dieta Mediterránea de las mujeres gestantes la aparición temprana de síntomas precursores de asma en los lactantes a los 3 meses de edad. La conclusión fue que no se observaron diferencias en los patrones dietéticos de las madres cuyos hijos fueron clasificados como casos o controles y tampoco se observó una asociación entre un bajo grado de adherencia a la dieta Mediterránea y un mayor riesgo de aparición de síntomas precursores de asma en la descendencia. Como conclusión final creemos necesario establecer o crear un índice único de adherencia a la dieta mediterránea específico para gestantes, permitiendo así reducir la heterogeneidad de futuras investigaciones.

Estos resultados plantearon la necesidad de profundizar en el estudio de la dieta de las embarazadas y su relación con los síntomas indicados anteriormente, centrándose en grupos de alimentos o nutrientes específicos que componen la dieta tales como verduras, frutas, legumbres, bollería, etc. Para ello se llevó a cabo el **estudio III.** Tras la realización de este estudio se concluyó que un mayor consumo de bollería y dulces en la mujer embarazada está asociado a un mayor riesgo de que la descendencia desarrolle síntomas precursores de asma a edades tempranas. Sin embargo, un alto consumo de café, té e infusiones durante el embarazo, debido a su carácter antioxidante, está asociado a un menor riesgo de aparición temprana de estos síntomas.

En el **capítulo 2**, se estudiaron los factores prenatales, perinatales y posnatales que pueden influir en la microbiota del lactante y sus metabolitos, así como su asociación con la aparición precoz de síntomas precursores del asma a los 3 meses de edad**.** Este capítulo comprende un único estudio piloto de tipo Caso-Control con las 200 parejas madre-hijo seleccionadas en función de si los lactantes habían presentado o no síntomas tales como sibilancias, eczema, dermatitis, tos seca, bronquitis y/ o bronquiolitis a los 3 meses de edad. En dicho estudio se realizó, mediante PCR cuantitativa, la caracterización de ciertas especies y grupos bacterianos presentes en las heces de los lactantes a los 3 meses de edad, clasificados por la comunidad científica como bacterias beneficiosas para la salud. Además, se analizaron y cuantificaron los ácidos grasos de cadena corta presentes en las heces de los lactantes a los 3 meses de edad mediante cromatografía de gases. Con los datos obtenidos se realizó

una comparativa entre el grupo control y el grupo casos. Posteriormente se realizaron diversos análisis estadísticos bivariantes y multivariantes para observar la influencia de distintos factores prenatales, perinatales y postnatales en la microbiota intestinal de los lactantes y los ácidos grasos de cadena corta. Por último, utilizando modelos de regresión logística, se estudió la posible relación entre la microbiota intestinal de los lactantes y su perfil de ácidos grasos, con la aparición de síntomas precursores de asma en los lactantes. Las conclusiones fueron que existen diferencias en la microbiota intestinal de los lactantes entre el grupo control y el grupo casos, así como en las proporciones molares de los ácidos grasos de cadena corta mayoritarios. Concretamente, la composición de la microbiota intestinal de los lactantes pertenecientes al grupo de casos se caracterizó por recuentos más bajos de *Atopobium, Bacteroides, B. breve, B. longum*, *Clostridium* cluster XIVa y cluster IVa y *Clostridium difficile*, y recuentos más altos de *Akkermansia muciniphila* en comparación con los controles. También se pudo concluir que las heces de los lactantes clasificados como casos presentaron mayores proporciones molares de ácido Acético y menores de ácido Butírico y Propiónico, ambos relacionados también con efectos beneficiosos para la salud. Otra conclusión fue que recuentos altos de *Akkermansia muciniphila* y *B. breve*, y recuentos más bajos de *Bacteroides-Prevotella, Clostridium* cluster IVa y cluster XIVa, y *B. longum,* se asociaron con una mayor probabilidad de desarrollar síntomas precursores del asma a los 3 meses de edad. En relación a los ácidos grasos de cadena corta mayoritarios y su posible asociación con la aparición temprana de síntomas precursores del asma en lactantes, una menor concentración de ácido butírico en las heces a los 3 meses de edad se asoció con una mayor probabilidad de aparición de estos síntomas.

List of abbreviations

AHEI-2010: Alternative Healthy Eating Index

AHEI-P: Alternate Healthy Eating Index modified for pregnancy

aMED: Alternative Mediterranean Diet Index

ARG: Antibiotic resistance gene

BFI: Breastfed Infant

BFI: Exclusively Breastfed Infants

BM: Breastmilk

BMI: Body Mass Index

CHILD cohort: Canadian Healthy Infant Longitudinal Development general population cohort.

CI: Confidence interval

CS: Caesarean section

CSDI: Caesarean section delivered infant

Ct value: Cycle threshold

DCs: Dendritic cells

DGGE: Denaturing Gradient Gel Electrophoresis

DHA: Docohexanoic fatty acids

DHM: Donated Human milk

DOHaD: Developmental Origins hypothesis for Health and Disease

EOAPS: Early Onset of Asthma Precursors Symptoms

EPA: Eicosapentaenoic fatty acids

EPIC cohort: European Prospective Investigation into Cancer and Nutrition cohort

FAMD: Factorial Analysis of Mixed Data

FFI: Formula fed Infant

FFQ: Food Frequency Questionaire

FID: Flame Ionization Detector

FISH: Fluorescence in situ hybridation

FOS: Fructo-Oligosaccharides

FTI: Full Term Infant

GBS: Group B *Streptococcus*

GOS: Galacto-Oligosaccharides

GPCRS: G protein-couple receptors

HDCAs: Histone deacetylases

HDM: House Dust Mite

HMOs: Human Milk Oligosaccharides

HMP: Human Microbiome Project

ICSD: Infants Caesarean Section Delivered

IEA: Infants Exposed to Antibiotics

IEIPA: Infants Exposed to Intra Partum Antibiotic

IEP: Infants Exposed to Probiotics

IF: Infant Formula

IFI: Infant Formula feeding Infants

IMGDMN: Infants Mother's Gestational Diabetes Mellitus No

IMGDMY: Infants Mother's Gestational Diabetes Mellitus Yes

INEA: Infants No Exposed to Antibiotics

INEIPA: Infants No Exposed to Intra Partum Antibiotic

INEP: Infants No Exposed to Probiotics

INMA: INfancia y Medio Ambiente

IPA: Intra partum antibiotic

IQR: Inter-Quartile Range

ISSAAC: Study of Asthma and Allergies in Childhood

IVD: Infants Vaginally Delivered

IVF: In Vitro fertilisation

L/C: Ratio of *Lachnospira* to *Clostridium neonatale*

LABs: Lactic Acid Bacterias

LPS: Lipopolysaccharides

MACs: Microbiota-accessible carbohydrates

MALDI-TOF: matrix-assisted laser desorption/ionization- Time of Fly

MD: Mediterranean diet

MDA: Mediterranean diet adherence

MDS: Mediterranean Diet Score

MGDM: Mother's Gestational Diabetes Mellitus

MLN: mesenteric lymphocytes node

MUFAs: Monounsaturated fatty acid

MXI: Mixed feeding Infants

N-3: Omega-3

N-6: Omega-6

NCIT: New Culture-independent Techniques

NELA cohort: Nutrition in Early Life and Asthma cohort

NGS: Next Generation Sequencing

NIH: National Institute of Health

OR: Odd ratio

OTUS: Operational Taxon Units

P: p- value

PAMPs: Pathogen-associated Molecular-Patters

PCR: Polymerase Chain Reaction

PTB: Pre-Term Births

PTI: Pre-Term Infant

PUFAs: Polyunsaturated Fatty Acids

qPCR: Quantitative Polymerase Chain Reaction

rMED: Relative Mediterranean Diet Index

Runx3: Runt-related transcription factor 3

SCFAs: Short Chain Fatty Acids

SEATON: Study of Eczema and Asthma To Observe effects of Nutrition

T1D: Type 1 diabetes

T2D: Type 2 diabetes

TGF-β: Transforming growth factor β

Th1: Type 1 immune response

Th2: Type 2 immune response

TLR: Toll-like Receptor

Treg: Regulatory T cells

VDI: Vaginally Delivered Infant

VDR: Vitamin D receptor

WHO: World Health Organisation

I. STATE OF THE ART

1. Transmission, colonisation and evolution of the infant gut microbiota.

1.1 Prenatal transmission and colonisation: sterile utero paradigm and its colonisation hypothesis.

Until recently, the in utero environment has been considered sterile under normal conditions, and the colonisation process was thought to begin at birth when the infant is exposed to the microbiota of the mother and the environment [1]. However, if “in utero colonisation hypothesis” proves correct, there would be major repercussions on our understanding of the establishment of the pioneer human microbiome, its role in human health and the role of environmental, lifestyle, and clinical factors that affect its assembly and function.

Since the studies of Theador Escherich in 1886 [2], who first describe the meconium (the earliest stool from an infant) to be free of viable bacteria, the idea that term foetuses are sterile in utero has been widely accepted [3]. A few years later, Tisser [4] established that bacterial colonisation of the newborn occurs when the baby initiates transit through the labour channel via contamination by maternal vaginal and faecal bacteria. The process continues after birth and progressively the intestinal lumen of the newborn becomes colonized by bacterial species. The abundance and variety suffer changes over time to culminate into a relatively stable microbial composition seen throughout adulthood [5]. According to this concept, which has been referred to as *the sterile womb paradigm* [6], microbes are acquired both vertically (from the mother) and horizontally (from other humans or the environment) during and after the birth [3]. The majority of the studies that established the sterile womb paradigm employed traditional culture-based methods and microscopy, which are still considered valid today despite their limitations because may fail to detect viable but non-cultivable microbes [7].

For more than a century, the hypothesis of Escherich was generally accepted despite the fact that other two additional, independent studies conducted in 1927 and 1934 (n=100 and n=50, respectively), suggested some microbial activity in

meconium which supposed a conflict with this idea. Specifically, Burrage (1927) [8] found 38% of meconium samples from healthy pregnancies to be positive for bacteria cultured and Hall and O'Toole in 1934 [9] observed positive bacterial cultures in 19 of the 50 test meconium samples, being *Micrococcus* spp. the bacterium mostly isolated. Interestingly, Hall and O'Toole disclosed a positive correlation between birth and meconium passage. Snyder in 1936 [10], using rectal cannulation and intra-rectal swabbing of newborns, found 6.3% of the samples to contain bacteria by direct culture and 36% by enrichment culture. Despite this, he attributed the susceptibility of the method to contamination of skin microbes in the enrichment process.

After the early studies on the meconium discussed above, further microbiological research on the meconium ceased for a period of over 60 years (reviewed by Koleva *et al*., [1]) until Jimenez *et al*., in 2008 [5]. They reported 100% (n=21) of meconiums to be positive for bacteria by culture techniques and therefore confirmed the non-sterility of infant meconium shown by earlier researchers. Two years later and using non-culture-based techniques, Mshvildadze *et al*., [11] provided further evidence on the presence of bacteria in meconium obtained from neonates born at 22 to 32 weeks gestational age. These authors detected microbial DNA in 91% of meconium samples and denaturing gradient gel electrophoresis (DGGE) profiling revealed an association between prematurity and reduced meconium microbial diversity.

However, this dogma of a sterile *in utero* environment has been challenged. There is now a multitude of recent studies employing next-generation DNA sequencing techniques that have challenged the traditional view of human microbiome acquisition. These studies propose that neither the foetus, the placenta, or the amniotic fluid are sterile, and that acquisition and colonisation of the human gastrointestinal tract begins in utero [5,12,13]. Madan *et al*., in 2012 [14], in a prospective longitudinal study, applied high throughput pyrosequencing of the hypervariable V6 region of the 16S rRNA gene to understand the gut microbial colonisation in prematurity. They found that the meconiums of all subjects (n=6) were

not sterile, and the predominant bacterial genera were *Lactobacillus*, *Staphylococcus* and *Enterobacteriaceae*. Gosalbes *et al*., [15] characterised the meconium microbiota in twenty term newborns from a Spanish birth cohort, to assess whether it contributes to the future microbiota of the infant's gastrointestinal tract, and to evaluate how it relates to lifestyle variables and atopic-conditions. For this, the authors used the high-throughput pyrosequencing of the 16S rRNA gene and their conclusion was that the meconium microbiota differed from the microbiota of faeces, vagina, and skin from adults but was similar to that of young infant faeces. Another conclusion of this study was that meconium microbiota has an intrauterine origin, which is influenced by maternal factor and may have consequences for childhood health.

A few years later, Moles *et al*., [16] aimed to characterize the evolution of gut microbiota during the first 3 weeks of life of 14 preterm neonates, including the meconium microbiota. The bacterial diversity and taxonomy were examined using culture-dependent and molecular techniques, including DGGE and Human Intestinal Tract Chip (HITChip) analysis of 16S rRNA amplicons. Both approaches showed that spontaneously released meconium of such neonates contains a specific microbiota that differs from those observed in early faecal samples [17]. The bacteria groups belonging to the phylum *Bacilli* and Firmicutes were the main bacteria groups detected in meconium, while Proteobacteria phylum was abundant in the faecal samples. Culture- based techniques revealed that *Staphylococcus* predominated in meconium and that *Enterococcus*, together with Gram-negative bacteria such as *Escherichia coli*, *Escherichia fergusonni*, *Klebsiella pneumoniae* and *Serratia marcescens*, was more abundant in faeces. In addition, molecular techniques showed the high prevalence of bacteria related to *Streptococcus mitis* and *Lactobacillus plantarum* in meconium, whereas in the third week faeces predominated those related to *Escherichia coli*, *Enterococcus*, and *Klebsiella pneumoniae* and *Yersinia*. Another study, carried out by Hu *et al*., in 2013 [18], aimed to assess the diversity of meconium microbiome and determine if the bacterial community of the meconium is affected by maternal diabetes status. The meconium was collected from 23 newborns stratified by maternal diabetes status and the

meconium microbiome was profiled using multi-barcode 16S rRNA sequencing followed by taxonomic assignment and diversity analysis. A diversified microbiota was found in all meconium samples, which was not affected by the mode of delivery. The meconium showed a lower species diversity, higher sample-to-sample variation, and enrichment of Proteobacteria and reduction of Bacteroidetes compared with adult faeces. The taxonomy analyses, among the meconium samples, suggested that the overall bacterial content in meconium significantly differed by maternal diabetes status. Specifically, in those samples of the diabetes group, the phyla Bacteroidetes and the genus *Parabacteroides* were enriched. Two years later, one study by Hansen *et al*., in 2015 [19], based on fluorescence in situ hybridisation (FISH) with 16S rRNA-targeted probes, showed evidences of microbial presence in 66% (10 of 15) of meconium samples, from 15 healthy full-term vaginally-delivered infants. The members who predominated in meconium belonging to *Bifidobacterium*, *Enterobacteriaceae*, *Enterococcaceae*, and *Bacteroides-Prevotella* [1].

Currently, the microbiome of the meconium remains a subject of study for many research providing novel information for the development of strategies to guide the formation of healthy microbiota. More specifically, whether maternal factor during pregnancy or the delivery modes could influence the microbiome composition of meconium. In 2018, Shi *et al*., [20] used a metagenomic sequencing technique to characterize the meconium microbiome from the faeces of a Chinese cohort of vaginally and C-section delivered infants (CSDI). In contrast to Hu *et al*., [18] the authors founded that the meconium microbiome diversity was higher in vaginally delivered infants (VDI) than that in CSDI. *Propionibacterium* species were most abundant in the VDI, whereas the CSDI group had high levels of *Bacillus licheniformis*. Moreover, different modes of delivery affected the antibiotic resistance gene (ARG) prevalence, what might influence the infant's health later in life. In this sense, Dominguez Bello *et al*., [21] also studied the impact of birth mode on bacterial communities in a rectal swab obtained at birth. They also found that the meconium microbiota composition of full-term infants (FTI) was influenced by the mode of delivery too [1].

Furthermore, Tapiainen *et al*., [22] investigated if maternal factors during pregnancy, such as the environment, influence the microbiome of the first stool more than immediate perinatal factors. Regions of the bacterial 16S rRNA gene were sequenced to characterize the microbiome of the first-pass meconium samples (n=212). With a relative abundance of 44 %, Firmicutes was the most abundant phyla, Proteobacteria, 28 %, and Bacteroidetes, 15 %. The diversity of microbiome is increased by the biodiversity of the home environment, whereas perinatal factors, such as the delivery mode or exposure to antimicrobials during the labour did not have an effect.

In summary, despite the fact that culture and molecular techniques have provided preliminary evidences for diverse group of bacteria in meconium from both FTI and preterm infant (PTI), it remains unclear the origin of the microorganisms. Meconium microbial communities have low species diversity and high inter-individual variability, thus being very similar to early faecal microbiota [16,18]. At the phylum level, meconium microbiota looks more closely like the gut microbes of infants than of adults [21]. Despite this similarity, when compared meconium with faecal samples at 3 and 12 months, meconium was found to be less abundant in *Bacteroides* and *Bifidobacterium* species and therefore more likely to be colonized with *Escherichia-Shigella* and *Enterococcus* [23]. Moreover, the high similarity between meconium and amniotic fluid microbes [24], and the fact that large quantities of amniotic fluid are swallowed by foetus in the last trimester of pregnancy [25], it leads us to the idea that the meconium microbiota may have an intrauterine origin. As we reviewed in detail in the next section, these findings contradict the classic dogma whose main idea is that the newborn comes from a sterile environment and suggest that the establishment of intestinal microbiota is initiated in the prenatal gut [1].

1.2 Which is the origin of meconium microbiota and their routes of transmission?

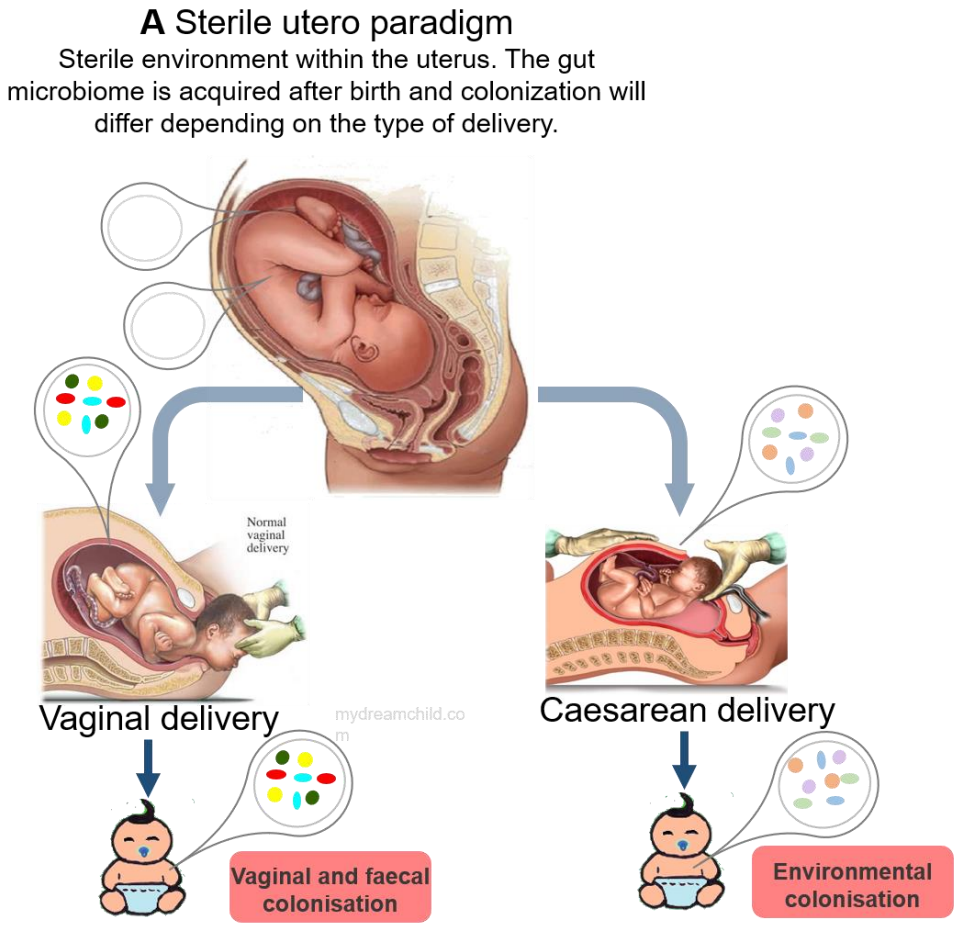
Until 20 years ago, the intra-uterine environment has been considered sterile under normal conditions during pregnancy. The presence of bacteria in amniotic fluid, foetal membranes and placenta tissue was only investigated when there are symptoms of infection or circumstances that may facilitate it, such as premature rupture of membranes or cervical dilatation.

In some measure, because of the sterile womb paradigm and their permanent influence, and also technical and ethical problems, relatively few researches have studied the uterine microbiota in healthy, full-term pregnant women. However, in some recent studies, thanks to the new advances in sequencing and molecular technologies, it has been possible to study the microbiome of areas considered sterile until then. Some of them have provided evidences of the presence of microbes in placental tissue [12], amniotic fluid [26,27], umbilical cord blood [28], foetal membranes [29] and meconium [13,16,18,20]. Some such research have studied the uterine microbiota in healthy, FTI without any sign of infection or inflammation and have shown the presence of microbes in the uterine environment [5,13,15,29] suggesting the existence of functional pathways that allow a bacterial exposure with the foetus during the stage of pregnancy [1]. On the other hand, other studies have concluded that there is no evidences of a placenta, amniotic fluid and/or early contact with microbes [30,31] and therefore, the *in utero* colonisation hypothesis continues to be the subject of debate [32]. Perez Muñoz *et al*., in 2017 [3] in their review concluded that current scientific evidence does not support the existence of microbiomes in the foetal healthy environment. With new molecular and sequencing techniques, it has been possible to overcome the limitation of culture-based methods but it is true that the data that support the *in utero* colonisation hypothesis must be taken with extreme caution, because of them were obtained with particular methodological limitations. For example, the bacterial DNA detected may belong to non-viable microorganisms, that is, dead microorganisms which cannot be detected by culture-based techniques. In the case of studies carried out in placenta this consideration is particularly important because the placenta plays a key role in

the elimination of microbes and other components that may be ubiquitous in the blood [3]. Another important methodological issue is that the highly sensitive molecular techniques employed to study the low microbial biomass may detected contaminating microbes and therefore produce false-positive results [7].

While vaginal microbes associated with preterm birth can get access to the uterine environment through an ascending route, the mechanisms by which gut bacteria reach this human niche (placenta, amniotic fluid and umbilical cord) are not well understood (**figure 1)**.

There are still many doubts about what could be the mechanisms by which intestinal bacteria gain access to the uterine environment. It has been suggested that microorganisms ascend from the vagina and are able to travel to the placenta through the bloodstream after translocation of the gut epithelium. It is known that one of the main roles of the intestinal epithelial barrier is to prevent microbial entry into the circulatory system, but dendritic cells can actively penetrate the intestinal epithelium to the intestinal lumen where bacteria are present, elevate these live bacteria and transport them through the body as they migrate to the lymphoid organs [17]. Furthermore, bacterial species that are normally found in the human oral cavity have also been isolated from the amniotic fluid and probably these bacteria, during periodontal infections, may have access to the bloodstream thanks to the inflammation of the gums. The main bacterial species found in amniotic fluid with an oral origin are *Fusobacterium nucleatum*, *Streptococcus spp.*, *Bergeyella spp.*, *Porphyromonas gingivalis*, *Rothia dentocariosa*, and *Filfactor alocis* [6]. To test whether maternal gut bacteria can be transferred to foetuses *in utero*, two pioneer studies investigated if oral administration of a genetically labelled *Enterococcus faecium* to pregnant mice resulted in its presence in amniotic fluid and meconium of term off-spring after sterile CS [5,28]. In both studies, *Enterococcus faecium* genetically distinctive strain was detected by PCR in the intestinal lumen of pups delivered 1 day prematurely by CS. Also, *E. faecium* with the genetic label was cultured from amniotic fluid and meconium of pups from inoculated mothers.



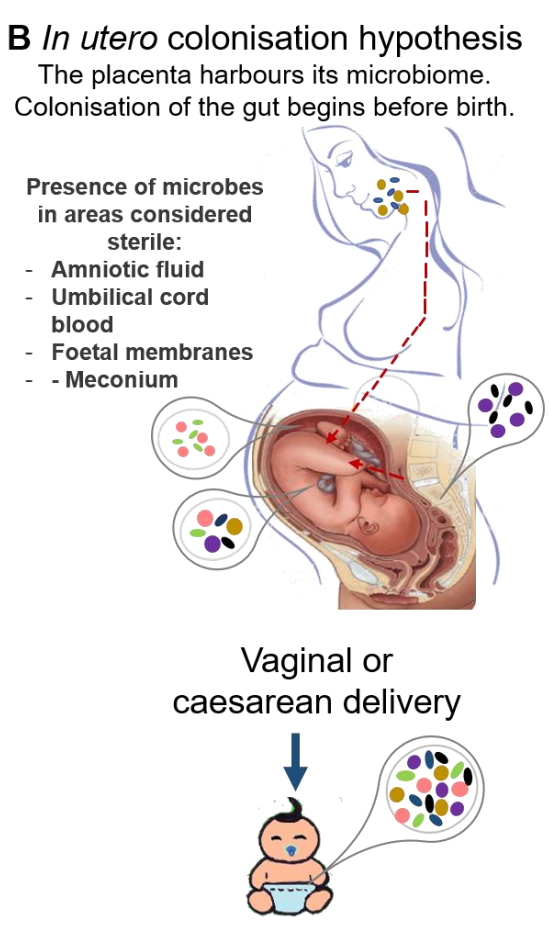


Figure 1. Schematic representation of the opposing concepts by which human microbiota is acquired early in life. A: sterile womb paradigm, B: The “in utero colonization hypothesis”. Modified from Perez-Muñoz, *et al.,* [3]

In contrast, it could not be detected in the samples obtained from a non-inoculated control group. As we have commented previously, the infant's microbiota is not only composed of bacteria, but also non-pathogenic viruses or archaea. About these microorganisms, we still don't know almost anything about how they may be transferred from mother to infant. Fortunately, culture-independent new techniques such as high-throughput sequencing, are a great resource for study this field and will hopefully lead to a characterization of the "foetal microbiome" *in the uterus* [6].

1.3 Microbial colonisation and their evolution during the first year of life: How is the microbiota establishment?

Microbial colonization during the prenatal stage can imprint the offspring microbiota and the immune system in preparation for contact with a much larger inoculum transferred during the post-natal stage, specifically during vaginal delivery and lactation [17]. It is from birth when the human microbial colonisation process begins to a greater extent and continues to develop and modulate in species abundance for about 3 years, until the microbiota becomes adult-like. A big number of research in both animal models and humans, suggests that the process of microbial colonisation begins at birth and its development is especially significant during early life, as this period constitutes a critical window for immunological and physiological development [33]. The great advances in metagenomic technologies during the last 20 years have allowed us to know more accurately the composition of the intestinal microbiota since early infancy [23,34,35].

Different genotype-based studies have provided definitive evidence of the transmission of specific microbes from mothers to the gut of their infants [21,23,36]. This concept of transmission between mother and infant is commonly known as vertical transmission or mother-newborn transmission (**figure 2**).

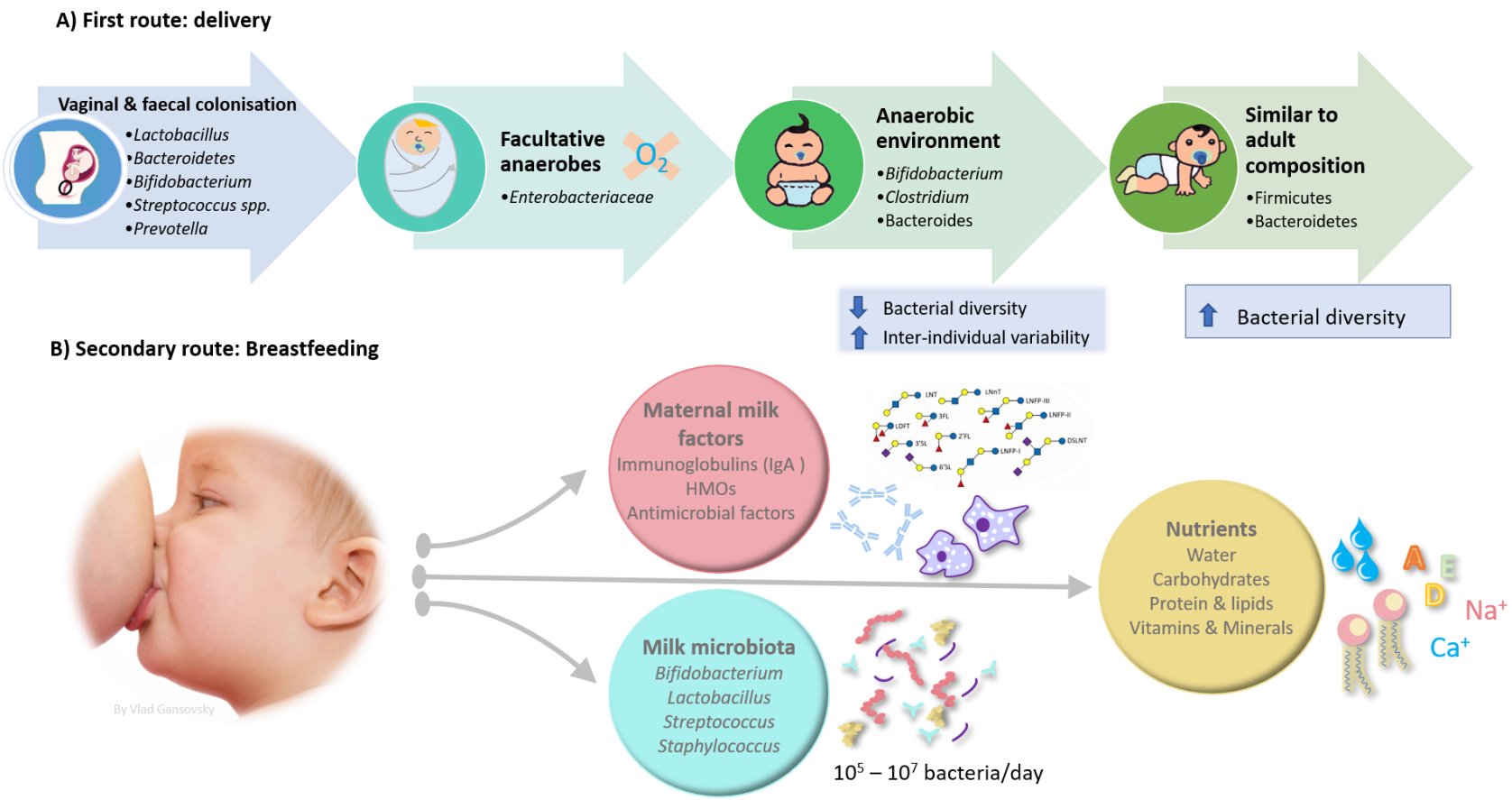


Figure 2. Schematic summary of the two main routes that comprise the vertical transmission or mother-newborn transmission. **A)** Route linked to the type of delivery. **B)** Route linked to breastfeeding. HMOs: Human Milk Oligosaccharides.

For example, Bäckhed *et al*., in 2015 [23], using metagenomic sequencing, compared the microbial species in 98 newborns and their mothers and found that 135 of 187 taxonomically annotated MetaOTUs present in vaginally delivered newborns also were found in their own mothers including important species such as *Escherichia/Shigella*, *Bifidobacterium longum*, *Enterococcus faecalis*, *Bacteroides fragilis*, *B. thetaiotaomicron* and *Bilophila wadsworthia*, reinforcing the concept of vertical transfer. Also, Dominguez-Bello *et al*., in 2010 [21] demonstrated that neonates born vaginally have a microbiota resembling the vaginal microbiome, enriched with *Lactobacillus* and *Prevotella* species, although other bacteria, such as the *Enterobacteriace* family, including *Escherichia* or *Klebsiella*, are also present [32].

During vaginal delivery, the infant gut becomes colonized by maternal vaginal and faecal bacteria (*Lactobacillus*, *Prevotella* of the Bacteroidetes phylum, *Sneathia* of the Fusobacteria phylum) [5]. First colonizers, facultative anaerobes such as members of the *Enterobacteriacea* family, create an anaerobic environment in the intestinal lumen which allows the grow of strict anaerobes as *Bifidobacterium spp.*, *Clostridium*, and *Bacteroides* [1,17]. The neonatal gut microbiota is characterized by low bacterial diversity and higher inter-individual variability as well as a relative dominance of the phyla Proteobacteria and Actinobacteria (mainly comprised of the genus *Bifidobacterium*), with the microbiota becoming more diverse with the emergence and dominance of Firmicutes and Bacteroidetes as time after birth increases, resembling the adult composition and diversity approximately between 2 and 5 years of age [17,23,37]. A secondary route of maternal microbial transmission is breastfeeding. This transmission occurs in two different ways, indirectly, via maternal milk factors that affect bacterial growth and metabolism such as human milk oligosaccharides (HMOs), secretory IgA, and anti-microbial factors and, directly, by the exposure of the neonate to the milk microbiota [38]. Breast milk provides a mix of nutrients and pro microbial and antimicrobial agents, which satisfies the nutritional requirements of the infant and confers protection against pathogens through the transmission of maternal antibodies (IgA) and other antimicrobial factors [7,39].

Traditionally, through the use of culture-dependent techniques has confirmed the presence of microbes in human milk. Most bacteria isolated from breast milk belong to *Staphylococcus*, *Streptococcus*, *Lactobacillus* and *Bifidobacterium* spp., [40]. It has been described that an infant can consume approximately 800 ml/day of milk, arriving to ingest between 10^5 and 10^7 bacteria per day, with human milk constituting one of the main sources of bacteria to the breastfed infant gut [41]. However, the composition of breast milk microbiota is not stable over time. With the development and application of culture-independent techniques and Next generation sequencing (NGS) platforms various studies have been able to determine that there is an evolution in the composition of the microbiota in breast milk throughout the period of breastfeeding. For example, Cabrera-Rubio *et al*., in 2012 [42] described this evolution and concluded that the colostrum microbiota has a higher diversity than mature milk, being dominated by *Weissella*, *Leuconostoc* (both lactic acid bacteria), *Staphylococcus*, *Streptococcus*, and *Lactobacillus* spp. One month later, the *Staphylococcus* level is dramatically reduced, and there are higher levels of *Veillonella*, *Prevotella*, *Leptotrichia* (typical inhabitants of the oral cavity), *Lactobacillus*, *Streptococcus* spp., and increasing levels of *Bifidobacterium* and *Enterococcus* spp. These techniques have confirmed the existence of a rich and diverse breast milk microbial community. Also, Ward *et al*., [43] carried on a shotgun metagenomics analysis of 10 pooled human milk samples by total DNA sequencing using Illumina NGS technology and reported that Proteobacteria (65 %) and Firmicutes (34 %) are the predominant phyla and *Pseudomonas* spp. (61,1 %), *Staphylococcus* spp. (33,4 %), and *Streptococcus* spp. (0.5 %) are the predominant genera. The origin of these commensal bacteria remains unclear but their presence starts during the third trimester of pregnancy and continues through lactation. Some reviews summarize the different studies carried out to determine the different routes and conclude that human milk microbiota could derive from colonization from mother's skin and the infant's oral cavity during suckling because breast milk flows back into the mammary ducts, which provides a route for bacteria found in their infant's oral cavity to enter the mammary gland. But a number of studies also support the entero-mammary pathway hypothesis, wherein bacteria from the maternal gut

may reach the mammary glands via maternal dendritic cells and macrophages [7,38,40,41].

In addition to shaping the composition of the microbiota, the practice of early feeding affects the metabolism of the microbiota. The microbiomes of newborns and breastfed are enriched in genes necessary for the degradation of HMOs. HMOs are the third largest component of breast milk and are structurally complex sugars unique to human breast milk. They are not digestible, promote the proliferation and growth of specific microorganisms, including the species *Bifidobacterium* and Bacteroidetes, and the metabolism of these substrates resulted in the production of lactate and Short Chain Fatty Acids (SCFAs), which in turn increased the acidity of the surrounding environment, an important factor in preventing the invasion of pathogens. For all of that, HMO are considered a type of prebiotic and exert positive effects on health [38,39,44].

Throughout the first year of life, gut bacterial diversity and richness continue to respond rapidly to changes in the infant diet. The first introduction of infants to solid foods occurs during weaning (complimentary feeding) when infants are exposed to a much larger array of non-digestible carbohydrates than those present in breast milk or formula. The presence of new substrates can significantly alter the gut microbiota, favouring the growth of polysaccharides fermenters such as *Bacteroides*, *Clostridium*, *Ruminococcus*, and *Faecalibacterium* [39,45]. During weaning the alpha diversity, concept that indicates how many different species could be detected in a microbial ecosystem, increases, resulting in the replacement of Proteobacteria and Actibobacteria by Firmicutes and Bacteroidetes phyla as the dominant members of the infant microbiota [7,46]. Bersdtröm *et al*., (2014) [47] found that *Lactobacillaceae*, *Bifidobacteriaceae*, *Enterococcaceae* and *Enterobacteriaceae* abundance decreased while *Lachnospiraceae*, *Ruminococcaceae* and *Bacteroidaceae* species increased during the period from 9 to 18 months, e.g., during the period characterized by transition from milk-based feeding to family diet. This was largely in agreement with a European study of 605 children from five European countries carried out by Fallani and colleagues in 2011

[46]. These authors found that weaning was associated with a decrease in *Bifidobacteriaceae*, *Enterobacteriaceae*, *Clostridiaceae* while increase in *Ruminococcaceae* and *Lachnospiraceae* species occurred from 6 weeks of age until 4 weeks after the introduction of solid foods, irrespectively of differences in geographic location, use of antibiotics, mode of delivery and milk feeding practices which is in agreement with another report carried out by Laursen *et al*., in 2016 [48]. A decrease in saccharolytic bacteria such as members of *Bifidobacteriacea* family and an increase in *Lachnospiraceae*, which are associated with breast milk, has been correlated with a higher protein intake. On the other hand, ingestion of fibre was demonstrated to be associated with higher levels of *Prevotellacea*. Interestingly, two species that are absent or present at very low levels during early infancy, *Faecalibacterium prausnitzii* and *Akkermansia muciniphila*, increase in abundance to adult levels at 12 months and 24 months respectively [7]. Both species are being widely studied because they are considered to promote a correct state of health in adulthood. Interestingly, the microbiota composition in African and European infants is very similar until the introduction of solid foods, indicating the dominant role of diet over other variables in shaping the microbial composition of the gut in early life [38].

Additionally, the introduction of solid foods also changes the metabolic function of the gut bacteria as genes involved in the degradation of sugars from breast milk are less needed and utilized. Instead, the microbiota adapts to the available energy source, and functionally matures to be able to degrade complex sugars and starch found in solid food [23]. By the end of the first year of life, the composition of the infant gut microbiota is more similar to the microbiota of an adult; however, a typical adult microbial profile is not established until 2-3 years of age [37]. The development of such a establish condition reaches a climax status represented by the establishment of a homoeostasis among all its members. A wide range of factors can cause shifts in this microbiota balance, thereby disrupting the gut microbiota homoeostasis and causing a so-called state of dysbiosis. There is a controversy on the exact meaning of dysbiosis, simply because of the lack of an accurate description of a “normal” or healthy microbiota. Dysbiosis is usually associated with harmful effects and may have long-term consequences leading to

disorders or diseases. The process of development and maturation of the intestinal microbiota is a dynamic and non-random process, in which both positive and negative interactions take place between the main microbial taxa [7]. Therefore, more research is needed to clarify what specific components of a solid food diet play the biggest role in the development of the infant gut microbiota and how it will affect the child's health in the long term.

2. Main influential factors in the microbial colonisation

Human microbial colonisation represents the *de novo* assembly of a complex microbial community, a process that is influenced by a number of different factors (both intrinsic and extrinsic) such as mode of delivery, type of feeding, and antimicrobial treatments. Also, as previously discussed, the diet during the pregnancy period and during the first years of life has an influence in this development. The mother's age, as well as environmental and life style, and family genetics have also been reported to impact the infant microbiota [49]. Various epidemiological studies have established a clear correlation between these factors that disrupt the gut microbiota during childhood on the one hand, and immune and metabolic disorders later in life on the other [7,50]. The following section highlights some perinatal, neonatal and postnatal factors that are thought to influence the development of infant gut microbiota and they have been summarized and schematized in **figure 3**.

2.1 Prenatal and perinatal factors

Prenatal factors during pregnancy can affect the maternal gut microbiota, which in turn can affect the infant *in utero* colonisation and even influence the future development and behaviour of the infant.

For example, Bailey *et al*., in 2004 [51] showed that infant monkeys born from mothers stressed during pregnancy had significantly lower counts of *Bifidobacterium* and *Lactobacillus*. Also, as a result of the consumption of probiotics (*Lactobacillus rhamnosus*) during the last stage of pregnancy, it has been observed an increased in faecal *Bifidobacterium longum* counts in their infants [52], although, it is not clear

whether these microbes were acquired from the mother during pregnancy, during birth, or after birth [53]. There are other factors that can influence such as the use of antibiotics in the perinatal period, maternal diet during pregnancy, gestational age or smoke during the pregnancy.

2.1.1 Antibiotics during pregnancy

Regarding the use of antibiotics, initial epidemiological evidence indicates that disrupting microbial exchange through the use of antibiotics in pregnancy may increase offspring risk of childhood obesity and asthma [54]. As many reviewed resume, antibiotic over usage can significantly disrupt the overall ecology of the gut microbiota, alter the abundance of resident gut bacteria, potentially bias the child toward certain diseases and conferring antibiotic resistance in infancy [39,55]. In one of the previous studies carried out in mice, it was observed that prenatal antibiotics decrease the diversity and structure of the microbiota [56]. In Western countries, treatment with antibiotics during pregnancy is widespread, representing 80% of the medications prescribed in pregnancy. However, although antibiotic treatment can sometimes save lives, it can also have harmful consequences. The use of antibiotics in the prenatal period, it has been associated with delayed colonisation by some microbes especially *Bifidobacterium* and *Lactobacillus* species [57–61]. Also, a study carry out by Muller *et al*., in 2015 [62] found that children exposed to prenatal antibiotics in the second or third trimester had 84% higher risk of obesity compared with unexposed children. Kuperman and Koren in 2016 [63] reported an increase in vaginal colonization by the species *Staphylococcus* due to the use of antibiotics during pregnancy and also observed potential microbial changes in other areas than the intestine which could be related to an increased risk of allergies and obesity.

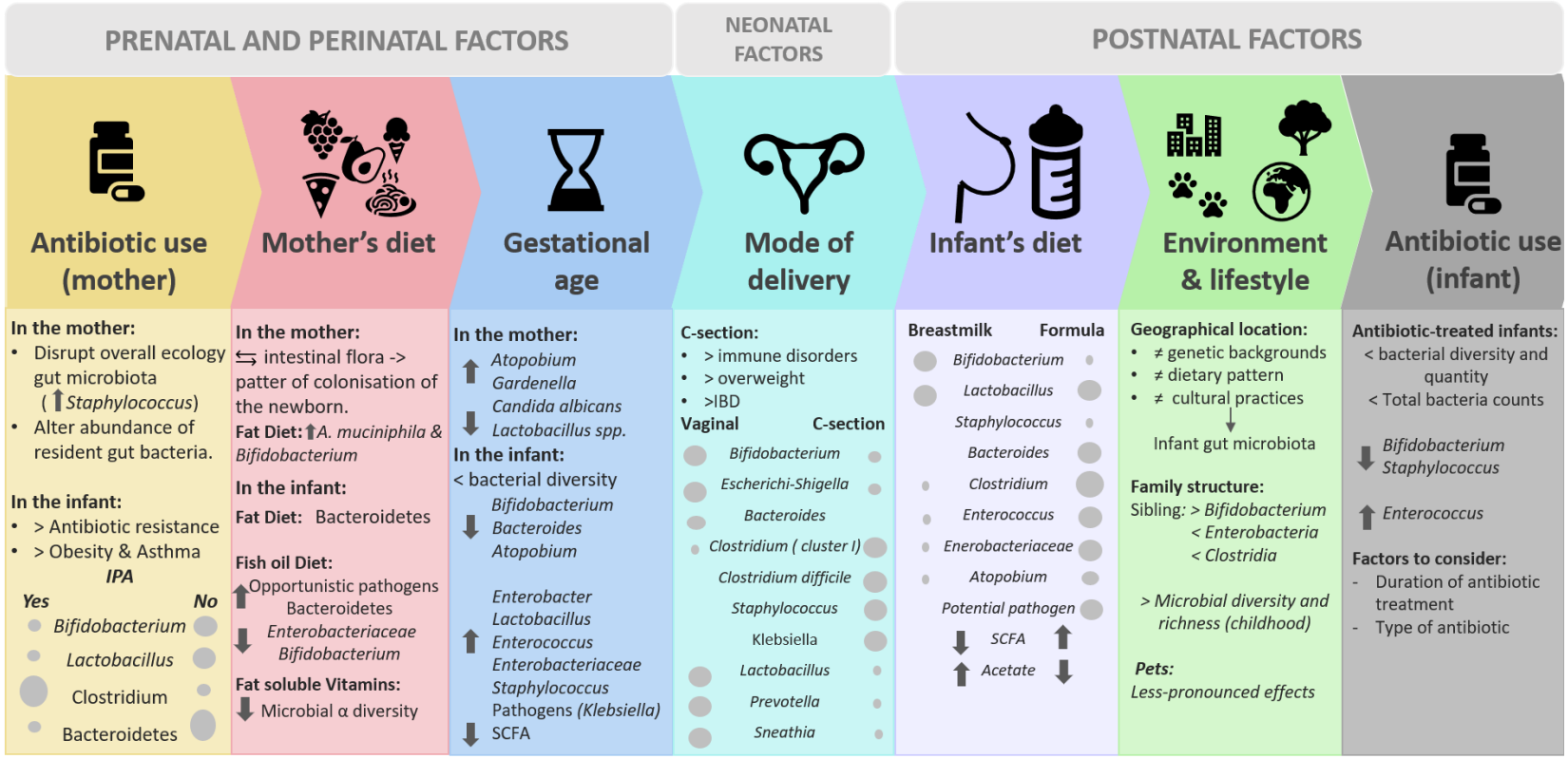


Figure 3. Main influential factors in the gut microbiota development. IPA: Intra partum antibiotic. SCFAs: Short chain fatty acids.

During the perinatal period the main cause of antibiotic exposure is the use of intrapartum antibiotic prophylaxis (IPA) in over 30 % of total deliveries [64]. Mothers are given IAP as standard treatment for prevention of vertical transmission of Group B *Streptococcus* (GBS) to neonates. This standard of care treatment is a window of opportunity to examine how antibiotics can affect the infant microbiome [65]. It has been reported in recent studies that IPA affects the development of later microbiota in the newborn [60,61,66–69]. In a longitudinal prospective birth cohort, the bacterial community in early life of infants exposed to IPA were different from that infant who were not exposure at 10 days and 6 week of age and these differences disappear at 12 weeks [70]. The authors observed that Actinobacteria had a delay pattern of colonisation in VDI exposed to IPA and in CS infants. Also, the time of exposure to treatment influences the pattern of colonisation, in this study there was a decrease of 7.2 % in the abundance of *Bifidobacterium* and a positive effect on the abundance of *Clostridium* for each hour of IPA administration during vaginal birth. Aloisio *et al*., in 2016 [71] observed significant differences in the microbial composition in newborns whose mothers received IPA during delivery, with increased level of Proteobacteria and a lower number of Actinobacteria and Bacteroidetes. A similar pattern of lower relative proportions of Bacteroidetes and Actinobacteria and a higher abundance of Proteobacteria and Firmicutes was also observed by Nogacka *et al*., [64]. These findings underscore the fact that the consumption of antibiotics by mothers during pregnancy can affect the gut microbiome of the newborn, which in turn can affect the health and development of the infant. Therefore, this factor must be carefully observed and controlled.

2.1.2 Diet and nutrition during pregnancy

It has recently been reported that the maternal diet during pregnancy has a key impact on both the maternal and infant microbiota in a delivery mode-dependent manner [72–74]. As we have commented previously, the maternal intestinal flora is the major source of a healthy microbiota for the newborn, and the diet during pregnancy is a factor that can modify that microbiota, and therefore the pattern of colonisation of the newborn *in the utero* and subsequently during delivery and early life. Moreover, the changes in the mother's microbial composition can alter the

abundance of genes that promote various metabolic processes during pregnancy [75] also affecting the microbial composition of the newborn. Several previous studies, in addition to demonstrating a clear association between the diet and the intestinal microbiome, have also successfully demonstrated the important role that maternal dietary modulations have since they can influence changes in the intestinal microbiome [74–76].

A high fat diet during pregnancy and its effects in offspring is one of the most studied diets among researchers [75–78]. In 2014, Ma *et al*., [77] concluded, in a primate model, that maternal consumption of high fat diet during pregnancy or post birth results in dysbiosis of the neonatal intestinal microbiome. They observed the dominance of Bacteroidetes and an increased in the abundance of *Prevotella* in the gut of dams who were fed with a high fat diet [77]. Gohir *et al*., in 2015 [75], in another study carried out in mice, studied how the pup's intestinal microbiota is affected by the maternal diet before and during pregnancy. The authors observed significant changes in the composition of the gut microbiota later in pregnancy in female mice fed on high fat diet compared to those fed on a normal food diet. More concretely, they observed higher levels of *Akkermansia muciniphila* and *Bifidobacterium* in mothers who were fed with a high fat diet before and during pregnancy. Similar to the results obtained in mice, Mann *et al*., [78] demonstrated in rat that during pregnancy, the gut microbiota is altered in a similar manner to that which occurs in women, and that these changes are further exaggerated by exposure to a high fat diet. Furthermore, Mann *et al*., [78] also found a significant increase in relative abundance of *A. muciniphila* in mothers who were fed with a high fat diet but not in *Bifidobacterium* levels. In 2016, Chu *et al*., [76] carried out a study in humans and demonstrated that independently of the maternal body mass index, a high fat maternal diet alters the neonatal gut microbiome. They observed a reduction in Bacteroides levels in infants born to mothers who consumed a high fat diet during pregnancy.

Some authors have focused their research on how intake or exposure to certain specific foods such as fish oil influence the microbiota. For example, Gibson

et al., in 2015 [79] compared the intestinal microbiota among offspring of rats born to mothers fed either n-6 polyunsaturated fatty acid (PUFAs), n-3 PUFAs or chow. The authors concluded that there is an alteration in the development of the intestinal microbiota and a general reduction in microbial richness due to maternal exposure to a diet rich in PUFAs. The authors also observed an increase in the level of Bacteroidetes in the gut microbiota of offspring born to mothers who had consumed a diet rich in fish oil and also a reduction of *Enterobacteriaceae* and *Bifidobacteria spp.* when compared to the chow group and an abundance of taxa of opportunistic pathogens like *Bacteroides fragilis*, *Bilophila wadsworthia* and *Enterococcus faecium* [79]. Mandal *et al*., [80] studied, in 60 women in the second trimester of pregnancy, the relationships between the intake of macro and micro nutrients in the diet during the pregnancy period through the use of food frequency questionnaires and observed, after delivery, taxonomic differences in their gut microbiota. They concluded that some of the most important modulators of the mother's gut microbiota are fats and fats soluble vitamins. Specifically, they observed that a higher dietary intake of fat-soluble vitamins, especially vitamin D, were associated with reduced microbial alpha diversity. In addition, vitamin D, mono unsaturated fat, cholesterol and retinol were associated with higher levels of Proteobacteria, a phylum known to be associated with multiple pathogens and to have pro-inflammatory properties. On the other hand, the authors observed that saturated fats, vitamin E and proteins were associated with a relative reduction in Proteobacteria levels. Newly, Meyer *et al*., in 2017 [81] in a longitudinal study, using two dietary treatment, showed that replacements in the mother's diet during pregnancy (such as changing the intake of fats to carbohydrates or changing the consumption of specific sugars) was related with significant changes in the milk microbial pattern and HMOs composition. Meyer and colleagues suggested a possible role of maternal food habits during and post pregnancy on infant gut microbiota development given that, as previously mentioned, the breast milk microbiome and HMOs come in direct contact with infants during breastfeeding and thus can modify their gut microbial composition.

As Kumbhare *et al*., reviewed recently [55], alcohol consumption during pregnancy has been associated, for more than 50 years ago, with various disorders

in the newborn and premature delivery. It is well known that alcohol consumption alters the intestinal microbial composition including the mother's intestinal microbiota during pregnancy. The initial intestinal colonization of the infant can be affected by such changes in the maternal intestinal microbial composition, making it more predisposed to infections and diseases later in life. In general, all these studies emphasize that the maternal diet during pregnancy is a very influential factor in the development of the intestinal microbiota of both the mother and the newborn, and therefore, a key in the health of the infant during the first years of life.

2.1.3 Gestational age

One of the most important perinatal factors in the establishment of the infant gut microbiota is the gestational age. The World Health Organisation (WHO) [82] defined preterm births as those occurring before 37 completed weeks of gestation and usually have very low birth weight. During the pregnancy period, microbial diversity within the vaginal microbiota decreases, while members of *Lactobacillus* species increase, potentially reinforcing their protective function. However, infections caused by bacteria, viruses, and fungi during the pregnancy period have been treated a leading cause of intrauterine growth restriction and preterm birth. Certain infectious agents can reach the amniotic fluid, establish an intra-amniotic infection and initiate an inflammatory response at the maternal and foetal tissues, which promotes pre-labour rupture of membranes and preterm birth [83]. Bacterial communities characterized by high levels of *Atopobium*, *Gardnerella* and *Ureaplasma* as well as lower levels of *Lactobacillus spp.* or a higher presence of *Candida albicans* have been found to be correlated with preterm birth (PTB) [83].

PTI have to overcome serious health challenges. In most cases, and depending on the degree of prematurity, PTI have a degree of immaturity in the digestive system and respiratory and immune and neurological problems. Because of that, in most cases, a hospitalisation is necessary for the application of treatments such as the intensive use of antibiotics and other medications, and/or even the use of artificial respiration and feeding such as sterile parenteral nutrition. Also, in most cases the births of premature infants are usually by C-section and therefore they

never come in contact with the mother's vaginal microbiome. All these factors can interfere in the process of colonisation and the correct development of the gut microbiota, resulting in an unusual establishment and a different or deviating composition with increased colonization by pathogenic microorganisms.

The patter of colonisation in PTI is characterized by a reduce in bacterial diversity [16,84]. Several authors have studied the gut microbial colonisation in preterm and FTI and reported that PTI showed a reduced level of strict anaerobes such as *Bifidobacterium*, *Bacteroides*, and *Atopobium*, and high levels of facultative anaerobes like *Enterobacter*, *Lactobacillus* and *Enterococcus*. Also, it has been observed an increased abundance of *Enterobacteriaceae* family and *Staphylococcus*, and a higher colonisation by pathogens, such as *Klebsiella* [11,66,84,85]. Additionally, Hill *et al*., in 2017 [34] in the INFANTMET cohort, using 16S rRNA amplicon Illumina sequencing and bacteriological culture, observed that at the phylum level Proteobacteria and Firmicutes are among the phyla that dominate in PTI when compared to FTI. As a result of the alterations in the composition of the intestinal microbiota, differences have also been observed in the main microbial metabolites, the SCFAs, whose concentration in faeces is higher in FTI than in premature neonates [85].

2.2 Neonatal factors

2.2.1 Mode of delivery

The mode of delivery has been recognized as an important driver of the early gut microbiota composition in FTI and possess a big impact on the type of microbiota ingested during the birth by the newborn. Several studies have shown that the human neonatal microbiota across all body habitats (skin, oral, nasopharyngeal, and gut) is influenced by their mode of delivery [21,86,87]. As we have indicated previously, recent studies suggest that during the pregnancy period occurs a transfer from the mother to the foetus *in utero* [38]. However, during the labour and immediately after birth, microbes from the mother and surrounding environment colonize the gastrointestinal tract of the infant leading to the development of a dense

complex microbiota, being the first major exposure of the neonate to microbes [53]. VDI come into contact with the maternal vaginal and faecal microbiota, which results in neonatal gut colonisation by vagina-associated microbes such as *Lactobacillus*, *Escherichia*, *Bacteroides*, *Bifidobacterium*, *Streptococcus spp.* and *Prevotella* [21,87–90]. In contrast, children born by CS are also exposed to their mother's microbiota, but initial exposure is most likely to non-maternally derived environmental isolates from equipment, air, and other infants, with the nursing staff serving as vectors for transfer (*Staphylococcus*, *Corynebacterium*, *Propionibacterium*) [21,53,86]. According to the numbers provided by the WHO, in Spain more than 25 % of births are performed by CS and globally, the number of CS has grown from 12 % in the year 2000 to 21 % in 2015 over the world [91]. The early colonisation patterns of CSDI differ greatly from children born vaginally and are less diverse than those delivered vaginally [21,39,86,92,93]. In particular, it was demonstrated that CSDI had minor amounts of *Bifidobacteria* [21,88], *Escherichia - Shigella*, and absence of *Bacteroides* [89,93] and are enriched with *Clostridium* (cluster I), *Clostridium difficile*, *Staphylococcus* species [87] and higher amounts of *Klebsiella* [94]. In contrast, VDI were characterized by *Bifidobacteria* [19], predominantly *B. longum* and *B. catenulatum* species [86,94] and vaginal-related microbes such as *Lactobacillus*, *Prevotella*, and *Sneathia* [21,86,87]. Also, Jakobsson *et al*., (2014) [93] showed that the gut microbiota of CSDI at 24 months of age is less diverse than those delivered vaginally. The authors hypothesize that this drop in diversity may be due to delayed colonisation of the gut by Bacteroidetes colonisation until 1 year of age. These differences between vaginally and CSDI gradually decrease, but CSDI remain more heterogeneous than VDI up to 12 months of life. In one recent research, that was interested in studying the longer-term impact of delivery mode on gut microbiota development over the first four years of life, has found that *Lachnospiraceae* was dominant in CSDI and *Parabacteroides* was found to be more unique to VDI at one and two years of age; however, by year four, *Lachnospiraceae* and other *Clostridium spp.* became more dominant in these VDI [95].

The differences observed in the microbiota between VDI and CSDI have been associated with the protective effect of vaginally delivered labour, particularly since it has been suggested that C-section has long-term health implications. In fact, C-section has been associated with an increased risk of immune disorders such as asthma [96–98], allergy [99], type 1 diabetes (T1D) [100], as well as an increased risk in overweight [101], a higher risk of development of inflammatory bowel disease [102] or an enhanced risk for developing celiac disease [103]. Moreover, different modes of delivery affected the ARG prevalence, what might influence the infant's health later in life [20]. The cause of this could be an inadequate development of the immune system since in several studies it has been observed that CSDI have a much lower level of Th1-related chemokines in their blood, which can translate into less protection [53].

On the other hand, there are authors who in their studies have not observed any association between CSDI and T1D or the development of celiac disease [50,103]. Despite these studies, it can be concluded that the relevance of early gut microbiota in the maturation and development of the host's immune system is supported by the finding that the mode of delivery influences the health status through adulthood, while the effects on gut microbiota composition decrease after the first years of life [7]. The most accepted explanation for the association of delivery mode with the development of disease is the gut microbial dysbiosis, but it is necessary to carry out more research to adequately support this hypothesis, as these remain association studies [39].

2.3 Postnatal factors

Some of the most important postnatal factors, that may influence the gut microbiota composition of a growing neonate are the mother's diet during breastfeeding, the mode of infant feeding, the introduction to solid food, environment and life style, the use of antibiotics and host genetics.

2.3.1 Diet during the first months of life

Mostly, breast milk (BM) is the first food to which the newborn is exposed and it is during breastfeeding when one of the most important links between the mother and the baby is formed. In spite of the fact that nowadays infant formulas (IF) are products increasingly similar to BM, if we compare them, large differences continue to be observed in all aspects, including nutritional value and composition. This is because BM has a very complex composition mainly due to the presence of various growth factors and enzymes, and also this composition changes over time (colostrum, transitional milk and mature milk) [42,104–107]. It has been reported, that BM and more specifically its bioactive compounds, such as HMOs, are beneficial for the infant since they not only help in a better development but also reinforce the immune system of the newborn [108,109], offer protection against allergies and asthma [110], and may also provide protection against different diseases such as coeliac disease, obesity, T2D, diarrhoea and many other metabolic disorders as Kumbhare *et al*., reviewed [55]. Initially the infants are fed with BM and/or IF and later it begins with the introduction of solid foods. The WHO recommends exclusive breastfeeding of infant up to six months of age in order to ensure that the growing infant receives the full nutritional benefits of BM [82].

The differences in the gut microbial composition between the infants who has been fed with BM and those who have been fed with IF are well documented [37,111]. The gut microbiome in formula-fed infants (FFI) exhibits higher diversity than that of breastfed infants (BFI) because are exposed to different carbohydrates, bacteria, and nutrients, causing different microbial colonisation patterns of the gut. In this context, different publications have reported that stools of BFI contain higher levels of *Bifidobacterium* species belonging to the phyla Actinobacteria, being *B. breve*, *B. longum*, *B. dentium*, *B. infantis* and *B. pseudocatenulatum* the most prevalent [23,112]. Also, BFI's stools contain higher levels of *Lactobacillus* and lower levels of potential pathogens than those infants feeding with IF. Infants exclusively feed with IF were more often colonized by *Staphylococci*, *Bacteroides*, *Clostridiidium* species, *Enterococci*, Enterobacteria, and the genus *Atopobium* [67,87,89,104,106,112–114]. As we mentioned earlier, a different pattern of

colonization also implies differences in microbiota metabolism and therefore SCFAs levels. The levels of SCFAs are also different in the stools of BFI versus FFI. Exclusive breastfeeding was associated with lower absolute concentrations of total SCFAs, acetate, butyrate, propionate, valerate, isobutyrate, and isovalerate, yet higher concentrations of lactate. Further, the relative proportion of acetate was higher with exclusive breastfeeding [115].

Although it is well established that early infant feeding has a major influence on the establishment of the gut microbiota, very little is understood about how the introduction of first solid food and the gradually replace of the milk-base diet influences the infant gut microbiota. As Laursen *et al*., reviewed in 2017 [116], a large number of longitudinal studies have showed a significant change in microbial composition around the stage of introduction to solid foods and cessation of milk-base diet but these longitudinal studies were performed in a limited number of infants. Bergström and colleagues in 2014 [47] carried out a large Danish cohort ("SKOT study") to study the faecal microbiota of 330 infants at 9, 18 and 36 months of age. The authors observed during the period characterized by transition from breastfeeding/formula feeding to a family diet, an increase in the levels of *Lactobacillaceae Bifidobacteriaceae*, *Ruminococcaceae*, and *Bacteroidaceae* species. Fallani *et al*., [46] in a European cohort formed by 531 infants from five different countries, observed similar results and demonstrate that, regardless of differences in geographic location, antibiotic use, delivery mode and milk feeding practices, there are consistent changes in the microbial composition of infants. Specifically, the authors observed a decrease in *Bifidobacteriaceae*, *Enterobacteriaceae* and *Clostridiaceae* while increasing in *Ruminococcaceae* and *Lachnospiraceae* species starting at 6 weeks up to 4 weeks after introduction of solid food. These results are in agreement with those found by Thompson *et al*., in 2015 [117] who also observed an increase in the diversity in the intestinal microbiota of infants both in exclusively BFI and non-exclusively BFI (n=10) after introduction of solid foods. In this study the authors observed a higher diversity and species richness in non-exclusively BFI compared with exclusively BFI. On the other hand, the pattern of exclusively BFI gut microbiota showed increased proportions of

Bifidobacterium and a decrease in abundance of Bacteroidetes and Clostridiales than non- exclusively BFI.

4.3.2 Environment and lifestyle

Geographical location has been described as a relevant environmental factor that may have an impact on the microbiota. Different ethnogeography populations have distinct genetic backgrounds, dietary patterns, and cultural practices. Of course, regions which have more resources also have access to better sanitation and healthcare than developing nations [39]. For example, De Filippo *et al*., in 2010 [118] compared the faecal microbiota of children from a rural African village of Burkina Faso and that of European urban children residents in Florence, Italy (EU). The reason to select these two populations was that the diet of the children from Burkina Faso is characterized by the consumption of grains, legumes and high-fibre diet of vegetables with the absence of processed food and furthermore, it represents a society similar to that of early human colony at the Neolithic era. EU children have a western diet, which is characterized by a high consumption of animal fats, sugars and a greater caloric intake. Burkina Faso children were dominated by Bacteroidetes and showed a depletion in Firmicutes population. In addition, they found significantly high levels of SCFAs in Burkina Faso than in EU children. Subsequently, further studies have continued investigating and comparing the differences between developing *vs*. developed countries including a large cohort of paediatric and adult samples from the Amazonas of Venezuela, rural Malawi and urban United States areas. *Bacteroides* predominated in the North American samples, whereas *Prevotella* predominated in Malawian and Venezuelan samples [37]. As well as, there were many more differences in the samples from USA than between Malawian and Venezuelan microbiota and these differences were evident in children older than 3 years of age. Three years later, Lin *et al*., [119] observed the same *Prevotella-Bacteroides* split when the authors compared American children living in wealthy neighbourhoods with children living in a Bangladesh slum. Another research comparing southeaster African and northern European infants reported a different bacterial group composition, with the group *Prevotella-Bacteroides* and the genus *Bifidobaterium* being present at a higher abundance in African children [120]. In

Spain, Echarri *et al*., [121] evaluated the microbiota of 40 breastfed FTI from two different Spanish locations, one in the northern Atlantic coast and the other in the south-east Mediterranean coast, at different stages (faecal samples were collected at 8, 30 and 90 days of life). The authors showed a high inter-individual variability on the levels of the different microbial groups. However, despite this variability their results showed statistically significant higher levels of *Bacteroides* and *Staphylococcus* at 8 days of age and lower levels of *Enterobacteriaceae* in infants from the Mediterranean coast than in infants from northern Spain. Similar levels of *Enterococcaceae*, Clostridia XIVa and IV clusters, *Atopobium*, *Bifidobacterium* and *Lactobacillus* were found between both groups at 90 days of life. When the different sampling times were not taken into account, lower counts of *Lactobacilli* and higher counts of *C. leptum group*, as well as significantly higher levels of *Bacteriodes* and *Staphylococcus* were observed in infants from the south of the country compared to those on the north coast. These differences in colonisation patterns have also been observed in comparative studies between different countries belonging to the same continent and not only have been observed between juxtaposed populations as developing *vs.*. developed countries. For example, it has been reported that infants from Northern areas from Europe have higher levels of *Bifidobacterium spp.* and some *Clostridium spp.* and *Atopobium spp.*, while Southern European infants had a higher abundance of *Eubacteria*, *Lactobacillus*, and *Bacteroides* [89]. Also, other authors have showed significant differences between the gut microbiota of European infants from different countries [122,123]. For example, between Estonian and Swedish infant [123] or between German and Finish ones, where German infants showed a higher abundance of the *Bacteroides-Prevotella* group and *Akkermansia muciniphila* but the proportion of *Bifidobacterium spp.* was higher in the Finish infants [122].

Among the geographical location, family structure and lifestyle may influence the model of colonisation in infant gut microbiota and have also been describe as a relevant environmental factor [17]. Despite this, it is necessary to establish more decisive evidence about the effects of family structure, size, and birth order in the pattern of colonisation [124]. Infants without sibling, who was recruited from the

Child, Parent and Health: Lifestyle and Genetic Constitution (KOALA) Birth Cohort Study, had slightly lower numbers of Bifidobacteria, compared with infants with older siblings [87]. These results agree with what was reported by Adlerberth *et al*., in 2007 [125] in another study as part of the ALLERGYFLORA study. In this study, infants with older siblings had lower proportions of Enterobacteria, other than *Escherichia coli*, as well as clostridia in the gut but also a higher anaerobe/facultative anaerobe ratio. In a recent study performed with a Danish cohort, the presence of older siblings was shown to be associated with increased gut microbial diversity and richness during early childhood, while the presence of household pets had less-pronounced effects on the gut microbiota [90]. Overall, geographical location (dietary patterns and lifestyle in a specific area and family structure (siblings) it has been seem that affect colonisation of the gut microbiota during early life, although more studies are needed to determined more accurately the factors that influence or contribute to a greater extent.

2.3.3 Antibiotics during early life

Antibiotic usage in early life, a period of critical window, can also disrupt the normal pattern of colonisation affecting the growth of otherwise dominant bacterial phyla in the human gut. These alterations can remain for long periods of time, spanning months and even years with partial or complete recovery [55] and lead to greater susceptibility to numerous diseases later in life [87,89,126–128] such as asthma [129,130]. Yassour *et al*., in 2016 [131] carried out a longitudinal study to research changes in the gut microbiome from 39 infants, half of whom received, during the first 3 years of life, multiple courses of antibiotics. The microbiota of antibiotic-treated children was less diverse in terms of both bacterial species and strains, with some species often dominated by single strains. In addition, they observed short-term composition changes between consecutive samples from children treated with antibiotics. In addition, to studying the effect of the use of antibiotics in the gut microbiota of infants, other authors have reported differences in the effect of the use of antibiotics depending on the type of antibiotic provided. For example, Korpela and colleagues [132] observed in 2-7 year-old Finnish children (n=142; sampled at two time points) that the use of macrolides is associated with a

change in the microbial composition, reducing diversity and metabolism of the microbiota that also proved to be durable. Specifically, after one year of administration of the course of antibiotics, the authors observed the restoration in the levels of *Bifidobacterium* and *Bacteroides* and antibiotic resistance and also concluded that Penicillin imprint a weaker mark on the microbiota in comparison with macrolides [132]. Another factor to consider is the duration of antibiotic treatment. About this theme, one study has investigated how short (≤ 3 days) or long treatment (≥ 5 days) may influence the gut microbiota of the infant in early life. Zwittink *et al*., [133] observed a depletion in infant gut *Bifidobacterium* levels after a short treatment of antibiotics until the third week of life. In long treatments, this depletion in *Bifidobacterium* remains decreased till sixth week postpartum. In both type of treatment with antibiotics, *Enterococcus* became the dominant genus of the microbial community. These results are in line with other authors [95,114,134]. Specifically Martin *et al*., [114] observed a slightly depletion in total bacterial counts and also decreased levels of *Bifidobacterium* and *Staphylococcus* in infants who received antibiotics. These results suggest that the first moths of life are indeed a critical window where the infant gut microbiota could be influenced by the use of antibiotics and link these changes with later diseases. However, more researches are necessary to understand the impact of antibiotic treatment and their long-term effects. Moreover, issues related with the increasing number of pathogenic strains and their antibiotic resistant should be taken into account.

3. The early appearance of asthma and their relationship with gut microbiota

3.1 Childhood asthma and the development of the immune system

Asthma is the major non-communicable disease affecting both children and adults, with high morbidity and relatively low mortality compared with other chronic diseases [135]. It is a disease with many clinical phenotypes and its principal characteristics include a variable degree of airflow obstruction, bronchial hyper-responsiveness, and airway inflammation that leads to recurrent episodes of

wheezing, breathlessness, chest tightness, and coughing, particularly at night or in the early morning [136,137], and severity ranges from occasional symptoms to disabling persistent symptoms and/or frequent life-threatening exacerbations [138]. However, in children <5 years of age, clinical symptoms of asthma are variable and non-specific [137]. Asthma and the allergic diseases of allergic rhinitis (hay-fever), atopic dermatitis (eczema) and immunoglobulin E-mediated food allergy are closely associated, the likelihood of developing asthma being increased by a personal or family history of allergic disease [138]. There are two types of asthma, non-allergic and allergic. Both of them have similar manifestations of airway hyper-responsiveness and inflammation, mediated by different mechanisms. By definition, allergic asthma is associated with an allergen sensitisation, hypersensitivity mediated by immunoglobulin E (IgE) and a type-2 immune response (Th2). On the other hand, non-allergic asthma is associated with a neutrophilic inflammatory response and a Type-1 (Th1) and Type-17 (Th17) cytokine profile. The mechanism of asthma is initiated by mast cell activation in response to allergen binding IgE receptor and Th2-cells [136]. This leads to mast cells producing and releasing cytokines and recruiting eosinophils and other inflammatory cells. The activation of these cells triggers the release of mediators, among which is interleukin 4 (LTC4), which contracts the smooth muscle of the airways. Current therapy for asthma is based on the achievement of several objectives, such as relaxation of the smooth muscle of the airways and the prevention and reversal of inflammation [139]. Epidemiologic data have shown that over the last few decades the prevalence of asthma has increased at an incidence of 1.4 % per year in the paediatric population (0-17 years) and in general, the risk of developing asthma is highest for children during the period between birth and four years of age [140,141]. Asthma is estimated to affect approximately 300 millions of people worldwide and it is likely that by 2025 a further 100 millions may be affected [142]. Asthma also incurs significant health care expenditure, and is one of the most common chronic diseases. Uphoff *et al*., in 2017 [143] examined variations in prevalence rates of childhood asthma, wheeze and wheeze with asthma in different countries around Europe and the authors found that asthma prevalence varied from 1.72 % in Germany to 13.48 % in England at 4

years of age, and the prevalence of wheeze varied from 9.82 % in Greece to 55.37 % in Spain. In the International Study of Asthma and Allergies in Childhood (ISAAC), the global prevalence of wheeze was estimated to be 11.6 % for 6–7-year-old children [144].

The hygiene hypothesis and the “lost microbe” hypothesis contemplate that the loss of specific bacteria from the modern day human microbiota has been due to our increasingly hygienic lifestyle, C-section deliveries, and excessive use of antibiotics [145,146]. Due to the dramatic changes in lifestyle in the last century [145], the human microbiota has suffered a loss of diversity and that loss is one of the causes in the recent increase in the prevalence of asthma [141]. However, it fails to explain the coexisting epidemic in autoimmune disease or the high rates of asthma. This limitations has motivated the continued search for alternate explanations such as the Developmental Origins hypothesis for Health and Disease (DOHaD) or “perinatal programming” which proposes that nutritional and other environmental stimuli can “program” developmental, metabolic and immune pathways during critical periods of prenatal, and postnatal development, and subsequently induce long-term changes in metabolism and susceptibility to chronic diseases including asthma [147]. It is known that the development of the immune system begins in utero and continues during the first years of life. A Th2 response is promoted in the foetal immune system by the maternal environment during pregnancy, which are thought to protect the foetus from immunologic rejection by the mother, and it is after birth when occur a transition to a non-allergic Th1 phenotype. If during early postnatal life this transition is delayed or damaged, there is an increased risk of atopic disease including atopic dermatitis, allergic rhinitis, allergic conjunctivitis, anaphylaxis and asthma [147]. Gut microbes have been shown to induce regulatory T-cells that help guide the host’s Th1/Th2 balance, and recognition of microbiota-derived peptides by mucosal receptors has been shown to enhance systemic innate immunity [148] also two studies in germ-free mice show how, during the postpartum stage, the immune system produces an immune response directed by Th2 cells but after restoration of the intestinal microbiota, there is a shift towards a normal immune phenotype, dominated by type cells Th1 and

Th17 [149]. All of this suggests that the intestinal microbiota plays a key role in establishing the balance between the Th1/Th2 phenotypes during early stages of life [149,150]. Microbial metabolites, including SCFAs, could influence the development of asthma, since their composition in maternal and infant feeding has been associated, however inconsistently, with childhood asthma [151]. A growing body of epidemiological and microbiological literature supports the hypothesis that the genesis of allergic disease, and by extension asthma development, may lie at least in part in the communities of microbes that exist in the gastrointestinal tract [140]. Some factors has been identified as related to atopic disease development in childhood such as, intrauterine exposure, specifically exposure of antibiotics and Mediterranean diet adherence (MDA) [152], early life antibiotic exposure [127], C-section [50,98,99,153–155], formula feeding or lack of exposure to pets during pregnancy.

3.2 Gut-lung Axis: Mourine and longitudinal human studies define the early life critical window

Within the scientific community, a new concept has been established, the “gut-lung axis” which attempts to mechanistically define how microbes in the gut might influence immune function in the lung [156]. As Stiemsma and colleagues reviewed [157], one of the main connections occurs through interactions of the intestinal microbiota with pattern recognition receptors of the innate immune system. It is well known that Toll-like receptor (TLR) signalling can be stimulated by pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS) and peptidoglycan. This stimulation confers downstream activation of many genes that regulate inflammation and innate immune responses. Dendritic cells (DCs) are also the intermediaries of gut microbiota-immune cell cross talk in a similar way to the antigen-recognition and IgE-mediated hypersensitivity pathways, since they regularly take samples of gut microbes in the intestinal lumen or lymphoid tissues. The detection of intestinal microbiota PAMP by DCs promotes immune tolerance in the intestine, but also produces phenotypic changes in DCs and its migration to the mesenteric lymph node (MLN) to promote the preparation of T cells. In the MLN, T

cells acquire targeting molecules (e.g., CCR4, CCR6), which initiate migration to other parts of the body, including the respiratory mucosa of the respiratory tract. Therefore, it is possible that varying phenotypic changes occur in CDs due to interactions with specific gut microbes, through their corresponding PAMPs, which would produce subsequent effects on lymphocyte priming/homing and therefore, changes in anti-inflammatory responses in the airways.

Another area of gut-lung axis research involves microbial-derived metabolites, such as SCFAs. These metabolites are known to modify gene expression through inhibition of histone deacetylases (HDACs), cytokine and chemokine production, and cell differentiation, proliferation, and apoptosis [151]. SCFAs functions as HDACs inhibitors and ligands, predominantly agonist, of G protein-coupled receptors (GPCRs). The ability of SCFAs to inhibit HDACs generally promotes a tolerogenic, anti-inflammatory cell phenotype necessary for the maintenance of immune homeostasis [158]. SCFAs can also modulate T cells, particularly regulatory T cells (Tregs), through HDAC inhibition [157]. Diverse studies, characterizing the ability of specific SCFAs to regulate the quality of the colonic Treg cell pool, have shown that propionate and butyrate induce FoxP3 (a transcription factor which, following exposure to allergens, is critical for the conservation of immune system homeostasis and responsible for the suppression of Th2 responses [159]) in an HDAC-dependent manner, while acetate is less effective [160,161]. Clostridia species are large producers of SCFAs and their production of butyrate has been associated with the generation of peripheral Tregs in the colon [161]. In a house dust mite (HDM)- mouse model of experimental asthma, both acetate and propionate, after HDM exposure, were capable of reducing cellular infiltration into the airways [162]. In a later study using the same asthma mouse model, maternal intake of acetate was shown to reduce allergic airways disease in the adult offspring of mice [163]. In both studies the authors demonstrated that a reduced severity of allergic airway inflammation in offspring were associated with an increase in the intake of fermentation of microbiota-accessible carbohydrates (MACs), during the pregnancy period.

Animal model studies have made significant advances in the search to understand the gut-lung axis and in many of them it has been found that the result is time sensitive, which leads us to the concept " early life critical window". These studies, to understand the gut-lung axis, have been based on the identification of large-scale changes in intestinal microbial compositions in asthma and allergy-induced mice, and in the manipulation of the intestinal microbiome with antibiotics, increasing the severity of these diseases [164–167]. Cahenzli *et al*., [167] demonstrated that an increased microbial diversity in early life is required to regulate IgE production and decrease disease severity in a mouse-model of antigen-induced oral anaphylaxis. Lyons *et al*., [164] showed that perinatal exposure to *B. longum* induces Tregs and protects against allergic airway inflammation in adult mice and Russell *et al*., [165,166] showed that perinatal exposure to antibiotics differentially exacerbates lung disease in adult mice. Specifically, the authors showed that perinatal (in utero and up to 21 days after birth) versus strictly prenatal (in utero) vancomycin treatment of ovalbumin (OVA)-challenged mice exacerbate asthma-related immune responses.

The gut-lung axis also is supported by different longitudinal studies conducted (**figure 4**), such as in the Canadian Healthy Infant Longitudinal Development (CHILD) cohort, in which it has been shown that intestinal microbiota profiles differ between infants who develop or not asthma [33,168,169]. Nylund *et al*., in 2013 [170] in a prospective study, found lower abundance of Bacteroidetes and greater abundance of *Clostridium* clusters IV and XIVa at 18 months in children subsequently diagnosed with eczema. Similar results were founded by Abrahamsson *et al*., [168] who using 454-pyrosequencing in faeces from a Swedish cohort of 40 infants associated atopic eczema at 2 years and asthma development at school age with a lower diversity of Bacteroidetes at 1-week and 1-month of age. Recent findings from the CHILD cohort study, using NGS, identified a higher ratio of Enterobacteriaceae to Bacteroidaceae at 3 months of age to be predictive of food sensitization at 1 year [171]. Arrieta *et al*., [33] in a nested case-control study of the same CHILD cohort, examined the gut microbiota of 319 infants and found that children with a high risk of asthma at school age (classified as those with atopy and

wheeze at 1 year) exhibited transient gut microbial dysbiosis during the first 100 days of life. Specifically, they identified decreases in the abundances of four bacterial genera, *Lachnospira*, *Veillonella*, *Rothia*, and *Faecalibacterim* in the 3-months faecal microbiota, which were associated with atopy and wheeze at 1 year. However other studies have identified shifts in specific bacterial taxa in early life. For example, In the KOALA Birth Cohort of nearly 1.000 infants, colonization with *C. difficile* at 1 month of age was associated with and increased risk of eczema, recurrent wheeze, allergic sensitization, and asthma by 7 years of age [172]. Also in other studies colonization with the pathogen *Clostridium difficile* has been associated with increased future risk of wheeze or asthma [173,174]. In 2016, Stiemsma *et al*., [175] found that *Lachnospira* remained decreased in the 3-month faecal microbiota while one particular bacteria species, *Clostridium neonatale* was increased in asthmatics at this time-point. The authors calculated a ratio of *Lachnospira* to *C. neonatale* (L/C) and showed that children with lowest L/C ratio (quartile 1) were 15 times more likely to be diagnosed with preschool-age asthma than children in the other L/C quartiles [175]. Interestingly, both in the studies carried out by Arrieta *et al*., [33] and Stiemsma *et al*., [175] the authors identified the gut microbial changes only in the first 3 months of life, highlighting this time frame as the early life critical window during which gut microbial dysbiosis is most influential in promoting asthma and atopic disease in humans. With all these studies, it is becoming more evident that gut microbial dysbiosis is promoted in human atopic diseases and that is characterized by taxa-specific shifts in abundance at the family, genus, and even species level and not by global changes to the composition of the intestinal microbiota [33,169,175], and also that the “critical window” to identify these intestinal microbial changes in humans could only cover the first 100 days of the new-born’s life. To complete this idea and as we showed above, several factors can modify the profile of the intestinal microbiota and therefore cause a state of intestinal microbial dysbiosis, being only some of them proposed to explain the increasing prevalence of asthma. In the next section we will focus on some of those factors that can modify the intestinal microbiota profile and therefore favour or inhibit the risk of asthma during later stages of life such us the diet during the pregnancy period and the adherence to a

Mediterranean diet, early life antibiotic exposure and breastfeeding during the first three months of life.

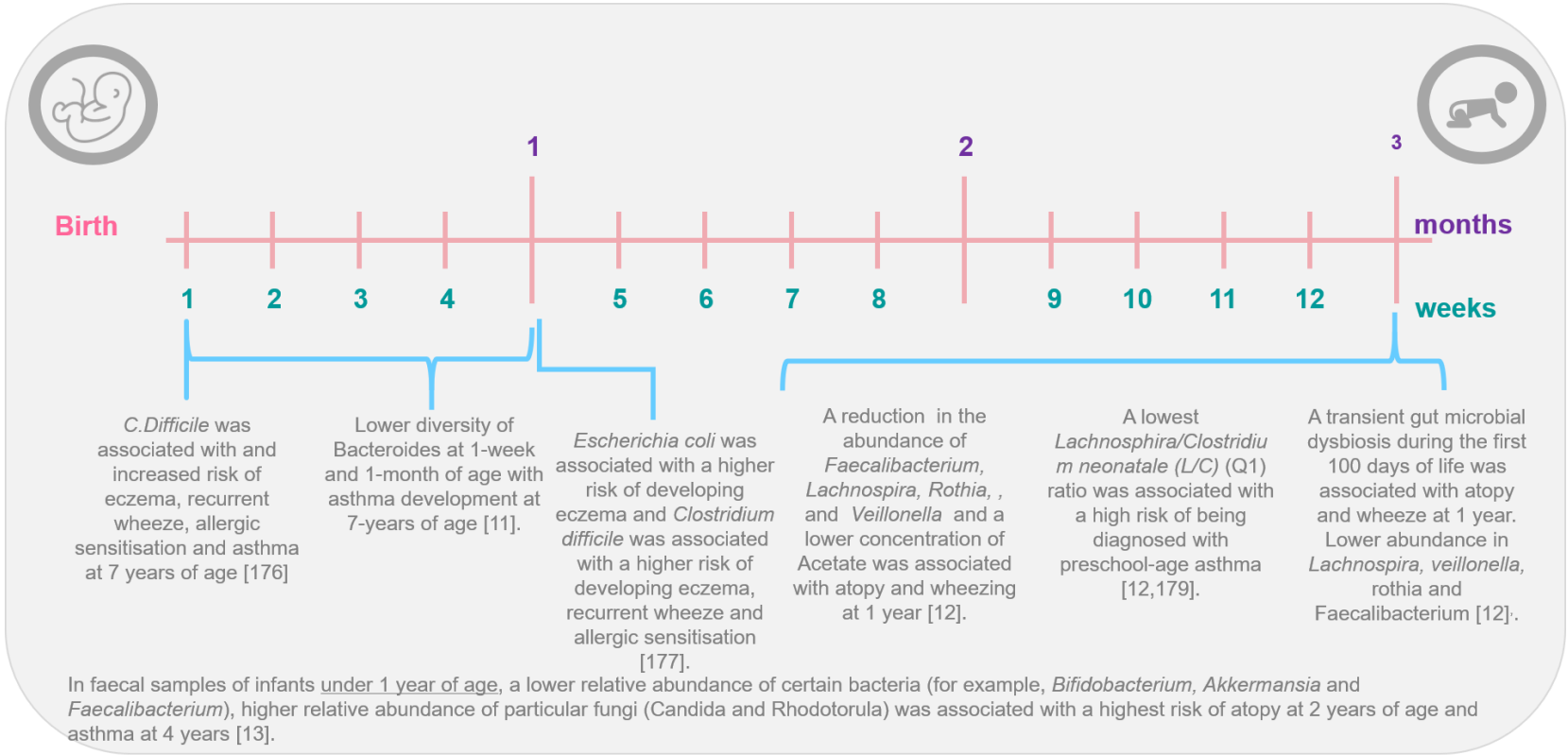


Figure 4. The gut-lung axis: Defining the critical window in early life. Modified from Stiemsma *et al.,* [157]

3.3 Factors related to allergic asthma development during early life

3.3.1 Influence of maternal diets during the pregnancy period and adherence to Mediterranean diet (MD) on the development of asthma

The relationship between a mother's diet during pregnancy and the child's subsequent risk of developing asthma or atopy has become a topic of growing investigation. Amati *et al*., [176] in their review defined the MD and MDA as a "diet characterized by a high intake of fruits, vegetables, whole grain cereals and bread, legumes, fish and nuts; low-to-moderate consumption of dairy products and eggs, and limited amounts of red meat. It is low in saturated fats and high in antioxidants, fibre and mono and polyunsaturated fatty acids (PUFAs) mainly derived from extra virgin olive oil and oily fish (n-3 PUFAs)". The MD is known to have many beneficial effects and a protective effect against diseases, demonstrated in numerous reviews [177–179], high-quality studies and meta-analyses, making it the most widely studied and evidence-based dietary approach to healthy eating and disease prevention [180–182]. For example, in 2008, Chatzi *et al*., [152] found that adherence to a MD during pregnancy was associated with protection from persistent wheeze and atopy in children. Nurmatov *et al*., [178] concluded that adherence to a MD was protective for persistent wheeze and atopy and García-Marcos *et al*., [177] in another systematic review, assessed whether adherence to MD was associated with the prevalence of 'current wheeze'; 'current severe wheeze'; or 'asthma ever' and the authors concluded that Adherence to the MD tended to be associated with lower occurrence of the three respiratory outcomes. Castro Rodriguez *et al*., [183] in a review concluded that the adherence to MD by the mother during pregnancy showed some protective effect on asthma and/or wheeze symptoms in the offspring only during the first year of life, but not afterwards. Only very few studies showed a protective effect on wheezing, current sneeze, and atopy, and none on eczema. These results coincide with those found by Yin Zhang *et al*., in 2019 [179], in a systematic review and meta-analysis of observational studies. The authors concluded that a high adherence to the MD during pregnancy may have short-term effects on wheeze in in the first 12 months.

Focusing on the different food groups that make up the MD, a frequent maternal intake of fish during pregnancy also reduced the risk of food and possibly inhalant allergic sensitizations [184]. Miyake *et al*., in 2010 [185] investigated the association between maternal intake of vegetables, fruit, and selected antioxidants during pregnancy and the risk of wheeze and eczema in the offspring aged 16-24 months. Higher maternal consumption of green and yellow vegetables, citrus fruit, and beta-carotene during pregnancy may be protective against the development of eczema in the offspring. Higher maternal vitamin E intake during pregnancy may reduce the risk of infantile wheeze. Recently, in a study carried out by Baïz *et al*., in 2019 [186] the authors observed a significant inverse association between the consumption of cooked green vegetable before pregnancy and childhood asthma and for the first time, a significant positive association was found between meat intake during the pregnancy period and a risk of wheezing, allergic rhinitis and atopic dermatitis.

If we focus more specifically on macronutrients and micronutrients that make up a diet, there are current evidences for an association between the ingestion of certain nutrients during pregnancy and asthma, wheeze, or atopic conditions in childhood. One of the micronutrients present in MD are PUFAs. Omega-3 (n-3) long-chain PUFAs and omega-6 (n-6) long-chain PUFAs are mostly evaluated for the prevention of asthma. The n-3 PUFAs are found mainly in fish oil and n-6 PUFAs are mostly found in margarine and vegetable oil. It had been hypothesized that an intake of higher n-3 and lower n-6 PUFAs contributed to a decrease in the instance of asthma [138,187]. Also, it is known that n-3 PUFAs influence immune functioning and may affect the cytokine phenotype during its development. Salam *et al*., [188] conducted an study to test if maternal fish consumption during pregnancy may affect children's asthma risk by modulating early-life immune development. They authors found that in children born to mothers with a history of asthma, the Odd ratio (OR) of asthma was 0.20 when mothers ate oily fish at least monthly during pregnancy compared with no consumption. However, this effect was not appreciated in the group of children of non-asthmatic mothers. In contrast, fish stick (a source of trans-fats) consumption during pregnancy increased asthma risk in children. Miyake *et al*.,

[189] also carried out another study in 2008 related to the intake of certain specific types of fatty acids during pregnancy and their association with the prevalence of asthma symptoms. In this case, it was observed that the intake of n-3 and n-6 PUFAs, and linoleic acid (n-6) was independently associated with an increased prevalence of wheeze during childhood. Nevertheless, there was no a significant measurable relationship of consumption of alpha- linoleic acid (n-3), eicosapentaenoic acid (EPA) (n-3), docosahexaenoic acid (DHA) (n-3) or arachidonic acid (n-6) or the ratio n-3/n-6 PUFAs with the prevalence of wheeze in infants. The authors also found no relationship between wheezing and consumption of total fat, saturated fatty acids, mono-unsaturated fatty acids and cholesterol. One year later, in a randomized placebo-controlled trial in winch supplementation with n-3 PUFAs was prescribed to mothers during pregnancy and lactation, high levels of DHA and EPA in maternal and infant plasma were associated with lower prevalence of immunoglobulin E-associated disease [190]. In addition, maternal n-3 PUFAs supplementation was found to be associated with a lower risk of food allergy and immunoglobulin E-associated eczema during the first year of life in infants with family history of allergic disease. Because the findings about the prenatal intake of PUFAs and their relationship to the prevalence of wheezing and childhood asthma have been inconsistent, Rosa and colleagues in 2019 [191] tried to clarify the associations between prenatal PUFAs status during the pregnancy period (in second-trimester plasma) and child wheeze and/or asthma at age 4 to 6 years, and modifying effects of maternal asthma/atopy, child sex, and maternal race in a total of 1019 mother-child couples. As a result, the authors showed that a higher n-6 PUFAs were associated with increased risk of ever and current diagnosed asthma, whereas n-3 PUFAs were associated with lower risk of current diagnosed asthma. Also. higher n-6 PUFAs were associated with a higher risk of all respiratory outcomes among children born to women with asthma. All these results lead us to conclude that more studies are needed to clarify the therapeutic potential that PUFAs intake may have during pregnancy in asthma prevention. Taking into account not only the subtypes of PUFAs, but also the dose. Recently, Beckhaus and collages in 2015 [192], in a systematic review and meta-analysis including by 120 titles, abstracts, and citations,

and 32 studies (29 cohorts), concluded that current evidences suggests a protective effect of maternal intake of each of three vitamins or minerals (vitamin D, vitamin E, and zinc) against childhood wheeze but is inconclusive for an effect on asthma *per se* or other atopic conditions. Years later, the SEATON (Study of Eczema and Asthma To Observe effects of Nutrition) birth cohort, carried on by Devereux *et al.,* in 2019 [193], was recruited in utero to identify associations between maternal diet during pregnancy and childhood asthma and atopic disease. The authors founded that lower maternal vitamin D and E intakes during pregnancy were associated with increased risk of children wheezing, being diagnosed with asthma in the first 10 years but not after puberty, suggesting that post-natal exposures predominate in the aetiology of incident asthma as children transition through puberty into adulthood. But also there are other studies in which their authors have concluded that maternal vitamin D intake from foods during pregnancy may be negatively associated with risk of asthma in childhood [194,195]. Vitamin C may also play an important role in the prevention of wheeze and asthma, however, Allen *et al*., in 2009 [196] in a systematic review and meta-analysis, reported no evidence of an effect of maternal vitamin C intake on infant wheeze in two included studies (cohorts).

Folic acid is an indispensable vitamin during pregnancy, and since the early 1990s, maternal folic acid supplementation has been recommended prior to, and during the first trimester of pregnancy. It belongs to the group of B vitamins and its function is to prevent neural tube defects, also plays and important role in nucleic acid and protein synthesis by donating methyl groups. Several authors have tried to clarify what role the folic acid intake might have during pregnancy and the early onset of asthma in the offspring [197–200]. For example, in a retrospective cohort study of 428 children, born 1996–2005, and their mothers, the authors concluded that children born to women which consume folic acid in the first trimester only, or first trimester and later, have increased relative OR of asthma compared with children born to women with no folic acid exposure [200]. No association was seen in children born to women exposed after the first trimester [200]. In another large prospective cohort of children born between 2002–2006 from the Norwegian Mother and Child Cohort Study, pregnant women taking supplemental folic acid at/or above the

recommended dose (400 µg/d of supplemental folic acid), combined with a diet rich in folate, reach a total folate intake level associated with a slightly increased risk of asthma in children [199]. But the findings are controversial because there are other studies whose result suggest that maternal folic acid intake during pregnancy is not associated with asthma development in offspring [201] or even that early folic acid or prenatal supplementation among atopic women may be important to prevent wheeze among offspring [202]. Recently, a meta-analysis, which includes a total of 10 studies with maternal folate intake and 5 studies with blood folate concentration, has been carried out to clarify the association between maternal folate intake and infant asthma risk [203]. The authors concluded that maternal folate intake during pregnancy was significantly related to the risk of infant asthma and similar results were found for different geographic regions (Europe and North America). This meta-analysis indicates that the intake of maternal folate during pregnancy could increase the risk of infant asthma and therefore, when supplementing the maternal diet during pregnancy with folic acid to prevent birth defects, adverse effect of this supplement should not be ignored in childhood asthma [203].

Another important issue in addition to identifying the possible macronutrients and micronutrients that can affect the onset of childhood asthma, it is also necessary to understand what is the mechanism that causes its influence. For example, as Yong *et al*., [187] reviewed, traditional natural products such as garlic, curcumin, and broccoli could modify the epigenetic program, such as DNA methylation and histone acetylation, and in this way perhaps prevent allergic disease in the perinatal stage. In addition, the authors reviewed that the supplementation with dietary methyl donor, such as folic acid, might modify the epigenetic mechanism by increasing DNA CG methylation, which suppresses the expression of immune genes, such as runt-related transcription factor 3 (Runx3) expression, and promotes the development of asthma. Runx3 is a specific gene that negatively regulates allergic airway disease [187]. In the case of vitamin D, which have been mentioned above, the mechanism of action on the development of childhood asthma is being studied because there are still aspects that are not clear. Sandhu *et al*., [204] concluded that vitamin D have an influence on the functions of Th1, Th2, and Treg, thereby enhancing the secretion

of interleukim-10 (IL-10), at the same time, it plays an essential role in cellular metabolism and differentiation via its nuclear vitamin D receptor (VDR) that may enhance epigenetic modifications enzymes. In the case of vitamin E, it is known to be part of a larger family of fat-soluble compounds that includes the tocopherols (alpha-, beta- and gamma-tocopherol) and tocotrienols. During the oxidation of fats, it stops the production of reactive oxygen species that are formed in that process, being considered an antioxidant compound. Precisely, its antioxidant action may be the key of the mechanism of preventing asthma, since the antioxidant effect is known to change Th2 differentiation towards Th1 differentiation [138,187].

3.3.2 Influence of mode of delivery on the development of asthma

As mentioned above, the hygiene hypothesis suggests that the immune system of newborns is polarized toward Th2 cells because of a rise in the caesarean section rate, could diminish initial microbial exposure and thereby alter Th1/Th2 development and affect the risk of developing atopy [205]. To test this hypothesis, different studies have examined whether mode of delivery at birth influences the risk of developing subsequent atopic conditions and asthma, because bacterial colonization of the gut in CSDI differs from that in VDI [21,87–89,114,173]. The results have been inconsistent; some studies reported a relatively increased risk of asthma in CSDI, along with a lower frequency of atopic sensitization and allergy development in the VDI [98,99,205–209], whereas others disputed it [210–212].

Jakobsson *et al*., in 2014 [93] studied the postnatal intestinal colonisation pattern in 24 infants, VDI (15) or by CS (9). The intestinal microbiota was characterised using pyrosequencing of 16S rRNA genes at 1 week and 1, 3, 6, 12 and 24 months after birth and the authors measured Venous blood levels of Th1- and Th2-associated chemokines at 6, 12 and 24 months. The result showed that CSDI had lower total microbiota diversity during the first 2 years of life, lower abundance and diversity of the Bacteroidetes phylum and were less often colonised with the Bacteroidetes phylum. Also, CSDI had significantly lower levels of the Th1-associated chemokines CXCL10 and CXCL11 in blood. Adeye *et al*., in 2018 [213] examined whether C-section increased the risk of wheeze or food allergy in early

childhood compared with vaginal delivery and whether these associations were mediated by breastfeeding. The study population was the Upstate KIDS cohort (2008–2010) of 5,753 mothers and infants. The authors concluded that emergency CD (n = 1,356) was associated with elevated risk of wheeze (adjusting for pregnancy complications, maternal atopy, gestational age, birth weight, and smoking during pregnancy) and an increased risk of food allergy (adjusting for maternal atopy, pre pregnancy body mass index, smoking during pregnancy, and parity). However, no significant associations were found in any of the results in the case of planned CD (n = 1,565 infants).

After observing all the discrepancies that exist between the different studies reviewed, we believe that it is necessary to carry out further research into this topic, taking into account all possible confounding factors that can influence, e.g., breastfeeding.

3.3.3 Protective effect of breastfeeding on asthma development

Following birth, the development of infant immune system continues through breastfeeding. As Waidyatillake *et al*., reviewed [214], breastfeeding has been shown to favour lung growth and improve lung function. Furthermore, breastfeeding may be related to good respiratory system health through several potential mechanisms, including modulation of gut microbiota, epigenetics, immunity, and lung development [108]. Also, breast milk contains a wide variety of bioactive factors that support the development and maturation of the infant gut. Among others, a complex mixture of HMOs, lactoferrin, lysozyme, immunoglobulins and lipids all contribute to an intestinal microbiome in breastfed infants that is clearly distinct from that of FFI [215]. In addition, one of the main cytokines present in breast milk is the transforming growth factor β (TGF-β), which is involved in maintaining intestinal homeostasis, inflammation regulation, and oral tolerance development [110]. Another important issue related to breastfeeding is the skin-to-skin contact that occurs between the mother and the infant during the breastfeeding process which may provide an additional source of protective maternal microbes to the nursing infant. The diverse and personalized prebiotic and probiotic components found in

human milk are not found in commercial infant formulas, and therefore cannot optimally support the natural assembly and development of the infant gut microbiota, which may lead to impaired immune system development and increased predisposition to asthma in adulthood in FFI [168].

Focusing on the microbiota of breast milk, it contains a rich microbiota composed of viable bacteria from the skin and non-skin bacteria. Funkhouser *et al*., in 2013 [6], identified, by high throughput sequencing of breast milk from 16 healthy women, 100-600 species of bacteria in each sample with nine genera present in every sample: *Staphylococcus*, *Streptococcus*, *Serratia*, *Lactococcus*, *Pseudomonas*, *Corynebacterium*, *Ralstonia*, *Propionibacterium*, *Sphingomonas* and *Bradyrhizobiaceae*. The breast milk microbiota confers a beneficial gut microbiota to infants, including increased colonisation by Bifidobacteria and reduced the prevalence and abundance of *C. difficile* and *E. coli* compared to FFI [87,89]. Breast milk microbes and other maternal milk factors are implicated in infant immune system development, resistance against infection, and protection against the development of allergies and asthma later in childhood [6,41,216]. In a meta-analysis of 117 studies, Dogaru *et al*., in 2014 [217] found that breastfeeding was associated with a 22 % reduced risk of asthma, and detected that the greatest effect was observed during early childhood. As we mentioned above, HMOs are the main milk components that favours the growth of Bacteroides, *Bifidobacterium* and *Lactobacillus*, which are fermenting bacteria, and therefore, can increase the levels of gastrointestinal and circulating SCFAs in the infant. Also, due to their prebiotic character, the HMOs from breast milk have been attributed beneficial effects on the immune system [7,147]. As Bode *et al*., reviewed in 2012 [218], HMOs can modulate the balance between Th1/Th2 immunity and provide essential nutrients for brain development and cognition. Moreover, higher proportions of certain HMOs have been associated with a decreased risk of respiratory disease in infants [39].

One of the most important maternal milk factors are antibodies (including IgG and IgA), providing passive immunity to the infant during infancy. Immune cells, such as neutrophils and macrophages, and cytokines are also present in breast milk,

along with bactericidal enzymes and antiviral factors [107,147]. Nutrients, and growth factors in breast milk have been shown to regulate the innate immunity, while fatty acid composition, such as PUFAs, can modulate neonatal cytokine responses [110,219]. The content of PUFAs, such as n-6 and n-3, in breast milk and its impact on the differentiation of the immune system has been particularly well studied, considering that PUFAs of type n-3 are inflammatory in addition to providing a protective effect on the intestinal barrier [109]. It has been show that low amounts of n-3 fatty acids present in breast milk are directly correlated to a high risk of atopy in infants [220].

Despite the many protective factors transmitted in breast milk, the debate remains open whether breastfeeding is protective against childhood asthma development and their impact on lung function [217,221,222]. Several studies have shown that asthma risk is reduced in breastfed infants [105,221–232]. More specifically, in the CHILD study, the authors observed that breastfeeding is associated with lower rates of wheezing in the first year of life [229] and lower odds of possible or probable asthma by three years of age [223], consistent with other cohorts in Canada, Sweden, United States and Australia [225,230]. However, others works claim there is no association or even an increased risk of asthma in breastfed children [233–239]. Rosas-Salazar *et al*., in 2019 [240] examined, in a prospective study of 1,456 healthy infants enrolled in a population-based birth cohort, the interactions between mode of delivery and breastfeeding on gut microbiomes in infancy and the development of recurrent wheeze in infants. They showed that there was a significant interaction between mode of delivery and breastfeeding on recurrent wheeze. In VDI, breastfeeding decreased the odds of recurrent wheeze by ~40 % but there was no effect of breastfeeding on recurrent wheeze in CSDI. These results demonstrate that the protective effect of breastfeeding on the development of recurrent wheeze is only seen in children born by vaginal delivery and these findings could explain discrepant results of prior studies of breastfeeding and childhood asthma risk. Bigman *et al*., in 2020 [241] used data from 1,177 mother-infant pairs who took part in the Infant Feeding Practices Study II in 2005-2007 and the Year 6 Follow-Up Study in 2012, provided evidence that exclusive breastfeeding

for the first 3 months may reduce the risk of respiratory allergies and asthma in children 6 years of age, but concerning asthma, statistical significance was reached only in children without a family predisposition to asthma.

As Miliku *et al*., reviewed in 2018 [242], infant feeding can be described and measured in many ways, making it difficult to compare results between studies and therefore may be one of the reasons causing confusion. In order to describe exclusive, complete, predominant, partial and mixed breastfeeding, different terminologies and criteria are applied inconsistently. The WHO [82] defines exclusive breastfeeding as feeding human milk only (including donor human milk), without any food, water, or other fluids, although vitamin and mineral supplements or medicine syrups are allowed. Most studies do not capture enough information to apply the definition described by the WHO, and many do not document the total duration of breastfeeding (age of the infant at weaning); therefore, systematic reviews have been limited to comparing breastfeeding in the terms “ever versus never” or breastfeeding “more versus less” [217,222]. As a result, it is not possible to assess the “dose effect” correctly without information on the exclusivity and duration of breastfeeding, being a useful data to study its causality [242].

Today, breastfeeding has evolved and there are other factors related to it that may be potentially important and are often ignored in epidemiological studies. For example, it is the case of the method of feeding with breast milk. There is the possibility of breastfeeding directly from the breast or also breastfeeding with expressed breast milk, bagged, in some cases frozen and thawed, and supplied with a bottle. It is also important, in infants who are fed with mixed breastfeeding, to consider the type of complementary feeding since it can be infant formulas, solid food or semi-solid food. Even in this sense, the relative proportion of breast milk ingested by the infant compared to other nutritional sources is also important. Finally, it is essential to consider whether the breast milk supplied is from the mother or from donors because if it comes from milk banks, breast milk is pasteurized to ensure its sanitary quality. All of these details are relevant to asthma research and should be considered. There are already studies that have observed how the protective effect

against wheezing during childhood is decreased due to the supplementation of breast milk with infant formulas. On the contrary, in this same study, this effect was not observed when supplementation was performed with solid foods [229].

Focusing on the mode of breastfeeding, several authors have reported that breastfeeding directly by suckling appears to be more protective than bottle-fed breast milk [223,243]. In relation to this, a recently study carry out by the CHILD has reported a dose-dependent reduced risk of wheezing [229] and asthma [223] among breastfed children. But these associations were less strong among infants fed with pumped breast milk compared to those fed at the breast, although both were superior to infant formula [223]. One of the potential reasons for this difference is the possible alteration of the bioactive components of milk during the extraction, refrigerated storage, freezing and thawing of breast milk [244–246]. Another possibility is the transfer to breast milk of asthmagenic chemicals or contaminants from breast pumps or storage containers, such as bisphenol A present in plastics [247–249]. It should also be borne in mind that breastfeeding provides skin-to-skin contact between the mother and the infant, being a source of exposure to protective microorganisms present on both the breast skin, nipple and maternal halo. Lastly, the suckling exercise at the breast by the infant can stimulate lung growth due to the effort that must be made to dispose of the food [250].

Current available scientific evidence supports the use of donated human milk (DHM) for very premature or sick newborns when their own mother’s milk is not available or is insufficient [251,252]. The first milk banks in the world were created in the first decade of the 20th century, and their practice has been increasing. In addition to increasing the number of banks, there has also been a large increase in the collection and donation of milk. According to the Spanish Association of Human Milk Banks (https://www.aeblh.org), in 2009, 745 litres of milk were collected and 258 newborns benefited while in 2017, the quantity collected increased to 9,280 litres and 2,800 children were benefited. The large increase in the use of pasteurized DHM is evident, but is rarely reported or studied in relation to respiratory health. This is relevant because DHM is submitted to pasteurisation to ensure its microbiological

safety in human milk banks but this treatment affects some of its bioactive compounds reducing the concentrations and biological activities of all immunoglobulins (Igs) classes. Among Igs, IgA is the most abundant in human milk and its concentration reduced by 20–60 % after pasteurisation [253]. These findings point us, regardless of the mechanism, that the mode of feeding (exclusive breastfeeding, with formula, donated milk, etc.) is a very important factor to consider in the investigation and it will be important to document these exposures and study their impact on immunological outcomes in the infant [242].

3.3.4 Influence of the use of antibiotics on the development of asthma

3.3.4.1 Prenatal exposure to antibiotics

During the last decade, different epidemiological studies have reported a link between allergic disease later in life and antibiotics exposure during prenatal period [126,130,254–256]. It has been repeatedly shown that prenatal antibiotics exposition may be associated with the occurrence of asthma during childhood [126,256].

One prospective study on asthma in childhood carried out by Stensballe *et al*., in 2013 [256] showed an increased risk of exacerbation in asthma if mothers had used antibiotics during third trimester in a clinical study of a birth cohort. Also, they replicated these findings in an unselected national birth cohort and confirmed increased risk of asthma hospitalisation, and inhaled corticosteroids in the children if mothers used antibiotics any time during pregnancy. This increased risk of asthma was also observed in the subgroup of mothers using antibiotics for non-respiratory infection. Metsala *et al*., in 2015 [130] conducted a study in which they also reached the same conclusion. In this study, the authors also observed the effect of different types of antibiotics most used. in this context the strongest association was observed for cephalosporins. One year later, Mulder *et al*., [254] conducted a study in which children with asthma (n=1,228) were compared to siblings without asthma. Their conclusion was that antibiotic use in the third trimester of pregnancy was associated with a small increased risk of asthma in preschool children (both the case–sibling and case–control analysis) and this association was robust to time-invariant confounding or exposure time trends. Moreover, Chu *et al*., [126] observed that

maternal use of penicillin or chloramphenicol shows an association with later infant asthma. In 2019, Lijun Bai *et al*., [257] conducted a meta-analysis to evaluate the risk of the use of antibiotics in each of the three trimesters that make up the gestation period independently on childhood asthma or wheeze. In this study the authors founded a positive association between prenatal antibiotic use in every specific trimester and risk of childhood asthma or wheeze. However, adjustment for confounders, such as infections during pregnancy and sibling, decreased the relative risk estimates, supporting the concept that these associations are, at least in part, because of confounding factors. Referring to this question, currently available research gives insufficient attention to confounding factors, such as maternal asthma history, smoking, mode of delivery, birth weight or even breastfeeding, which has been determined as a protective factor. But it should also be taken into account, as Milliken *et al*., reviewed [258], that in most of the studies, there is a lack of control for the number of antibiotic courses administered, the indication for antibiotics, the use of broad spectrum or narrow range antibiotics, the timing of use, the dose-dependent nature of the effect, the class of antibiotics used, or if the mother suffered the same affection.

3.3.4.2 Antibiotic exposure in early life

Epidemiological studies in humans indicate that broad-spectrum antibiotic exposure may play a role in the development of asthma and atopy. Marra and colleagues in 2009 [259], founded that antibiotic exposure in the first year of life was associated with a small risk of developing asthma in early childhood after adjusting for a great number of confounders factors. Also, the authors concluded that as the number of courses of antibiotics increased, this was associated with increased asthma risk, with the highest risk being in children who received more than 4 courses. Finally, all antibiotics were associated with an increased risk of developing asthma, with the exception of sulphonamides. Later, Muc *et al*., in 2013 [260] conducted a questionnaire-based study and also found that antibiotic exposure in the first year of life plays a significant role in the development of asthma and allergic rhinitis in children. Additionally, Hoskin-Parr *et al*., [261] assessed data from 4,952 children enrolled in the Avon Longitudinal Study of Parents and Children and found

a dose-dependent association between antibiotic usage during the first 2 years of life and the development of asthma at 7.5 years of age. Recently, H *et al*., in 2019 [262], used administrative linked health data in a total of 2,644 children from the CHILD cohort to examine the association between antibiotic prescribing (age <1 year) and asthma incidence (ages 1-4 years), concluded that antibiotic exposure in the first year of life increases the risk of being diagnosed with asthma in childhood while this risk was reduced with increased gut microbiota α-diversity. Thus, proving that the gut microbiota, represented by diversity and differentially expressed taxa, was a significant mediator between antibiotics and asthma. The use of antibiotics disturbs the balance of the microflora skewing the developing immune system toward hypersensitivity.

4. Techniques used to characterize the gut microbiota

It is not the objective of this thesis/review to discuss all the methods to study the microbiota since there are already reviews dedicated to this topic [263,264]. Instead, we describe the most relevant techniques used in this work to analyse the gut microbiota, including targeted approaches such as 16S rRNA gene NGS, as well as the advantages and disadvantages of these different methods.

From the beginning of the 20Th century, culture-based techniques were the only ones used in the study of the intestinal microbiota. Since then, progress has been made in the development of different techniques for phenotyping isolates based on fermentation profiles and growth requirements *in vitro*. Although bacterial identification using culture techniques is quite economical, these techniques require a lot of work and time. It should also be added that culture techniques provide a limited view of the intestinal microbiome diversity because only up to 30 % of the microorganisms that comprise the intestinal microbiota have been cultivated to date. It is important to note that microorganisms from the intestinal microbiota that have not been cultivated are not necessarily uncultivable. In fact, most could be cultivated, but growth conditions suitable for these microorganisms have not yet been developed or discovered [263].

During the last 20 years, cultivation techniques have been in second place due to the use of molecular techniques such as NGS. It has been described that only 56% of gut bacteria detected by NGS approaches have cultured homologs [7]. With the development and rise of new cultivation techniques, called culturomic techniques [265], this difference is being reduced. As Lagier *et al*., [266] described, "culturomics consists of the application of high-throughput culture conditions to the study of the human microbiota and uses matrix-assisted laser desorption/ionization–time of flight (MALDI–TOF) or 16S rRNA amplification and sequencing for the identification of growing colonies, some of which have been previously unidentified". In the new culturomic techniques also high-performance culture conditions are used, which involve the formulation of complex growth media, allowing the isolation and cultivation of a considerable number of new gut microorganisms from human faeces. In addition, as Fraher *et al*., [263] reviewed, culture techniques have been improved thanks to the development of new methodologies such as the use of microbial culture chips ('microPetri plates') and gel micro-drops. These techniques are characterized by having a high efficiency allowing the cultivation of previously uncultivated organisms. However, a symbiotic relationship between some microorganisms that make up the intestinal microbiota has been described, which is dependent for example on the metabolic activity of other microorganisms for their growth, which limits the usefulness of all these new pure culture techniques.

4.1 Culture-independent techniques: PCRs & High-throughput sequencing methods

For many years, for the identification of bacterial species, culture methods and biochemical tests for typing have been used as main standards. However, from the nineties, culture-independent techniques have not only significantly improved our understanding and knowledge about the intestinal microbiota, we also understand better how it interacts with the host and its relationship with the appearance of human diseases. Nowadays, with the use of these techniques, microbial diversity can be studied in a much more complete way. These techniques also provide us qualitative and quantitative information on the different bacterial species and finally, they allow

us to study factors that produce modulations in the gut microbiota and its possible relationship with the state of health in humans. Examples of these techniques are Polymerase Chain Reaction (PCR) or quantitative PCR (qPCR), denaturing gradient gel electrophoresis (DGGE), fluorescence *in situ* hybridization (FISH), DNA microarrays, and NGS of the 16S rRNA gene or its amplicons [263] (**figure 5**).

All these techniques need a previous phase of sample preparation which is mainly based on DNA extraction. Generally, most of the extraction methods used share a series of basic steps whose final objective is the separation of DNA from the rest of cellular components. However, we should add that no extraction protocol works equally well on all sample types [264]. The basic steps are:

- Cellular lysis by mechanical, physical and chemical means.
- Removal of lipids and proteins.
- DNA precipitation.
- Centrifugal separation.

In general, the lysis step receives the most scrutiny, as the intensity of the lysis can result in bias towards a particular taxonomic group. Regardless of the extraction protocol used, steps must be taken to minimize contamination from 'foreign' source. This will involve using sensible precautions (such as operating in as sterile an environmental as possible and using clean equipment) and using commercially available kits or laboratory reagents that have been thoroughly tested to be free of contaminating DNA. Likewise, care should be taken to minimize contamination during sample collection [264].

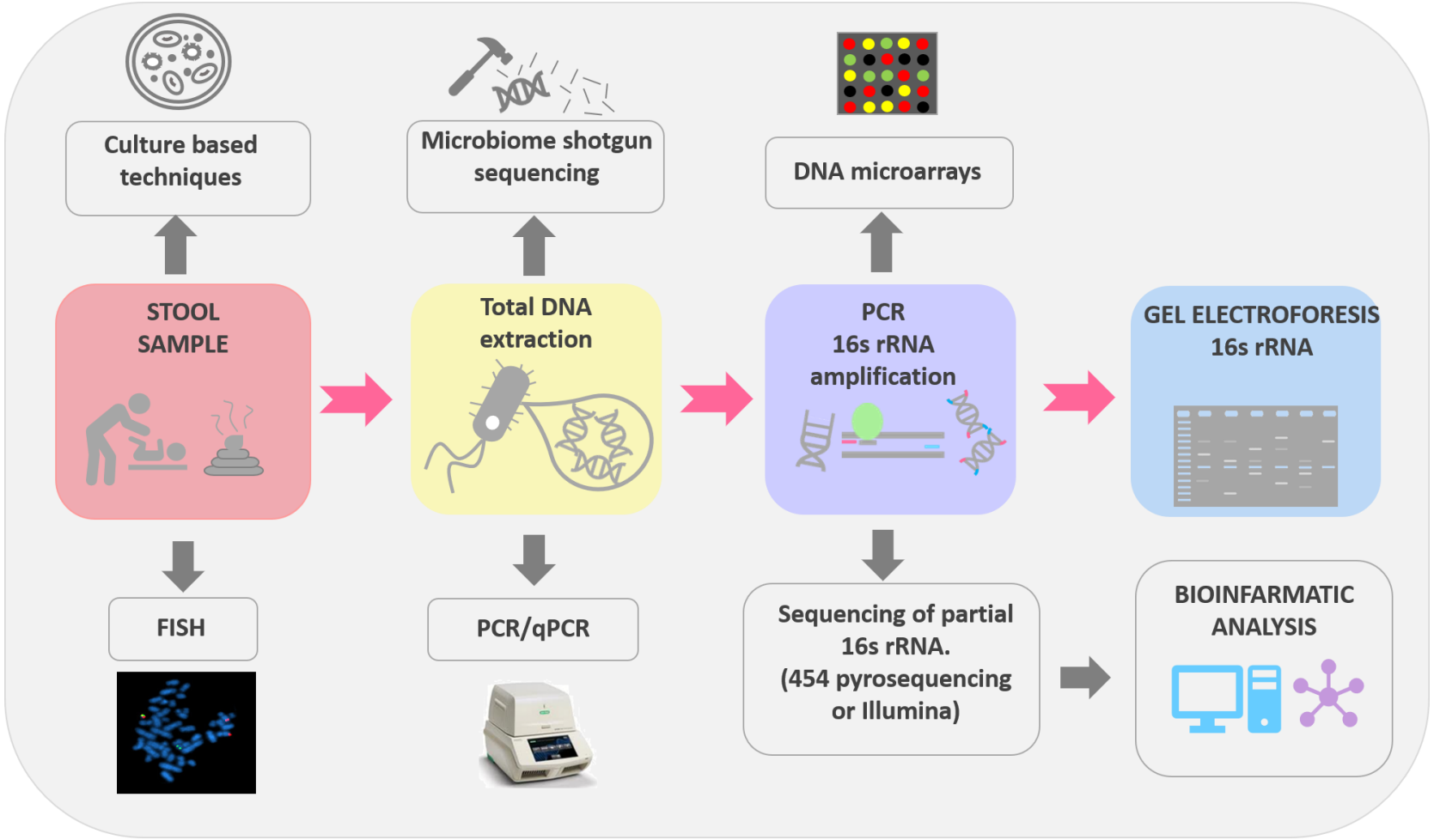


Figure 5. Techniques used to characterize the gut microbiota: An Overview

Following successful extraction of DNA, the next step in most of the techniques described is the amplification of the 16S rRNA gene by PCR using universal primers. The reason why the 16S rRNA gene is amplified is because this gene encodes several conserved regions that are unique to all bacteria and hypervariable regions that confer specificity to a large number of bacterial species. Within the 16S rRNA gene, although there is still no consensus on this, the use of PCR primers that amplify the V4 region are the most used, since at the taxonomic level it provides the most informative sequences [39]. The product resulting from 16S rRNA gene amplification is known as 16S rRNA gene amplicons.

The PCR alone amplifies the DNA and gives us information about the presence or not of a certain microorganism. Therefore, PCR alone does not allow quantification of the DNA present in a sample; qPCR, also known as real-time PCR, in addition to amplifying DNA also reliably quantifies the amount of DNA present. Among the main advantages of qPCR are the capacity for phylogenetic discrimination, the speed of analysis and the ability to analyse specific species thanks to the design of primers to target specific species [267]. When the qPCR techniques are combined with other molecular techniques, such as DGGE or DNA microarrays, which are semi-quantitative techniques, more detailed information on the diversity and abundance of the intestinal microbiota may be obtained. In addition, like DNA extraction kits, qPCR kits are widely available commercially and are a very useful tool in laboratories that do not have access to the other techniques mentioned. Although PCR or qPCR have been a great technical advance, they also have certain limitations and disadvantages. Both, the extraction protocol used and the choice of primers to amplify the target region of the 16S gene, may introduce bias to the results. In addition, PCR or qPCR protocols may be laborious and quite complicated at the technical level, and these techniques lack the ability to identify new species [263].

NGS is the gold standard for taxonomic identification at the species level and describes massive parallel sequencing technologies. Currently, there are two main sequencing technologies being used by most research groups: the 454

Pyrosequencing® (GS, FLX, and FLX Titani) (Roche Diagnostics GMBH Ltd, Mannheim, Germany), which allows reading DNA fragments of up to 500pb, and Illumina® (Miseq and Hiseq2000) (Illumina, San Diego, CA, USA). Illumina platform provides sequencing of DNA fragments of up to 300pb in length but it sequences 10-100 more samples, at a much higher sequencing depth and lower cost that the 454 pyrosequencer [9]. The typical analysis of the 16S community involves DNA or RNA extraction, amplification, standardization, library construction (addition of indexes to encode samples), sequencing and subsequent bioinformatic analysis. One of the main characteristics of this technique is the possibility of sequencing a large number of samples in parallel at the same time and in the same reaction set-up. For example, 454 Pyrosequencing®, in a single run, can sequence 500 million bases with a high accuracy (99 % or better). Because the length of the sequences is shorter, these techniques allow a greater number of readings thus allowing the detection of bacteria that are in low abundance. The main advantages of NGS techniques include: phylogenetic identification; the detection and/or identification of unknown bacteria; speed; and the ability to collect quantitative data at relatively low cost and fast speed.

Thanks to the use of 16S rRNA technology, our understanding of the composition of the intestinal microbiota has advanced a lot and has allowed us to compare between different microbial profiles, such as healthy and diseased ones. However, this technology does not provide us any functional information and therefore an association between a microbial pattern and a disease state cannot be inferred, allowing us to establish whether differences in the pattern are cause or effect of the disease. However, metagenomics, the most recent technology in the study of the intestinal microbiota, is able to address this issue allowing to obtain functional biochemical information. The metagenomic technique was first described by Handelsman in 1998 [268]. Since then, metagenomics has been used in various studies such as the Human Microbiome Project (HMP; funded by the National Institute of Health (NIH)) [269]. This technique is unique and involves random fragmentation of DNA present in a representative sample, sequencing of all genomic DNA fragments using metagenomic technique, also known as shot-gun sequencing, assembly in a continuous sequence by reconstruction of overlapping sequences and

finally, compare these sequences with databases of known genes, such as the NIH Genbank [263]. In the sequencing process, in the metagenomic techniques, the same NGS platforms discussed above are used. One of the main advantages of the microbiome shot-gun sequencing is the possibility of collecting data about genetic diversity and also about the functions of the intestinal microbiota, allowing correlations between the microbiome and healthy and disease states [270]. On the other hand, there are also limitations because the shot-gun sequencing is an expensive technique where the analysis of the large amount of data generated is computationally intense and requires specialized personnel and laboratories [39,263].

As we have commented previously, once the DNA sequences are acquired, the analysis and interpretation are necessary. Due to the large amount of data produced with the use of these modern techniques, the use of sophisticated analysis tools is necessary. In the 16S rRNA studies, the first step is to apply quality noise filtering and elimination algorithms in the raw sequences to minimize the effect of artefacts. Once filtered, the resulting readings are grouped into operational taxonomic units (OTUs), which represent similar organisms, allowing the deduction of phylogeny and taxonomic identity. It is at this stage when data from other studies can be incorporated or continue with the processing of our data individually. Through multivariate analysis and graphic representation, we can study the abundance of the different OTUs and thus obtain a description of the structure and sponsors of the microbial communities. In the case of metagenomic studies, functional annotation databases are used that help us identify important metabolic pathways. It also allows us to compare with other metagenomic studies.

Throughout this section we have commented the different advantages and main disadvantages of each technique but we must also take into account other disadvantages or limitations that may arise throughout the analysis. For example, one of the main limitations, both in DNA-based PCR and sequencing methods, is its inability to detect viable, cultivable and metabolically active microbes. These techniques allow us to detect very low amounts of DNA but organisms may be dead.

Therefore, to overcome this limitation and to be able to demonstrate some hypotheses successfully it is necessary to demonstrate the microbial viability, since the sites can be considered sterile even if the presence of bacterial DNA is detected. Another very important issue is precisely the ability of these techniques to detect very low levels of DNA. Assays performed on DNA from low samples of microbial biomass, such as placenta, amniotic fluid and meconium, are extremely prone to confusing results due to the presence of contaminating DNA. In fact, several studies have shown that the results obtained from sequence analysis, from samples with low levels of DNA, are unreliable due to bacterial DNA in reagents, consumables and components of DNA extraction kits (reviewed by Pérez-Muñoz *et al*., [3] and Salter *et al*., [271]. Negative controls also play an important role in studying the intestinal microbiota using NGS. Today, many researchers report the use of negative controls in their work, both during DNA extraction and in sequencing processes but despite this, even these controls have been classified as insufficient since, for example, Salter *et al*., [271] provided a list of bacterial taxa commonly present in reagents and consumables that are detected in negative controls and show that the smaller the amount of bacterial DNA in a sample, the greater the proportion of sequences that can be attributed to the contamination.

II. GENERAL MATERIAL AND METHODS

1. NELA study design

The Nutrition in Early Life and Asthma (NELA) study (http://www.nela.imib.es/) is a population-based prospective birth cohort study set up in Murcia (Spain), composed of 738 families. The objective of the NELA study is to investigate the impact of nutrition during pregnancy and early postnatal life on health outcomes in children of the Region of Murcia (Spain). The study is specially focused in the role of nutrition in the prenatal and postnatal lifetimes in the respiratory health during childhood, as well as in the origin of asthma and allergy. The recruitment period begun in March 2015 and has finished in 2018. Pregnant women who meet the inclusion criteria, described below, were recruited in the Virgen de la Arrixaca Clinical University Hospital (Murcia) before week 20 of gestation and followed up until delivery. Their children were enrolled at birth and have been followed up until 7 years of age.

As NELA is a multidisciplinary study, both outcome variables and exposures are entrusted to different working groups with specific expertise in the area. There is also a working group responsible for the recruitment, coordination and implementation of visits and their corresponding questionnaires, measurements and samples collection.

The inclusion criteria of the mothers are: women of Spanish origin, belong to the study area, women aged between 18 and 45 years, plan to live in Murcia at least the next two years, plan to give birth at the reference hospital, healthy women (women with asthma or allergies are not excluded), not to have followed any program of assisted reproduction (IVF techniques are excluded), single pregnancy (multiple pregnancies are excluded), no major foetal malformations and not to have communication problems.

For the implementation and achievement of this cohort, it was necessary the collaboration, participation and coordination of many research and clinical groups with specific expertise in Obstetrics, Paediatrics, Nutrition, Immunology, Genetics, Air Pollution, Bioinformatics, Psychology or Microbiology among other.

1.2 Recruitment and participation

Among the 1350 women invited to participate, 738 (54 %) were finally enrolled in the study. Reasons for non-participation were as follows: 37 % did not want to participate, 33 % said they did not have time, 2 % reported no interest, and 28 % did not offer any explanation. Excluding 16 withdrawn, 1 miscarriage and 1 stillbirth, there were 720 eligible mother-offspring pairs at birth.

1.3 Data collection and follow-up time points

Mothers had three follow-up examinations, one between 20 and 24 weeks of gestation, one between 32 and 36 weeks of gestation, one at delivery. Children have been followed at 3 and 18 months of age so far. They will be followed at 5 and 7 years of age; and until adolescence and beyond. **Figure 6** summarises the times for the different visits and the interventions performed or planned at every one of them.

1.4 Outcome variables

The main outcome variables were lung function and exhaled volatile organic compounds profile at three months of age of the children; and asthma (symptoms and/or diagnosis) at any point of the follow up visits, which initially took place at 18 months, 5 (currently starting), and 7 years of age. In each visit a set of measurements and a specific questionnaire is used to obtain the information of both, mother and child.

1.5 The NELA biobank

Samples and data from participants included in this study were provided by the BioBank “Biobanco en Red de la Región de Murcia” (PT17/0015/0038 & PT20/00109), integrated in the Spanish National Biobanks Network (B.000859) and they were processed following established protocols.

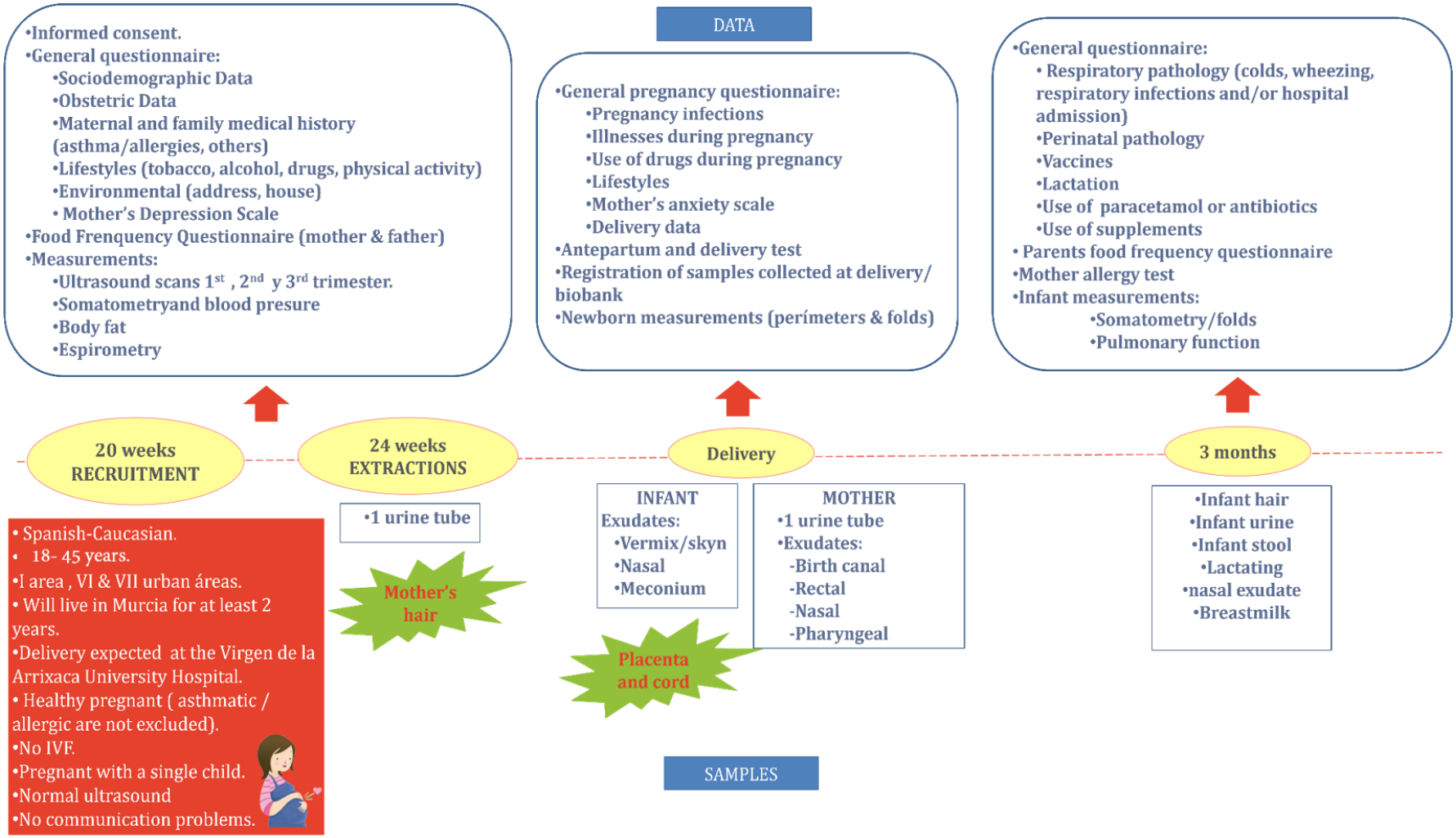


Figure 6. Summary of the experimental design of the study for the NELA cohort (up to 3 months of age of the child). Data collection and samples at each stage, as well as inclusion criteria. IVF: *In vitro* fertilisation.

2. Controls *vs.* cases study

A subset of 200 mother-infant couples belonging to the NELA cohort has been selected to conduct a case-control study on the link between infant faecal microbiota and SCFAs, and other perinatal and postnatal factors such as the adherence to a Mediterranean diet and exposure to antibiotic during the pregnancy period, the diet of the infant or the exposure to antibiotics during the first 3 months of life and the predisposition to the appearance of early symptoms indicative of asthma in the early age. On the basis of the sample availability, we selected 106 mother-child couples, case group, with available 3-month stool samples which present symptoms indicative of asthma (wheezing, dry cough, chest infection, bronchitis and bronchiolitis, atopic skin, dry skin and / or atopic eczema) and 110 controls mother-child couples without any of the symptoms described above were defined as healthy. The parents replied to questionnaires concerning breastfeeding, use of antibiotics, mother's diet during the pregnancy period and allergic symptoms.

This study was approved by the Research Ethics Commission (CEI code number: 1942/2018) and by the Experimentation Biosafety Committee (CBE code number: 125/2018) of the University of Murcia, Spain (**Annex 1**). Written informed consents were obtained from all participants prior to study enrolment.

2.1 Cases definition

Infant cases and their respective mothers were classified as cases when they presented at 3 months of age with one of the following clinical symptoms or episodes: wheezing, eczema, dry cough, dry skin, bronchiolitis or bronchitis.

- Wheezing was defined as a positive answer to the question "In the first 3 months how many episodes of wheezing or chest whistles has your child had?". "Recurrent wheezing" was defined as one or more episodes of wheezing in the first year of life" (reported in the 3 months NELA health questionnaire).
- Atopic dermatitis or eczema at 3 months was defined as a positive answer to the question "Has any doctor diagnosed your child atopic eczema during their first 3 months of life?" and the question "Does your child have dry

skin in general?" (reported in the 3 months NELA health questionnaire). "NO" defined as no diagnosis.

- Dry cough at 3 months was defined as a positive answer to the question "In the first 3 months, how many episodes has your child had a dry cough that was not due to a cold or flu that lasted more than 3 weeks?".
- Chest infection at 3 months was defined as a positive answer to the question “In the first 3 months, has a doctor ever told you that your child had a chest infection?”.
- Bronchitis or bronchiolitis at 3 months was defined as a positive answer to the question “In the first 3 months, has a doctor ever told you that your child had bronchitis or bronchiolitis?”.
- Atopic skin and dry skin at 3 months were defined as a positive answer to the question “At de moment, does your child have any of these skin conditions: itchy red patches on the skin and / or dry skin?”.

2.2 Sample collection

Two infant’s stool samples were collected by the parents (n= 216) at three months of age. For the collection of two samples, the parents received two faeces’ tubes together with a sterile depressor and an instruction form. Parents took the samples directly from the diaper into two collection tubes, being storage at -20ºC. Parents delivered the collected samples at the three-months-visit, transporting them in a refrigerated container. The stool samples were stored in the Biobank Platform of the Research Institute Biosanitary Virgen de la Arrixaca Clinical University Hospital (IMIB-Arrixaca) in Murcia at -40ºC after aliquoting the samples in a sterile way.

III. RESEARCH STUDIES

CHAPTER 1. Dietary characterization of mothers belonging to the NELA cohort: Mediterranean diet and their association with the early onset of asthma precursors symptoms in early life.

1. Introduction

1.1 Mediterranean diet adherence during the pregnancy period

The period of prenatal life is considered to be a critical window for both maternal and offspring's health, and specifically maternal diet during pregnancy has been proposed as one of the prenatal factors that has long-term implications and influence on the development of the placenta [272] and on the risk of developing gestational diabetes [273]. Prenatal diet is also associated with complications at birth including premature birth [274,275] or pre-eclampsia [276], low birth weight [274], and may influence the correct development and response of the foetal immune system and consequently the risk of developing allergies or asthma in childhood [177,277–280]. Traditionally, the isolated effects of foods or specific nutrients consumed during pregnancy on health and their possible role in the development of diseases in offspring have been investigated. However, this type of analysis can omit relevant information and be inaccurate since foods are consumed together in the context of a diet, creating synergies between them [184,185].

Mediterranean diet (MD) and Mediterranean diet adherence (MDA) have been investigated due to its beneficial effects and protective role against diseases, demonstrated in numerous high-quality studies, reviews [178,179,277] and meta-analyses [180,181,281,282], making it the most widely studied and evidence-based dietary approach to healthy eating and disease prevention [180–182]. One of the last and most correct definition of the MD during pregnancy is the one described by Amati *et al.,* [176] in their review in 2019; the authors described the MD in as a "diet characterized by a high intake of fruits, vegetables, whole grain cereals and bread, legumes, fish and nuts; low-to-moderate consumption of dairy products and eggs, and limited amounts of red meat and red wine. It is low in saturated fats and high in antioxidants, fibre and mono and polyunsaturated fatty acids (MUFAs) (PUFAs) mainly derived from extra virgin olive oil and oily fish (n-3 PUFAs)". Beyond nutritional guidelines, the MD represents a balanced and healthy lifestyle that includes physical activity, adequate rest, traditional and simple ways of cooking, and sociability at the table as the main habits registered in the latest available edition of the Pyramid of MD,

published by the Mediterranean Diet Foundation [283]. Olmeda-Requena *et al.,* in 2014 [284], carried out a research to study the factors associated with a low adherence to a MD pattern in healthy women and concluded that a younger age, a low social class, a low educational level and an unhealthy lifestyle (smoking and lack of exercise) were associated with a low MDA.

In 2003, Trichopoulou *et al.,* [285] were the first researchers to quantify MDA using a 10-point numerical scale, which is the well-known Mediterranean Diet Score (MDS). They reported an inverse association between the score obtained and total mortality. The higher the MDA, the lower the mortality rate, both from coronary heart disease and cancer. In relation to the numerous indices that have been developed to measure the MDA in the adult population, Olmedo-Requena *et al.,*[286] studied the degree of correlation between the five different indices that have been developed up to date applied to the same population (healthy adults), concluding that concordance between them (including Alternative Mediterranean Diet index (aMED) [287]) and Relative Mediterranean Diet index (rMED) [288]) was moderate or low. The authors reported the existence of a different classification of the subjects due to variations in the food groups included.

Furthermore, in most of the cohorts that study the pattern of adherence to the MD of adults or pregnant women, a single index has been used and there is no consensus when applying one or the other index. To date, no comparison has been made of the two most widely used scores (rMED and aMED), applied to the same population of pregnant women, and the sociodemographic and lifestyle factors that influence this degree of adherence to check if reach the same conclusions, this being a complementary objective of this work.

1.2 Maternal dietary patter and their association with the offspring's health

As we commented previously, the period of prenatal life is considered to be a critical window for future health of mother and child, and maternal diet during pregnancy has been proposed as one of the prenatal factors that can influence the correct development and response of the foetal immune system, promoting T-helper-2-cell (Th2) response and affecting airway development. As a result, this

might increase predisposition to allergic or asthma manifestations during the infant's first year of life [280]. Recently, several studies have shown that early exposure to nutrients or to certain foods specifically during pregnancy, influences early immune development and potentially prevents asthma or other precursor symptoms of it such as recurrent wheezing, eczema or atopy [176,277,278,280]. Therefore, the study of the relationship between maternal diet during pregnancy, more specifically the MD, and the consequent risk of developing asthma or atopy has become a topic of increasing research during the last 15 years [278,280]. For example, Chatzi *et al.,* in 2008 [152], carried out a birth-cohort study in Menorca and found that a high adherence to a MD during pregnancy was protective for persistent wheeze, atopic wheeze and atopy in children at age 6.5 years. But the same authors in 2013 [278], in another mother-child cohort studies (INMA and RHEA cohort) and meta-analysis approach, concluded that MDA was not associated with the risk of wheeze and eczema. In the same year, in a systematic review and meta-analysis carried out by García-Marcos *et al.,* [177] the authors found that adherence to MD tended to be associated with lower occurrence of 'current wheeze'; 'current severe wheeze' and 'asthma ever'. These results coincide with those found by Yin Zhang *et al.,* in 2019, [179] in a systematic review and meta-analysis of observational studies. The authors observed that a high adherence to MD during pregnancy may have short-term effects on wheeze in the first 12 months. Recently, Bédard *et al.,* in 2020 [289] reported that adherence to a MD during pregnancy may be associated with increased small airway function in childhood, but the authors did not found evidence for a reduced risk of asthma or other allergic outcomes. In the same way, Lange *et al.,* in 2010 [290] did not found any association between the MD score or Alternate Healthy Eating Index (AHEI-2010), modified for pregnancy, and recurrent wheeze in the offspring at 3 years of age. This last conclusion coincides with others systematic reviews in which the authors have not found evidence about a high index of adherence to MD and a protective effect on asthma symptoms and/or wheeze or eczema in the offspring [277,291]. Further, Lange *et al.,* [290] also conclude that perhaps the consumption of specific nutrients individually by the mother during pregnancy may be more important determinants than dietary pattern itself. All these contradictory conclusions may be due to the great heterogeneity observed

in the studies, and the existing variability when it comes to establishing the criteria that define asthma symptoms.

2. Objectives

2.1 General objectives

To establish the association between the maternal healthy diet during pregnancy, such as the adherence to a Mediterranean diet pattern, and a lower appearance of early asthma symptoms in infants at 3 months of age.

2.2 Specific objectives

- To evaluate the adherence to MD in all pregnant women that make up the NELA cohort and to identify related lifestyles and socio-demographic factors which could change this pattern.
- To investigate in a subsample population of 200 mother-infant pairs classified as controls and cases, the possible association between adherence to MD of gestational women of the NELA cohort during pregnancy and the risk of wheezing, atopy and eczema in their offspring at 3 months of age.
- To investigate, in a subsample population of 200 mother-infant pairs classified as controls and cases, the possible relationship between the consumption of certain food groups and specific nutrients in gestational women of the NELA cohort during the 1st trimester of pregnancy and the risk of wheezing, atopy and eczema in their offspring at 3 months of age.

3. Material and Methods

3.1 Study design.

The objectives of this chapter were achieved through three studies (I, II and III), all of them based on the NELA cohort. The first one (**study I)** is a characterization study applied to the entire cohort, the second and third (**study II** and **III)** are Cases-Controls studies to determine if exist an association between diet or food groups and the early appearance of asthma precursor symptoms (EOAPS). The experimental design of each of the studies is represented in **figures 7,8** and **9**.

In the **study I** (**figure 7)**, the degree of adherence to a healthy diet pattern such as the Alternative Healthy Eating Diet-2010 (AHEI-2010) and to the Mediterranean diet during pregnancy was evaluated in pregnant mothers belonging to the NELA cohort. In addition, in the study of the degree of adherence to the Mediterranean diet, two different indices were applied for later comparison. Finally, a multivariate analysis was carried out in order to evaluate which were the sociodemographic and lifestyle factors that could be associated with a lower level of adherence to the Mediterranean diet or to a healthy diet pattern during pregnancy.

In order to carry out this study, it was necessary to create and design a food frequency questionnaire (FFQ) adapted to the habits of consumption of pregnant women and validated it. All pregnant women recruited in the study were interviewed for the collection of clinical, sociodemographic, lifestyle and dietary information during the visit to the hospital at week 20 of gestation.

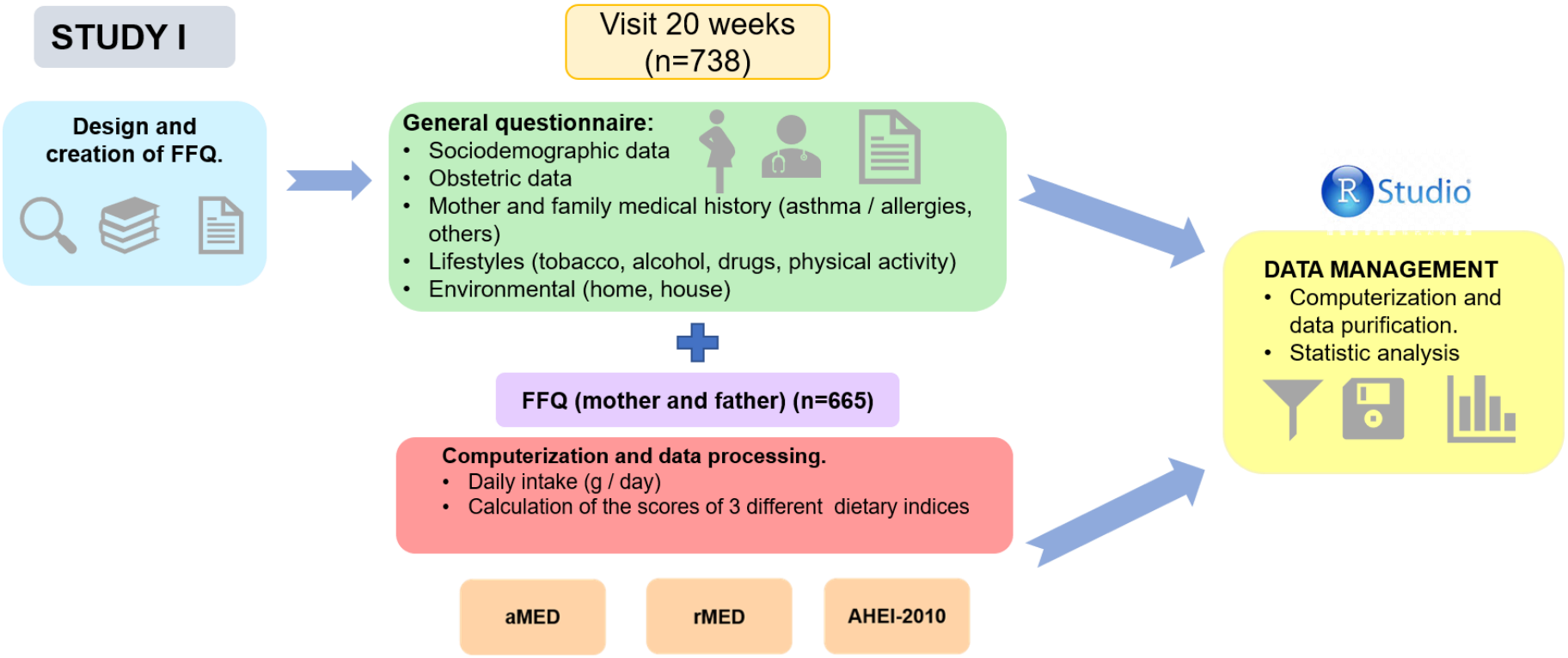


Figure 7. Experimental design of chapter 1, study I. FFQ: Food Frequency Questionnaires; aMED: Alternative Mediterranean Diet index; rMED: Relative Mediterranean Diet index; AHEI-2010: Alternative Healthy Eating Diet Index-2010.

In the **study II** (**figure 8)**, the possible association between the degree of adherence to DM of pregnant women of the NELA cohort during the 1st trimester of pregnancy and the risk of EOAPS in offspring at 3 months of age was evaluated. For this, a population subsample of 200 mother-infant pairs was selected, subdivided and classified as controls and cases depending on whether the infants had wheezing or atopic skin, episodes of bronchiolitis or bronchitis, and eczema at 3 months of age. In order to carry out the statistical models, other confusing variables of a sociodemographic, lifestyle and clinical history of the parents were also taken into account, all of which were collected through interviews and the completion of questionnaires in different phases of the cohort study (20 weeks of gestation, delivery and 3 months after delivery).

In the **study III** (**figure 9**), the possible association between the consumption of different food groups or specific foods, by pregnant women of the NELA cohort, and the risk of EOAPS in the offspring at 3 months of age was evaluated. For this, the same cases and controls groups. To carry out the statistical models, other confusing prenatal, perinatal and postnatal variables were again taken into account, such as sociodemographic characteristics, lifestyle and medical history of the parents, all of them collected through interviews and the completion of questionnaires in different phases of the cohort study (20 weeks of gestation, delivery and 3 months after delivery).

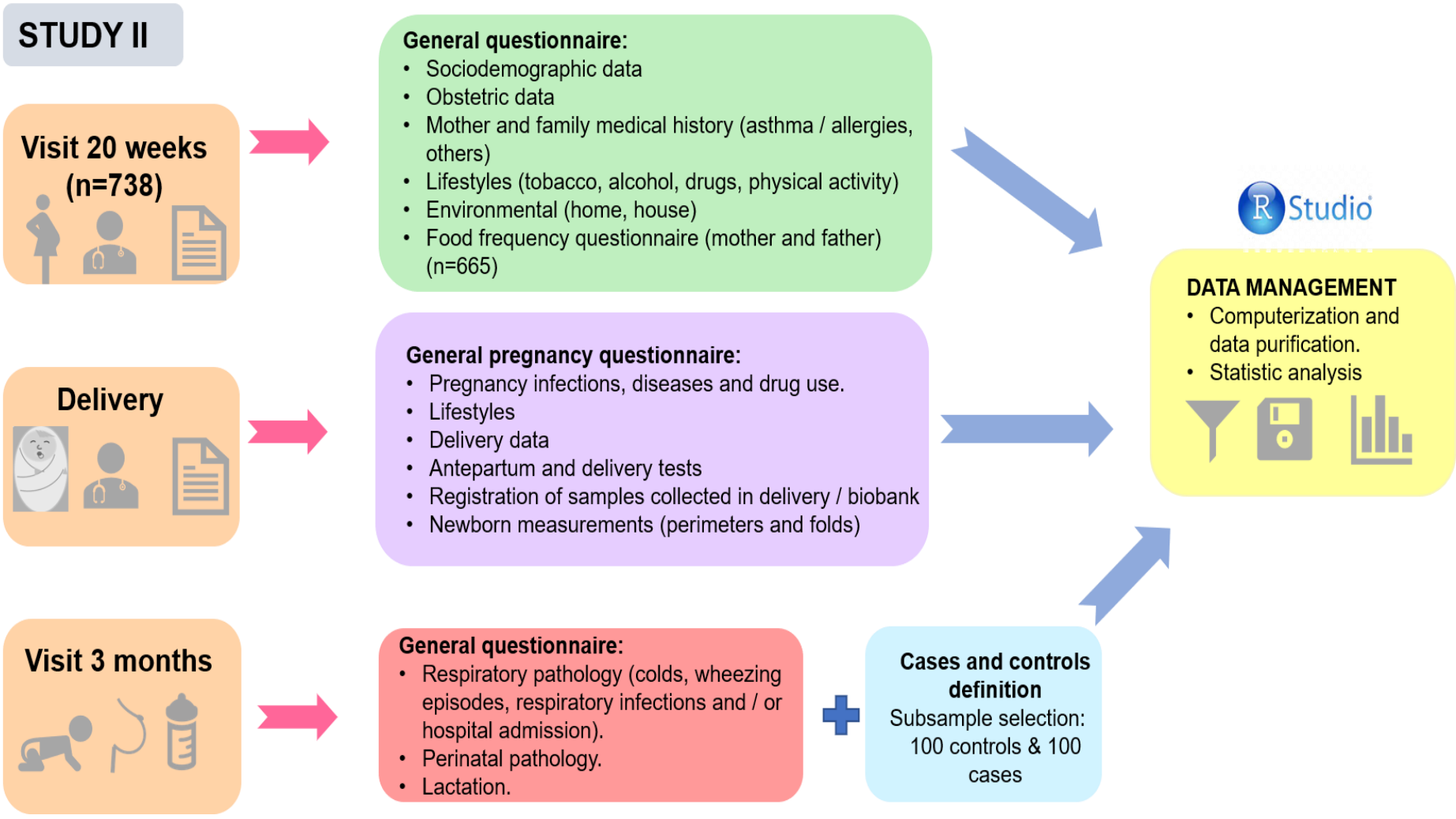


Figure 8. Experimental design of chapter 1, study II.

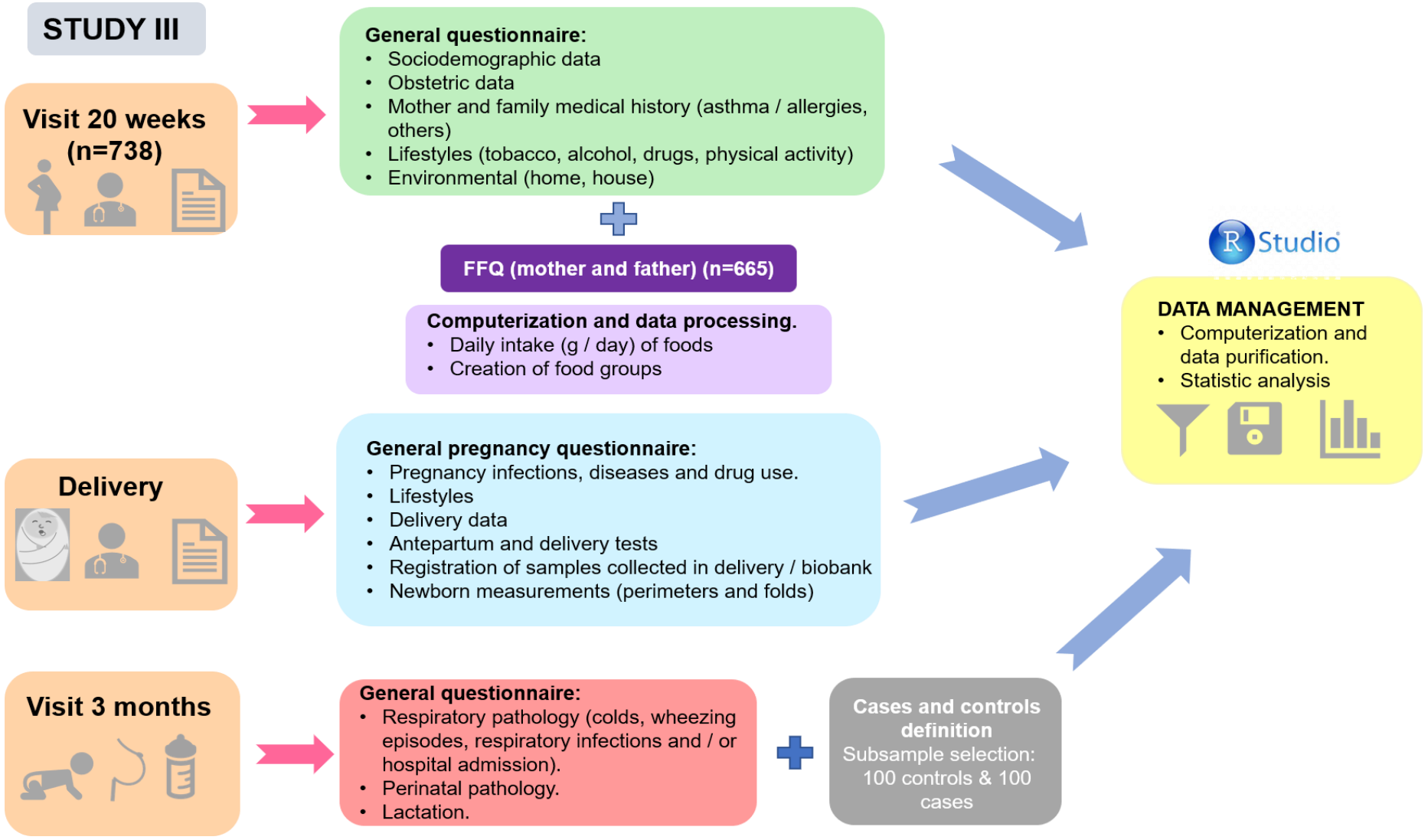


Figure 9. Experimental design of chapter 1, study III. FFQ: Food Frequency Questionnaires**.**

3.2 NELA cohort: subjects

To achieve **study I**, we worked with the population of pregnant women belonging to the NELA cohort. This cohort and the characteristics of the population have been previously described in the **section II. General material and methods** of this doctoral thesis.

3.3 Maternal dietary intake assessment and development of Mediterranean diet scores

Among the 738 women finally enrolled in the study, only 665 mothers completed the dietary information.

The dietary information on usual daily food intake was collected at 20 weeks of gestation, using a FFQ previously validated among pregnant women of the INMA prospective cohort study [292] which was administered by trained interviewers. The FFQ included 123 items, of which 112 were semi-quantitative, to assess usual food and nutrient intakes during the first 20 weeks of gestation, and 11 qualitative to collect information about the use of dietary supplements and organic food consumption. For each food item, the questionnaire asked how often, on average, the participants had consumed a particular amount of specific type of food from the beginning of pregnancy until the time of the interview. For each food item, standard units or reference serving sizes were specified. The questionnaire had nine possible intake frequency categories, ranging from ‘less than once per month or never’ to ‘6 or more times per day’. Nutrient values and energy intakes were obtained from the US Department of Agriculture Food Composition Tables [293], as well as other published sources for Spanish foods, portion sizes and their content for some specific nutrients such as folic acid [294,295]. The intake frequency for each food item was converted to the average daily intake for each participant. With the 78 foods of the FFQ, a total of 21 food groups (including water) were created based on the sum of the daily intakes (g/ day) that make up the different groups (**Annex 2)**.

3.3.1 Diet quality scores

To evaluate the degree of adherence to a MD during pregnancy, we used two scores: aMED [287]) and rMED [288]; both are a modified version of the

Mediterranean Diet Score (MDS) proposed by Trichopoulou and colleagues in 1995 [296]. As Chatzi *et al.,* reported in different publications [152,278] , we did not include alcohol consumption to calculate the scores because the population in the present study involved pregnant women and both scores had been developed for adults.

The rMED score was proposed by Bukcland *et al*., (2009) for the EPIC-Spain cohort [288]. This indicator has 8 components (originally 9, but alcohol was excluded): vegetables (excluding potatoes), fruits, nuts and seed (but excluding fruit juices), cereals (including whole grain, refined flour, pasta, rice and bread), legumes, fish and seafood, dairy products (including low and high fat products, cheese, yogurt, and cream desserts), total meat (including white, red and processed meat) and olive oil. In addition, before assigning the scores to the rMED groups (except alcohol), the intakes were transformed into grams per 1,000 Kcals/day. To assign the scores, tertiles were used instead of medium and the values 0, 1 and 2 were assigned to the first, second and third tertiles of intake, except for meats and dairy products that were assigned on the contrary, being the highest tertil scored with a 0. The possible scores ranged from 0 units (minimal adherence) to 16 units (maximum adherence). An rMED score of 0–5 was labelled as ‘low’, 6–11 as ‘medium’ and 12–16 as ‘high’ MD adherence by calculating the corresponding tertiles.

The aMED is also a version adapted by Fung and colleagues in 2005 [287] and used by Chazti *et al.,* (2013) [278] in different publications. This indicator has 8 components (9 originally), and a range from 0 to 8. For beneficial components (vegetables, fruits, fish and seafood, nuts, legumes and whole-cereals), women whose consumption was below the median (cohort-specific median) were assigned a value of 0 and women whose consumption was at or above the median were assigned a value of 1. For components presumed to be detrimental (red meat, liver, hamburgers and processed meats), the computation was inversed. For fat intake (the eight-food category) we used the ratio of daily consumption of mono-unsaturated lipids to saturated lipids. The total MD score was categorised to reflect three levels of adherence: (1) ≤ 3, Low; (2) 4-5, Medium and (3) 6-8, High MD quality in each index separately.

Table 1. Characteristics of Mediterranean diet pattern adherence indexes applied in pregnancy.

Food groups	Alternative Mediterranean score (aMED)	Relative Mediterranean score (rMED)
Scoring criteria	Ratios/day	Energy density= g*1000 Kcal/day
Vegetables	0 points ≤ median; 1 point > median	Tertile 1= 0 points; Tertile 2= 1 point; Tertile 3= 2 points
Legumes	0 points ≤ median; 1 point > median	Tertile 1= 0 points; Tertile 2= 1 point; Tertile 3= 2 points
Fruit	0 points ≤ median; 1 point > median	(Excluding fruit juice) Tertile 1= 0 points; Tertile 2= 1 point; Tertile 3= 2 points
Nuts	0 points ≤ median; 1 point > median	Included in fruit group
Fish	0 points ≤ median; 1 point > median	Tertile 1= 0 points; Tertile 2= 1 point; Tertile 3= 2 points
Cereals	only whole grain 0 points ≤ median; 1 point > median	Tertile 1= 0 points; Tertile 2= 1 point; Tertile 3= 2 points
Meat	(Red and processed meat) 0 points ≥ median; 1 point < median	(All type of meats) Tertile 1= 2 points; Tertile 2= 1 point; Tertile 3= 0 points
Dairy products	Not included	(Including skimmed) Tertile 1= 2 points; Tertile 2= 1 point; Tertile 3= 0 points
Mono/Saturated fats ratio	0 points ≥ median; 1 point < median	Not included
Alcohol	Not included	Not included
Potatoes	Included in vegetable groups	Not included
Olive oil cooking	Included in mono/saturated fats ratio group	Tertile 1= 0 points; Tertile 2= 1 point; Tertile 3= 2 points
Poultry	Not included	Included in meat group
Score range	0-9 points	0-16 points
Adherence category	Low= 0-3 points; Medium= 4-5 points; High= ≥ 6 points	Low= 0-5 points; Medium= 6- 11 points; High= 12- 16 points

Table 1 shows the main items of each of the scores used to determine adherence to MD, as well as their differences. The main difference between the two scores was that the aMED separated fruits and nuts into two independent groups and did not take into account the intake of dairy products in the indicator. Also, it included only whole grains in the cereal component and white meat was not included in the group of meats and processed meats. Finally, the ratio of mono-unsaturated to saturated fat was included as a fat source.

3.3.2 AHEI-2010

The AHEI-2010, which is a variation of the AHEI created by Fung *et al.,* in 2005 [287], has been associated with lower mortality and lower risk of diseases. This index was created by Chiuve *et al.,* in 2012 [297] and was also used in our study. It is a measure of diet quality based on American dietary guidelines and with modified recommendations from the US Department of Agriculture [293]. This index originally consists of 11 components, one of them being alcohol consumption, and has a range from 0 to 110. When it comes to applying this index to our study (pregnant women), an adaptation of the AHEI-2010 was performed eliminating the alcohol component. Due to this modification the score range changed from 0 to 100 points, as each of the components, 10 in total (instead of 11), contributes with 10 possible points (**table 2**)**.** For intermediate intakes, the proportional part between 0 and 10 was calculated by multiplying the number of daily rations consumed by 10, and then dividing by the criterion for a maximum score for that food group [290]. For example, for zero servings of fruit per day, a score of 0 was assigned; for one serving per day a score of 2.5 was assigned, as 4 servings per day was considered to be ideal. In this index, as in the case of the aMED, only the whole grain was contemplated within the group of cereals, the nuts instead of being included in the fruit component, were included together with the legumes. Regarding meats, the component was composed of the conscious between red meat and processed meats. Finally, polyunsaturated fats were divided into several items and sodium consumption was considered. For the transformation of the continuous variable into 3 degrees of adherence, the quintiles of the scores of the study population were calculated and the 3 intervals were established: low (42-58 points), medium (59-64 points) and high (65-81 points).

Table 2. The AHEI-2010 scoring method and mean scores at baseline among pregnant women in the NELA cohort Study.

Component	Criteria for minimum score (0)	Criteria for maximum score (10)
Vegetables, *servings/d*	0	≥5
Fruit, *servings/d*	0	≥4
Whole grains, *g/d*	0	75
Sugar-sweetened beverages and fruit juice, *servings/d*	≥1	0
Nuts and Legumes, *servings/d*	0	≥1
Red and processed meat, servings/d	≥1.5	0
Trans Fat, % of energy	≥4	≤0.5
Long-chain (n-3) fats (EPA* + DHA**), *mg/d*	0	250
PUFA†, *% of energy*	≤2	≥10
Sodium, *mg/d*	Highest decile	Lowest decile

EPA: Eicosapentaenoic acid.
DHA: Docosahexaenoic acid.
PUFA: Polyunsaturated fatty acid.

3.4 Potential determinants of adherence to Mediterranean dietary patterns

Of the 665 mothers who completed the dietary information, only 659 also completed all the information regarding sociodemographic and lifestyles patterns.

Information on the following sociodemographic characteristics and lifestyles patterns, which have an established or a potential association with a lower level of adherence to a MD in pregnancy, was collected through questionnaires administered in person during pregnancy: maternal age; parity (0, nulliparous; *vs.*. 1 or more, no nulliparous); gestational diabetes (yes/no); maternal education level (incomplete secondary or less, complete secondary, and university); maternal social class (defined as maternal occupation during pregnancy by using a widely used Spanish adaptation of the international ISCO88

coding system: I–II, managers/technicians; III, skilled; IV–V, semiskilled/unskilled; and unemployed) [298]; maternal body mass index (BMI Kg/m^2) based on height and pre-pregnancy self-reported weight (kg/m^2) (categorized as normal BMI<25, overweight BMI 25-29.99, and obesity BMI ≥ 30), maternal smoking and consumption of alcohol during the first 20 weeks of gestation (yes/no); mother's residential area (urban, residential and rural). Also, questions about physical activity or sedentary lifestyle during the pregnancy period were included in the questionnaire, focused on sedentary activity time (3 possible answers: <1 hour a day, 1-2 hours per day or ≥ 3 hours per day), sports activity time (3 possible answers: no exercise, up to 1h per week or ≥ 2 hours per week), and overall physical activity (self-report), defined as "sedentary/low active", "moderately active" and "strong active".

3.4 Study population: control *vs.* cases

A subset of 200 mother-infant pairs were selected, from the total of 738 pairs recruited in the NELA study, to conduct a pilot cases-controls study on the link between the mother's adherence to a MD during the pregnancy period and the predisposition to the appearance of early symptoms of asthma in their offspring. Nevertheless, we excluded 4 mothers because of missing diet assessment data on the basis of the sample availability. At the end, we selected a total of 197 participants: a) 96 mother-infant pairs as cases group when children presented precursor symptoms of asthma and b) 101 mother-infant pairs as control group without any of the symptoms described above. In addition to completing dietary questionnaires during pregnancy by gestational women, data and information about anthropometric measures (height, weight and body mass index) was obtained.

3.5 Statistical analysis

Data analysis were performed using RStudio version 1.2.5001 (RStudio Team (2019), Boston, MA) [299]. The median and interquartile range (IQR) were calculated for the quantitative variables of the study, and relative frequency distribution were estimated for qualitative variables. The non-parametric Kruskal-Wallis test or U-Mann Whitney test was performed for statistical comparisons

between the median aMED, rMED and AHEI-2010 scores of the study population according to the sociodemographic and lifestyle factors of the population. The Spearman correlation test was used to study the correlation between the two MD indices.

3.5.1 Study control vs. cases: population characteristics

The non-parametric Kruskal-Wallis test or U-Mann Whitney test was performed for statistical comparisons between controls and cases according to the sociodemographic and lifestyle factors of the population. The same statistical test was performed for statistical comparisons between controls and cases according to the daily intake of food groups or specific foods consumed.

3.5.2 Multivariate logistic regression models

In the total cohort, the three indices were modelled as continuous variables through multivariate linear regression as categorical variables, and through multivariable logistic regression (low *vs.* medium-high adherence) analysis to identify factors associated with probability of low MDA (≤ 3 points aMED; ≤ 5 points rMED) and low level of adherence to a healthy diet (≤ 58 points AHEI-2010). Medium and high level of adherence to aMED, rMED and AHEI-2010 was taken as the reference category.

In the controls *vs.* cases study, other multivariate logistic regression models were carried out to identify if the level of adherence to MD (in numerical and also categorical scale: high, medium, low), the daily intake (g/ day) of food groups or specific foods during pregnancy were associated with a higher risk of wheeze, atopy, atopic eczema, bronchitis and/or bronchiolitis in infants at 3 months of age. Other variables (maternal age, maternal pre-pregnancy BMI, maternal smoking, parental clinic history, mother's education, parity, type of lactation, infant's sex and newborn birth weight) were considered important confounders and for this reason were included to adjust all the models. Significance for all the tests carried out was set at p- value <0.05.

4. Results and discussion

4.1 Study I: NELA cohort Mediterranean diet adherence and potential determinants associated with this dietary pattern.

4.1.1 Baseline characteristics of mothers belonging to the NELA cohort.

The distribution of baseline characteristics of the mothers participating in the Spanish NELA cohort at 20 weeks of gestation are in **table 3**. The median age of the mothers at the time of recruitment was 33 years with a median weight of 62 Kg and BMI 23.03 Kg/m^2. 49.6 % were primiparous; and 8.2 % had gestational diabetes. More than a half of the cohort (71.7 %), lived in urban areas, 55.6 % had completed university studies and 18.8 % uncompleted secondary or less education. 16.1 % of the mothers reported to smoke during pregnancy and 5.7 % reported alcohol consumption. 37.4 % belonged to a high social class and 20.9 % were unemployed. A 57.9 % of the mothers reported that within their leisure time they spent an average of 1 or 2 hours a day doing sedentary activities such as watching television, computer or reading. 56.8 % of mothers did not practice any kind of sport, and within the group that did some sport activity majority (60.2 %) reported a sedentary/ low active lifestyle.

Table 3. Baseline characteristics of pregnant women at week 20 of pregnancy. NELA birth cohort study (n= 665).

	n	***%***	***Median***	***IQR***	***Min***	***Max***
Maternal age (years)			33.00	(30.00-36.00)	18.00	45.00
Anthropometric measures (n=661)						
Height (m)			1.64	(1.60-1.68)	1.47	1.82
weight (kg)			62.00	(56.00-70.00)	40.00	119.00
Body mass index (kg/m^2)			23.03	(20.83-25.88)	16.23	42.44
Normal weight (< 25)	456	69.0	21.59	(20.25-23.15)	16.23	24.98
Overweight (25-29.99)	143	21.6	26.71	(25.77-28.05)	25.00	29.86
Obese (≥ 30)	62	9.4	33.89	(31.27-37.16)	30.00	42.44
Parity, nulliparous	330	49.6				
Gestational diabetes (n=649)	53	8.2				
Area						
Urban area	477	71.7				
Residential	96	14.4				
Rural	92	13.8				
Maternal education						
Incomplete secondary or less	125	18.8				
Complete secondary	170	25.6				
University	370	55.6				
Maternal Smoking	107	16.1				
Maternal alcohol consumption	38	5.7				

Table 3. (continuation)

	n	***%***	***Median***	***IQR***	***Min***	***Max***
Maternal social class						
I-II	249	37.4				
III	146	22.0				
IV-V	131	19.7				
Unemployed	139	20.9				
Diet Index (20 weeks)						
aMED			4.00	(3.00-5.00)	0.00	8.00
rMED			8.00	(6.00-10.00)	2.00	15.00
AHEI-2010			61.00	(55.00-65.00)	42.00	81.00
Use of probiotics (n=622)	108	17.4				
Sedentary activity time (hours/day) (n=663)						
<1 hour per day	76	11.5				
1-2 hours per day	384	57.9				
≥ 3 hours per day	203	30.6				
Sports activity time (hours/week)						
Not exercise or sports	378	56.8				
up to 1 hour per week	88	13.2				
≥ 2 hour per week	199	29.9				
Physical activity (self-report)						
Sedentary/ low active	400	60.2				
Moderately active	235	35.3				
Strong active	30	4.5				

IQR: Interquartile range.

4.1.2 Level of adherence to the Mediterranean diet of mothers belonging to the NELA cohort using three different dietary indices.

The median adherence of the cohort corresponded to a score of 4.00 (Inter-Quartile Range (IQR): 3.00-5.00) and 8.00 (IQR: 6.00-10.00) for the aMED and rMED indices respectively. Both scores on the 3-level categorical scale (low, medium, and high) would be equivalent to a medium level of MDA. Even though there was a significant correlation between the two indices aMED and rMED (**figure 10**), significant differences were observed in the MDA distribution when applying the two indices to the study population (**figure 11**) (P <0.01). Specifically, in the case of the aMED index, 43.2 % and 19.1 % of the mothers presented a low and high degree of adherence respectively compared to 17.4 % and 8.9 % when the rMED index was applied.

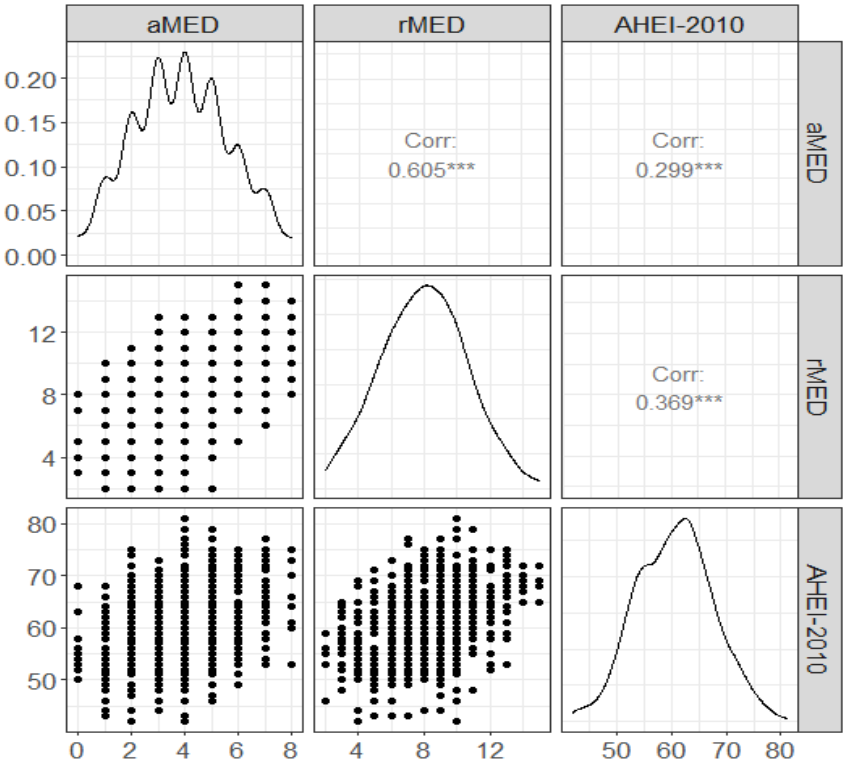


Figure 10. Correlation study between the 3 indices used to evaluate the pattern of adherence to the Mediterranean diet (aMED and rMED) and adherence to a healthy diet pattern (AHEI-2010) in the NELA cohort. *** (p-value<0.001); Corr: degree of correlation among indices.

In our study, the median score obtained by applying the AHEI-2010 index was 61 points (IQR; 55.00-65.00) (**table 3**), corresponding to a medium level of

adherence. However, 37.4 % of the mothers were classified with a low degree of adherence compared to 28.5 % of the mothers who were classified with a high adherence to a healthy diet (**figure 11**).

Despite the high degree of correlation that has been observed between the two MD indices (aMED and rMED), statistically significant differences have been observed in the distributions of the 3 groups into which the population can be divided according to their degree of adherence. In general, while previous studies have applied a single index of adherence to study the MD in pregnant women, it is the first study, to our knowledge, in which two different scores have been applied to characterize the MDA in pregnant women along with a third index which represent a healthy diet pattern.

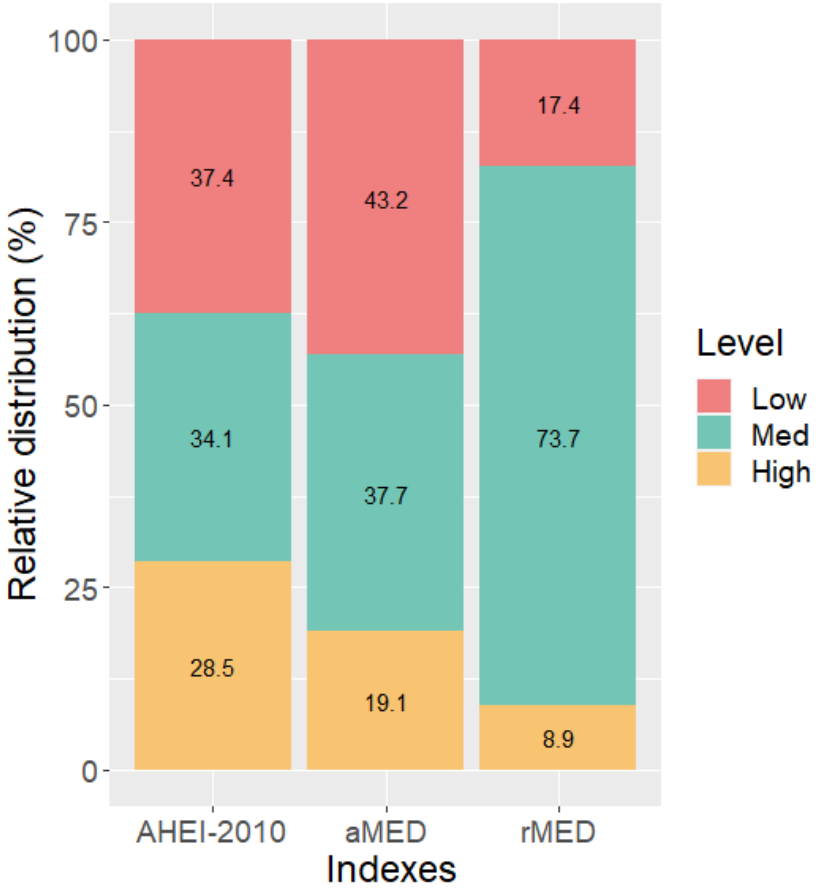


Figure 11. Distribution (%) of the study population according to the degree of adherence to the Mediterranean diet (aMED and rMED) and degree of adherence to a healthy diet pattern (AHEI-2010) during pregnancy in the NELA cohort.

The level of adherence to MD (aMED) as well as that of the healthy-style diet of pregnant women belonging to our study was in line with that observed in the work carried out by Lange *et al*., in 2010 [42], whose study population was characterized by presenting a medium level of adherence for both indices (4.6 points in the aMED index and 61 points in the AHEI-2010 index). Our results are also very similar, in reference to the percentage of mothers who present a low and high MDA index (using the aMED index) with the results observed by Chatzi *et al*., in 2012 [43] (42 %, and 15 % respectively) for a population residing in the Mediterranean area. However, in another study also carried out by Chatzi *et al.,* in 2013 [7], a lower proportion of mothers with a high level of adherence (approx. 7,5 % in Valencia population) (applying the aMED index) were found, compared to our population (19.1 %) despite the closeness between both areas (southern of Spain). On the other hand, we consider necessary to highlight the trend observed in recent years towards a worsening of adherence to the MD by Spanish adult population, being classified as low adherence and adopting a less healthy diet (typical of Western countries) [37,44]. It would be interesting to carry out studies to ascertain if this trend is observed in Spanish pregnant women, since it is a period in which mothers tend to be more careful in the choice of foods they eat, and avoid certain habits that could be harmful to their health and the health of their offspring.

The differences observed in the distributions of the population according to the MDA between both indices may be due to two issues: a) the differences in the determination of the foods that make up the single group; b) the different criteria that exist when trying to differentiate the food groups that conform to the MD pattern, such as olive oil, red meat, dairy products and cereals. In the case of olive oil, one of the main foods that define MD, the aMED index does not take this ingredient into account as an individual group, while the rMED index does, considering it positive. As a result of these differences, in other cohorts formed in the United Kingdom [45] or the United States [42], where MD is also studied using the aMED index, a lower percentage of low adherence (39.1 %) and a higher average MDA value (4.6 points) has been observed respectively, compared to our cohort, despite being geographic areas where following an MD is not the most common.

It must be considered that our population group has different nutritional requirements from that of a normal adult. Pregnant women suffer certain alterations during the first months of pregnancy that modify the dietary pattern. Besides, certain foods are also eliminated from the diet, or their intake is reduced because they are not recommended, such as the consumption of fish with high mercury content, while others are encouraged, such as dairy products as a source of calcium. About the last group, dairy products, there is no consensus when it comes to applying the indices or modifying them in pregnant women. Some studies include dairy as a positive food group [7,32,45], while others apply the rMED index without modifications, considering its consumption as negative due to their high fat content and because they do not differentiate between whole and skimmed dairy products, as in our case, following the recommendations [6,30,46].

4.1.3 Descriptive analysis of the level of adherence according to the sociodemographic and lifestyle characteristics in the NELA cohort.

Table 4 presents the analysis of the association between socio-demographic and lifestyle factors and the two MDA scores applying (aMED and rMED).

When the aMED index was applied, it was observed that the mothers who had an older age, non-smokers, as well as a higher educational level, higher social class and a who practice sports activities ≥ 2 hours per week, had a higher level of adherence to MD ($P < 0.01$). In the case of the rMED index, older non-smokers mothers, with a higher educational level, higher social class, who practice sports activities ≥ 2 hours per week and /or gestational diabetes, had a higher MDA ($P < 0.05$). The same analysis was carried out applying the AHEI-2010 index (**table 5**) and we observed that non-smokers mothers with an older age, a higher educational level, a higher social class, a strong active (self-report) lifestyle and /or practice sports activities ≥ 2 hours per week, had a higher AHEI-2010 diet pattern ($P < 0.05$) (**table 5**). No association with adherence was observed for the remaining variables, including, BMI and sedentary activity.

Table 4. Sociodemographic and lifestyle characteristics of women at week 20 of pregnancy according to the Alternative Mediterranean Diet and Relative Mediterranean Diet indexes score distribution. NELA birth cohort study (n= 665).

			aMED index score (points)			rMED index score (points)		
	n	***%***	***Median***	***IQR***	***P***	***Median***	***IQR***	***P***
Maternal age (years)					**<0.0001**			**<0.0001**
≥40	46	6.92	4.50	(3.00-5.75)		10.00	(7.25-11.00)	
35-39	191	28.72	4.00	(3.00-5.50)		8.00	(7.00-10.00)	
30-34	288	43.31	4.00	(3.00-5.00)		8.00	(6.00-10.00)	
25-29	109	16.39	3.00	(2.00-5.00)		7.00	(5.00-9.00)	
<25	31	4.66	2.00	(1.00-4.00)		5.00	(4.00-8.00)	
Body mass index (kg/m^2) (n=661)					0.30			0.73
Normal weight (< 25)	456	69.0	4.00	(3.00-5.00)		8.00	(6.00-10.00)	
Overweight (25-29.99)	143	21.6	4.00	(2.50-5.00)		8.00	(6.00-10.00)	
Obese (≥ 30)	62	9.4	3.00	(2.25-5.00)		8.00	(5.25-10.00)	
Parity (number of previous deliveries)					0.06			0.66
Nulliparous	330	49.62	4.00	(3.00-5.00)		8.00	(6.00-10.00)	
1 or more previous deliveries	335	50.38	4.00	(3.00-5.00)		8.00	(6.00-10.00)	
Gestational diabetes (n=649)					0.25			**0.028**
No	596	91.83	4.00	(3.00-5.00)		8.00	(6.00-10.00)	
Yes	53	8.17	4.00	(3.00-6.00)		9.00	(7.00-11.00)	
Area					0.21			0.50
Urban area	477	71.73	4.00	(3.00-5.00)		8.00	(6.00-10.00)	
Residential area	96	14.44	4.00	(3.00-5.00)		8.00	(7.00-10.00)	
Rural	92	13.83	4.00	(2.75-5.00)		8.00	(6.75-10.00)	
Maternal education					**<0.0001**			**<0.0001**
Incomplete secondary or less	125	18.80	3.00	(2.00-4.00)		7.00	(5.00-9.00)	
Complete secondary and superior	170	25.56	4.00	(2.25-5.00)		7.50	(6.00-9.00)	
University	370	55.64	4.00	(3.00-5.00)		8.50	(7.00-10.00)	

Table 4. (continuation)

			aMED index score (points)			rMED index score (points)		
	n	***%***	***Median***	***IQR***	***P***	***Median***	***IQR***	***P***
Maternal social class					**<0.0001**			**<0.0001**
I-II	239	35.94	4.00	(3.00-6.00)		8.00	(7.00-10.00)	
III	150	22.56	4.00	(3.00-5.00)		8.00	(6.00-10.00)	
IV-V	127	19.10	3.00	(2.00-5.00)		8.00	(6.00-9.00)	
Unemployed	149	22.41	3.00	(2.00-5.00)		7.00	(5.00-9.00)	
Maternal Smoking					**0.009**			**0.001**
No	558	83.91	4.00	(3.00-5.00)		8.00	(6.00-10.00)	
Yes	107	16.09	3.00	(2.00-5.00)		7.00	(5.00-9.00)	
Maternal alcohol consumption					0.20			0.17
No	627	94.29	4.00	(3.00-5.00)		8.00	(6.00-10.00)	
Yes	38	5.71	3.50	(2.00-4.75)		7.00	(6.00-9.00)	
Use of probiotics (n=622)					0.16			0.10
No	514	82.64	4.00	(3.00-5.00)		8.00	(6.00-10.00)	
Yes	108	17.36	4.00	(3.00-5.00)		8.00	(7.00-10.25)	
Sedentary activity time (hours) (n=663)					0.31			0.16
<1 hour per day	76	11.5	4.00	(3.00-5.00)		8.00	(6.00-10.00)	
1-2 hours per day	384	57.9	4.00	(3.00-5.00)		8.00	(6.75-10.00)	
≥ 3 hours per day	203	30.6	4.00	(2.00-5.00)		8.00	(6.00-10.00)	
Sports activity time (hours)					**<0.0001**			**<0.0001**
Not exercise or sports	378	57.1	3.00	(2.00-5.00)		8.00	(6.00-9.00)	
up to 1 hour per week	88	13.3	4.00	(2.00-5.00)		8.00	(6.00-10.00)	
≥ 2 hour per week	199	30.1	5.00	(3.00-6.00)		9.00	(7.00-11.00)	
Physical activity (self-report)					**<0.0001**			0.05
Sedentary/ low active	400	60.4	4.00	(2.00-5.00)		8.00	(6.00-10.00)	
Moderately active	235	35.5	4.00	(3.00-5.00)		8.00	(6.00-10.00)	
Strong active	30	4.5	5.00	(4.00-6.00)		8.50	(6.00-10.75)	

Bold values mean "statistical significance" (p -value<0.05). Numbers were expressed as median and interquartile range (IQR) for the quantitative variables. Statistical caparisons using non-parametric U-Mann Whitney test or Kruskal-Wallis test.

Table 5. Sociodemographic and lifestyle characteristics of women at week 20 of pregnancy according to the Alternative Healthy Eating index 2010 score distribution. NELA birth cohort study (n= 665).

	AHEI-2010 score		
	Median	*IQR*	*P*
Maternal age (years)			**<0.0001**
≥ 40	63.00	(59.00-66.75)	
35-39	62.00	(57.00-66.00)	
30-34	61.00	(56.00-66.00)	
25-29	58.00	(55.00-63.00)	
<25	55.00	(52.50-62.50)	
Body mass index (kg/m^2) (n=661)			1.00
Normal weight (< 25)	61.00	(55.00-66.00)	
Overweight (25-29.99)	61.00	(56.00-65.00)	
Obese (≥ 30)	60.00	(55.00-65.00)	
Parity (number of previous deliveries)			0.05
Nulliparous	60.00	(55.00-65.00)	
1 or more previous deliveries	61.00	(56.00-66.00)	
Gestational diabetes (n=649)			0.66
No	61.00	(56.00-66.00)	
Yes	60.00	(55.00-64.00)	
Area			0.23
Urban area	60.00	(55.00-65.00)	
Residential area	62.00	(57.75-65.00)	
Rural	62.00	(56.00-66.00)	
Maternal education			**<0.0001**
Incomplete secondary or less	58.00	(54.00-64.00)	
Complete secondary and superior	59.50	(55.00-64.00)	
University	62.00	(57.00-66.00)	
Maternal social class			**0.002**
I-II	62.00	(58.00-66.00)	
III	60.50	(55.25-65.00)	
IV-V	59.00	(54.00-64.00)	
Unemployed	60.00	(55.00-65.00)	
Maternal Smoking			**<0.0001**
No	61.00	(56.00-66.00)	
Yes	58.00	(54.00-63.00)	
Maternal alcohol consumption			0.08
No	61.00	(56.00-65.50)	
Yes	59.50	(53.25-63.75)	
Use of probiotics (n=622)			0.51
No	61.00	(56.00-66.00)	
Yes	60.00	(55.00-64.25)	
Sedentary activity time (hours) (n=663)			0.65
<1 hour per day	62.00	(55.75-66.00)	
1-2 hours per day	61.00	(56.00-65.00)	
≥ 3 hours per day	61.00	(55.00-65.00)	

Table 5. (continuation)

	Median	***IQR***	***P***
Sports activity time (hours)			**0.014**
Not exercise or sports	60.00	(55.00-65.00)	
up to 1 hour per week	61.00	(58.00-66.00)	
≥ 2 hour per week	62.00	(56.00-66.00)	
Physical activity (self-report)			**0.019**
Sedentary/ low active	60.50	(56.00-65.00)	
Moderately active	61.00	(55.00-66.00)	
Strong active	64.00	(61.25-67.00)	

Bold values mean "statistical significance" (p-value <0.05). Numbers were expressed as median and interquartile range (IQR) for the quantitative variables. Statistical caparisons using non-parametric U-Mann Whitney test or Kruskal-Wallis test.

4.1.4 Sociodemographic and lifestyle factors that increased the risk of a low adherence to the Mediterranean diet in the NELA cohort.

For aMED and rMED indices, older age, and practicing regular physical activity ≥ 2 hours per week were positively associated with MDA scores (see **table 6**).

The higher the number of previous deliveries, the lower the aMED index score (β: -0.29; (95 % IC: -0.49; -0.09; P <0.01). Only in the rMED index, a university educational level of the mother was associated with a better score in rMED index (β: 0.85; (95 % IC: 0.22; 1.49; P <0.01). In both indices, younger age was significantly associated with a higher risk of low MDA. (aMED; OR: 0.92; 95 % CI: 0.88-0.96; P <0.01; rMED; OR: 0.88; 95 % CI: 0.83-0.93, P <0.01). The number of previous deliveries was directly associated with a greater risk of a low MDA for each previous child (aMED; OR: 1.40; 95 % CI: 1.08-1.82; P <0.05; rMED; OR: 1.38; 95 % CI: 1.00-1.89, P <0.05). Only, for the aMED index, it was observed that university education was significantly associated with a lower risk of low MDA (OR: 0.48; 95 % CI: 0.28-0.83; P <0.01). Regarding the mother's sports activity and MDA, in aMED index, a lower risk of low adherence was obtained as the weekly hours of sports activity were ≥ 2 hours per week (OR: 0.54, 95 % CI: 0.36-0.81, P <0.01). It was observed that living in residential and rural areas decreased the risk of a low MDA but only in the rMED index.

Table 6. Association between sociodemographic characteristics and life style factors and low adherence to the Mediterranean diet in pregnant women at 20 weeks of gestation (n= 659) in the NELA cohort study.

	aMED				rMED			
	β†	**(95 % CI)**	**OR‡**	**(95% CI)**	**β†**	**(95% CI)**	**OR**	**(95% CI)**
Maternal age (years)§	0.09	(0.06; 0.12) **	0.92	(0.88-0.96) **	0.14	(0.09; 0.19) **	0.88	(0.83-0.93) *
Body mass index (kg/m^2)								
Normal weight (<24.99)	Ref		Ref		Ref		Ref	
Overweight (25-29.9)	-0.08	(-0.41; 0.25)	1.07	(0.70-1.61)	0.08	(-0.39; 0.55)	0.83	(0.47-1.44)
Obese (≥30)	0.10	(-0.36; 0.56)	1.07	(0.60-1.93)	0.15	(-0.52; 0.82)	1.28	(0.64-2.50)
Parity (number of previous deliveries) §	-0.29	(-0.49; -0.09) **	1.40	(1.08-1.82) *	-0.20	(-0.48; 0.09)	1.38	(1.00-1.89) *
Area								
Urban area	Ref		Ref		Ref		Ref	
Residential area	-0.08	(-0.47; 0.30)	1.05	(0.64-1.72)	-0.08	(-0.63; 0.47)	0.41	(0.17-0.88) *
Rural	-0.16	(-0.54; 0.22)	1.08	(0.67-1.76)	0.41	(-0.14; 0.96)	0.41	(0.19-0.80) *
Maternal education								
Incomplete secondary or less	Ref		Ref		Ref		Ref	
Complete secondary and superior	0.21	(-0.21; 0.62)	0.67	(0.40-1.13)	0.10	(-0.50; 0.70)	0.93	(0.50-1.73)
University	0.37	(-0.08; 0.81)	0.48	(0.28-0.83) **	0.85	(0.22; 1.49) **	0.80	(0.40-1.59)
Maternal social class								
I-II	Ref		Ref		Ref		Ref	
III	-0.14	(-0.53; 0.24)	1.01	(0.61-1.64)	0.06	(-0.49; 0.62)	1.26	(0.62-2.52)
IV-V	-0.24	(-0.69; 0.20)	1.09	(0.62-1.92)	0.02	(-0.63; 0.66)	1.19	(0.55-2.51)
Unemployed	-0.08	(-0.51; 0.35)	1.08	(0.63-1.86)	-0.12	(-0.74; 0.50)	1.30	(0.63-2.68)
Maternal Smoking, yes	-0.02	(-0.39; 0.36)	0.94	(0.59-1.51)	-0.17	(-0.71; 0.36)	1.11	(0.63-1.92)
Maternal alcohol consumption, yes	-0.23	(-0.80; 0.33)	1.19	(0.58-2.44)	-0.31	(-1.13; 0.50)	1.18	(0.46-2.75)

Table 6. (continuation)

	aMED					rMED				
	β†	**(95 % CI)**		**OR‡**	**(95% CI)**		**β†**	**(95% CI)**		**OR**	**(95% CI)**
Sedentary activity time (hours)											
<1 hour per day	Ref						Ref			Ref	
1-2 hours per day	-0.17	(-0.60; 0.26)		1.30	(0.76-2.28)		0.05	(-0.57; 0.67)		0.73	(0.36-1.53)
≥ 3 hours per day	-0.28	(-0.75; 0.19)		1.35	(0.74-2.47)		-0.19	(-0.86; 0.49)		1.42	(0.68-3.11)
Sports activity time (hours)											
Not exercise or sports	Ref			Ref			Ref			Ref	
up to 1 hour per week	0.08	(-0.32; 0.49)		0.69	(0.41-1.14)		-0.01	(-0.59; 0.57)		1.36	(0.72-2.49)
≥ 2 hour per week	0.56	(0.25; 0.87)	**	0.54	(0.36-0.81)	**	0.82	(0.37; 1.27)	**	0.77	(0.43-1.35)
Physical activity (self-report)											
Sedentary/ low active	Ref			Ref			Ref			Ref	
Moderately active	0.23	(-0.05; 0.52)		0.71	(0.49-1.01)		0.09	(-0.32; 0.50)		0.89	(0.54-1.44)
Strong active	0.90	(0.25; 1.56)	**	0.45	(0.17-1.10)		0.10	(-0.85; 1.05)		0.59	(0.13-1.95)

Ref: Reference; CI: confidence interval; β: Regression coefficients; OR: Odds ratio.
§ Maternal age was introduced as a continuous variable.
aMED (Alternative Mediterranean Diet index score).
rMED (Relative Mediterranean Diet index score).
†Multivariate linear regression analysis. Indices were modelled as continuous variables.
‡Multivariate logistic regression analysis. Indices were modelled as categorical variables (medium-high adherence *vs.* low); aMED score: low (≤ 3 points) and medium-high (≥ 4 points); rMED score: low (≤ 5 points) and medium-high (≥ 6 points).
All models were adjusted for age, BMI, parity, residential area, maternal education and social class, smoking and alcohol consumption, sedentary activity time, sport activity time and physical activity.
*p-value<0.05; **p-value<0.01.

Regarding AHEI-2010 index (**table 7**), a younger age (OR: 0.92; 95 % CI: 0.88-0.96, *P* <0.01), and smoke (OR: 1.70, 95 % CI: 1.07-2.70, *P* <0.05) were associated with a higher risk of low AHEI-2010 dietary pattern. About sport activity, up to 1 hour per week of sports activity (OR: 0.56; 95 % CI: 0.32-0.96, P <0.05) decreases the probability of presenting a low AHEI-2010 dietary pattern.

In the current prospective cohort study, 3 different dietary indices, based on the data from FFQ collected at 20 weeks of pregnancy, were calculated. In relation to the sociodemographic and lifestyle factors we found a markedly protective effect on age for the 3 dietary indices. The older they are, the higher the chance of a good MDA or a diet associated with a lower risk of cardiovascular disease. Also, the educational level of the mother and fewer previous deliveries were other variables with a protective effect for MDA, decreasing the probability of poor adherence. These protective markers coincide with those observed by other authors [22,37]. In other studies, the authors observed that a healthy dietary pattern in pregnant women was positively associated with older age, a higher educational level, a higher social class and greater physical activity [22,38–41]. Other authors also observed a negative association between the number of previous deliveries and a healthy diet pattern [38,40,41]. In relation to smoking and BMI, contradictory results have been reported. In certain studies, smoke and BMI have been negatively associated with a healthy diet pattern [40,41], while in others overweight/obesity was positively associated [38].

Table 7. Association between sociodemographic characteristics and lifestyle factors and low adherence to AHEI-2010 score in pregnant women at 20 weeks gestation (n = 659) in the NELA cohort study.

	β†	**(95 % CI)**		**OR‡**	**(95 % CI)**	
Maternal age (years)§	0.31	(0.18; 0.44)	**	0.92	(0.88-0.96)	**
Body mass index (kg/m^2)						
Normal weight (<24.99)	Ref			Ref		
Overweight (25-29.9)	0.43	(-0.88; 1.74)		0.90	(0.59-1.37)	
Obese (≥30)	0.82	(-1.04; 2.67)		0.82	(0.44-1.50)	
Parity (number of previous deliveries) §	-0.24	(-1.04; 0.55)		1.01	(0.78-1.30)	
Area						
Urban area	Ref			Ref		
Residential area	0.77	(-0.76; 2.30)		0.78	(0.46-1.29)	
Rural	0.83	(-0.70; 2.35)		0.67	(0.40-1.10)	
Maternal education						
Incomplete secondary or less	Ref			Ref		
Complete secondary and superior	1.10	(-1.76; 1.57)		0.83	(0.49-1.40)	
University	0.75	(-1.02; 2.51)		0.70	(0.40-1.23)	
Maternal social class						
I-II	Ref			Ref		
III	-0.49	(-2.03; 1.05)		1.54	(0.93-2.54)	
IV-V	-1.23	(-3.02; 0.55)		1.68	(0.95-3.00)	
Unemployed	-0.17	(-1.89; 1.56)		1.11	(0.62-1.95)	
Maternal Smoking, yes	-1.26	(-2.74; 0.23)		1.70	(1.07-2.70)	*
Maternal alcohol consumption, yes	-1.49	(-3.75; 0.77)		1.34	(0.65-2.76)	
Sedentary activity time (hours)						

Table 7. continuation

	β†	(95 % CI)		OR‡	(95 % CI)	
<1 hour per day	Ref			Ref		
1-2 hours per day	0.10	(-1.61; 1.82)		0.82	(0.43-1.56)	
≥ 3 hours per day	0.15	(-1.74; 2.03)		1.16	(0.63-2.16)	
Sports activity time (hours)						
Not exercise or sports	Ref			Ref		
up to 1 hour per week	1.36	(-0.26; 2.97)		0.56	(0.32-0.96)	*
≥ 2 hour per week	0.80	(-0.45; 2.05)		0.72	(0.48-1.08)	
Physical activity (self-report)						
Sedentary/ low active	Ref			Ref		
Moderately active	-0.19	(-1.33; 0.95)		1.54	(1.07-2.23)	*
Strong active	2.64	(0.02; 5.27)	*	0.35	(0.10-1.00)	

Ref: Reference; CI: confidence interval; β: Regression coefficients; OR: Odds ratio.
AHEI-2010 (Alternative Healthy Eating Index score).
§ Introduced as a continuous variable.
†Multivariate linear regression analysis. Index was modelled as continuous variable.
‡Multivariate logistic regression analysis. Index was modelled as categorical variable (medium-high *vs.* low adherence); AHEI-2010 score: low (≤ 58 points) and medium-high (> 58 points).
Both models were adjusted for age, BMI, parity, residential area, maternal education and social class, smoking and alcohol consumption, sedentary activity time, sport activity time and physical activity.
*p-value<0.05; **p-value<0.01.

As possible limitations of our study, we must cite the observational nature of the study, with dietary information recorded retrospectively (diet during the first 20 weeks of pregnancy). This may involve some memory bias, possibly reinforced by the social desirability of avoiding the recording of a large intake of unhealthy foods. Such bias would mainly affect mothers with worse eating habits, reducing the variability of the sample and the probability of obtaining significant associations. As possible advantages of our study, it should be noted that: (i) the large sample size and the study population selected from healthy pregnant women in the reference area, so the results could be more easily extrapolated to a wider population; (ii) the use of a previously validated FFQ among pregnant women in Spain which may reduce the presence of bias [25]; (iii) the compilation of all the information obtained in the different questionnaires was carried out by trained interviewers; and (iv) results have been presented for three indices, which allows a better comparability with other studies that use only one index.

4.2. Study II: Association between adherence to MD of gestational women of the NELA cohort and the early onset of asthma precursors symptoms at 3 months of age.

4.2.1 Baseline characteristics of mothers-infant pairs recruited in the NELA cohort: Controls vs. cases.

Table 8 presents the baseline characteristics of the variables considered possible confounding factors for t pregnant women whose offspring presented or not symptoms, classified as cases and controls respectively. In both groups, the median age of the mothers at the time of recruitment was 33 years, median weight was 62.25 Kg in the control group and 62.00 Kg in the cases group. The median pre-pregnancy BMI was 23 Kg/m^2 for both groups. The median weight gained during pregnancy period was 12.90 Kg in the control group and 12.00 kg in the cases group. Regarding the weeks of gestation, in both groups 40 weeks of gestation was the median observed value.

Two-thirds of the cohort lived in the city or peripheral neighbourhoods and within this category, a 53.48 % of the mothers belonged to the control group and 46.51 % to the cases group. On the other hand, 53.13 % of control's mothers and the 46.88 % of cases' mothers lived in rural areas. The mother's educational level is one of the confounding factors considered in this study and of the total of 113 pregnant women with university studies, a 53.10 % belonged to the control group compared to 46.90 % of mothers in the cases group. The number of mothers who had incomplete secondary studies or less, was very similar in both groups. If we focus on tobacco, both groups presented mothers who had smoked for at least the first 20 weeks of gestation, being fewer smokers in the control group (n=13 (13,4 %)) with respect to the group of cases (n=17 (18.7 %)). Hence, of the total of smoking mothers (n=30), 58.06 % belonged to the cases group. In relation to alcohol consumption, the number of mothers who had consumed alcohol during the first 20 weeks of pregnancy was 14 (3.55 %) of which 8 belonged to the control group and 6 to the cases group.

Regarding the use of antibiotics throughout the pregnancy or in each of the trimesters, numbers of those who consumed were very similar in both groups, 35 mothers in control group and 36 in cases group. On the other hand, of the total

of mothers who consumed antibiotics in each of the 3 trimesters of pregnancy, higher percentages of mothers belonging to the cases group were observed (61.29 % in 1st trimester; 51.52 % 2nd trimester and 54.17 % 3rd trimester). Continuing with the antibiotics, specifically the use of intra-partum antibiotic (IPA), 29.14 % (n = 51) of the mothers were exposed to IPA, observing similar percentages if we divided the population according to controls or cases. Of all the variables discussed so far, no significant differences have been observed between the control group and the cases group.

Table 8. Baseline characteristics of the study participants among healthy Spanish women during pregnancy period (n= 197) recruited in the NELA (Nutrition in Early Life and Asthma) cohort whose infants have been classified into cases and controls.

	Controls (n= 101)				Cases (n= 96)				
	n	%	Median	IQR	n	%	Median	IQR	*P*
Maternal age (years)			33.00	(30.00-36.00)			33.00	(30.75-35.00)	
Anthropometric measures (pre-pregnancy) (n=195)									
Height (m)			1.64	(1.60-1.68)			1.65	(1.61-1.68)	
weight (kg)			62.25	(57.00-69.25)			62.00	(56.00-69.00)	
Body mass index (kg/m^2)			23.20	(21.38-25.23)			23.30	(20.80-25.85)	
Normal weight (< 25)	74	54.01	23.00	(22.00-24.00)	63	45.99	22.00	(21.00-23.00)	
Overweight (25-29.99)	18	40.91	26.00	(25.50-28.00)	26	59.09	27.00	(26.00-28.25)	
Obese (>30)	3	33.33	34.00	(33.25-35.00	6	66.67	32.50	(31.00-33.75)	
Body mass index (kg/m^2) (32 weeks) (n=156)			27.75	(25.77-29.85)			27.62	(25.33-29.89)	
Weight gain during pregnancy (Kg) (n=182)			12.90	(10.00-16.00)			12.00	(9.20-15.50)	
Weeks of gestation			40.00	(39.00-41.00)			40.00	(39.00-40.25)	
Parity, nulliparous	60	50.85			58	49.15			
Gestational diabetes (n=195)	6	42.86			8	57.14			
Paternal clinic history									
Asthmatic mother, yes	0	0.00			13	100.00			***
Atopic dermatitis mother, yes	0	0.00			9	100.00			***
Asthmatic father, yes	0	0.00			10	100.00			***
Atopic dermatitis father, yes	0	0.00			9	100.00			***
Area									
City	69	53.49			60	46.51			
Residential area	15	41.67			21	58.33			
Rural	17	53.13			15	46.88			
Maternal education									
Incomplete secondary or less	17	47.22			19	52.78			
Complete secondary and superior	24	50.00			24	50.00			
University	60	53.10			53	46.90			
Use of probiotics (20 weeks), yes (n=182)	24	66.67			12	33.33			*
Maternal Smoking (20 weeks), yes	13	41.94			18	58.06			
Maternal alcohol consumption (20 weeks), yes	8	57.14			6	42.86			
Weight of the newborn (Kg)			3.25	(2.99-3.59)			3.30	(2.99-3.54)	

Table 8. (continuation)

	n	%	Median	IQR	n	%	Median	IQR	*P*
Use of antibiotics, yes (n=182)									
1st trimester	12	38.71			19	61.29			
2nd trimester	16	48.48			17	51.52			
3rd trimester	11	45.83			13	54.17			
Use of antibiotics (all pregnancy), yes (n=190)	35	49.30			36	50.70			
Use of intra partum antibiotics (IPA), yes (n=175)	25	49.02			26	50.98			
Mode of delivery									
Vaginal	86	56.58			66	43.42			
Caesarean	15	35.71			30	71.43			**
Sex of the newborn									
Girl	48	53.93			41	46.07			
Boy	53	49.07			55	50.93			
Type of lactation									
Exclusive breastfeeding	48	51.61			45	48.39			
Mixed	43	51.19			41	48.81			
Exclusive artificial	10	50.00			10	50.00			

aMED (Alternative Mediterranean Diet index score); *p-value<0.05; **p-value<0.01; ***p-value< 0.001. Numbers were expressed as median and interquartile range (IQR) for the quantitative variables and relative frequency distribution were estimated for qualitative variables. U-Mann Whitney test for continuous variables or Person's X^2 test for categorical ones. Number of individuals belonging to this category (n); Of the total number of mothers within each category, the percentage of mothers belonging to the control group or the case group was calculated. (%).

Significant differences were observed between the two study groups regarding the parental clinic history related with asthma and atopic dermatitis, type of delivery and the use of probiotics during the first 20 weeks of gestation. Of the total study population, only mothers and fathers with a clinical history of asthma or atopic dermatitis were observed in the cases group. With respect to the mode of delivery, of the 45 mothers who gave birth by caesarean section, 71.43 % belonged to the cases group (p- value <0.01). On the other hand, 19.78 % (n=36) of mothers consumed probiotics, 66.67 % belonging to control group. Finally, about of offspring, no significant differences were found when comparing the weight and sex of the newborn between the case and control groups.

4.2.2 Level of adherence to a Mediterranean diet of mothers during the pregnancy period: Control vs. cases.

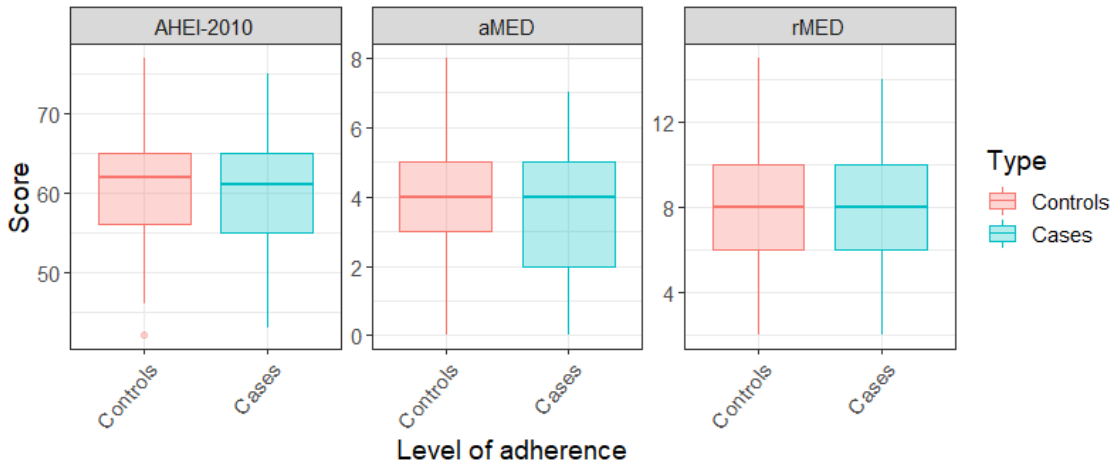


Figure 12. Comparison of the level of adherence to a Mediterranean diet pattern (aMED and rMED) and to a healthy diet pattern (AHEI-2010) among mothers of infant's controls and cases.

To respond the specific objective two, a comparative between control and cases with respect to the level of adherence to MD or a healthy diet pattern of the mothers, were performed (**figure 12**). No significant differences were found in the level of adherence between both groups (control or cases) for any of the 3 scores used (aMED, rMED and AHEI-2010). In the case of the control group, the scores obtained by applying the adherence indices aMED, rMED and AHEI were 4, 8 and 62 points, which would be equivalent to a medium level of adherence. The

cases group showed the same scores on the aMED and rMED indices and a similar score on the AHEI (61 points).

Table 9 represents the comparison of adherence levels to MD pattern between cases and controls groups applying aMED or rMED index based on different sociodemographic and lifestyle conditions. No significant differences were observed in the level of adherence between mothers belonging to the control or cases group for any of the sociodemographic or style life factors, when aMED or rMED index were applied, except in the case of mothers categorized as obese applying rMED index. It was observed that obese mothers in the control group had a significantly lower level of adherence than mothers from the cases group (control: 5.50 (4.75-8.00); cases: 8.50 (8.00-9.75); p-value <0.05).

Table 9. Comparison of the level of adherence to MD of pregnant women (n = 188), obtained by applying two different adherence indexes, classified according to the phenotype of the offspring as controls (n=97) and cases (n=91), based on confounding factors.

	aMED index score									**rMED index score**				
	Controls				Cases					Controls		Cases		
	n	%	Median	IQR	n	%	Median	IQR	*P*	Median	IQR	Median	IQR	*P*
Maternal age (years)														
≥40	6	75.00	4.50	(4.00-5.00)	2	25.00	5.50	(5.25-5.75)		9.50	(8.25-10.00)	10.50	(10.25-10.75)	
35-39	27	55.10	4.00	(3.00-5.00)	22	44.90	4.00	(3.00-5.75)		8.00	(6.00-10.00)	8.00	(7.00-10.00)	
30-34	43	46.74	4.00	(3.00-5.00)	49	53.26	4.00	(3.00-5.00)		8.00	(7.00-9.00)	8.00	(7.00-10.00)	
25-29	18	60.00	3.50	(2.25-4.75)	12	40.00	2.00	(1.00-4.00)		8.00	(6.00-9.00)	6.00	(4.75-8.00)	
<25	3	33.33	2.00	(1.00-3.00)	6	66.67	3.00	(2.25-3.75)		4.00	(3.50-7.50)	7.00	(5.25-8.00)	
Anthropometric measures (n=186)†														
Body mass index (kg/m^2)														
Normal weight (< 25)	71	54.20	4.00	(3.00-5.00)	60	45.80	4.00	(2.00-5.00)		8.00	(6.00-9.50)	8.00	(6.00-10.00)	
Overweight (25-29.99)	17	41.46	3.00	(3.00-4.00)	24	58.54	4.00	(2.75-5.00)		9.00	(7.00-10.00)	8.00	(6.75-9.00)	
Obese (>30)	8	57.14	3.00	(2.75-4.25)	6	42.86	5.00	(4.25-5.00)		5.50	(4.75-8.00)	8.50	(8.00-9.75)	*
Weeks' gestation (week)														
no premature	95	52.49	4.00	(3.00-5.00)	86	47.51	4.00	(2.00-5.00)		8.00	(6.00-10.00)	8.00	(6.00-10.00)	
premature (≤ 37 weeks)	2	28.57	3.50	(1.75-5.25)	5	71.43	4.00	-		6.50	(4.75-8.25)	9.00	(8.00-9.00)	
Parity, nulliparous	56	50.45	4.00	(3.00-5.00)	55	49.55	4.00	(2.00-5.00)		8.00	(6.00-10.00)	7.00	(6.00-9.00)	
Gestational diabetes, yes	5	38.46	3.00	(3.00-4.00)	8	61.54	4.00	(2.5-5.00)		8.00	(7.00-10.00)	9.50	(7.00-10.25)	
Area														
City	67	54.03	4.00	(3.00-5.00)	57	45.97	4.00	(2.00-5.00)		8.00	(6.00-10.00)	8.00	(7.00-10.00)	
Residential area	14	41.18	4.00	(3.00-5.00)	20	58.82	5.00	(3.75-6.00)		8.00	(7.00-9.00)	9.00	(7.00-10.00)	
Rural	16	53.33	4.00	(3.00-5.00)	14	46.67	4.00	(2.00-4.75)		8.00	(6.75-10.00)	7.00	(6.00-8.00)	

Table 9. (continuation)

	aMED index score									rMED index score				
	Controls				Cases					Controls		Cases		
	n	%	Median	IQR	n	%	Median	IQR	*P*	Median	IQR	Median	IQR	*P*
Maternal education														
Incomplete secondary	16	45.71	3.00	(2.75-5.00)	19	54.29	3.00	(2.00-5.00)		7.00	(5.00-8.00)	8.00	(5.00-9.00)	
complete secondary and superior	23	51.11	3.00	(2.00-4.00)	22	48.89	4.00	(2.00-4.75)		7.00	(6.00-8.00)	8.50	(7.00-9.75)	
University	58	53.70	4.00	(3.00-5.00)	50	46.30	4.00	(3.00-5.00)		8.00	(7.00-10.00)	8.00	(7.00-10.00)	
Use of probiotics, yes (n=182)†	22	64.71	4.00	(3.00-5.00)	12	35.29	3.50	(2.00-4.25)		8.50	(7.00-11.00)	7.50	(6.75-9.25)	
Maternal Smoking, yes †	13	43.33	4.00	(2.00-5.00)	17	56.67	4.00	(3.00-5.00)		8.00	(5.00-9.00)	7.00	(6.00-8.00)	
Maternal alcohol consumption, yes †	7	53.85	4.00	(3.00-4.00)	6	46.15	4.00	(3.25-4.75)		8.00	(7.00-8.50)	8.00	(7.25-8.00)	
Use of antibiotics (n=182)														
1st trimester	11	36.67	3.00	(3.00-4.50)	19	63.33	4.00	(2.00-4.50)		8.00	(7.00-9.50)	9.00	(5.50-10.00)	
2nd trimester	16	51.61	4.00	(2.75-4.25)	15	48.39	4.00	(2.00-5.00)		8.5	(6.00-10.00)	7.00	(6.50-8.50)	
3rd trimester	11	47.83	4.00	(4.00-5.00)	12	52.17	4.00	(2.75-5.00)		8.00	(7.50-10.00)	8.00	(6.50-9.00)	
Use of intra partum antibiotics (IPA), yes (n=167)	23	46.94	5.00	(4.00-5.50)	26	53.06	4.00	(2.00-5.00)		8.00	(6.50-10.00)	7.50	(6.00-9.00)	
Mode of delivery														
Vaginal	84	57.53	4.00	(3.00-5.00)	62	42.47	4.00	(2.25-5.00)		8.00	(6.00-10.00)	8.00	(6.00-10.00)	
Caesarean	13	30.95	4.00	(3.00-4.00)	29	69.05	4.00	(2.00-5.00)		7.00	(7.00-8.00)	8.00	(7.00-9.00)	
Type of lactation														
Exclusive breastfeeding	46	51.11	4.00	(3.00-5.00)	44	48.89	4.00	(2.00-5.00)		8.00	(6.00-10.00)	8.00	(6.75-10.00)	
Mixed	42	52.50	4.00	(3.00-5.00)	38	47.50	4.00	(4.00-5.00)		8.00	(6.00-10.00)	8.00	(7.00-9.75)	
Exclusive artificial	9	50.00	3.00	3.00	9	50.00	2.00	(1.00-4.00)		7.00	(4.00-8.00)	6.00	(6.00-7.00)	

aMED (Alternative Mediterranean Diet index score); rMED (Relative Mediterranean Diet index score). P: p-value; *p-value<0.05; **p-value<0.01; ***p-value< 0.001.†: Information obtain at 20 weeks of gestation. Numbers were expressed as median and interquartile range (IQR) for the quantitative variables. U-Mann Whitney test for continuous variables to compare two individual groups. Statistical analyses were not performed when the number of subjects was less than 3. Number of individuals belonging to this category (n); Percentage of total individuals belonging to this category (%).

Table 10. Comparison of the level of adherence to AHEI-2010 index of pregnant women (n = 188) classified as controls (n=97) and cases (n=91), based on confounding factors.

	Controls		Cases		
	Median	IQR	Median	IQR	*P*
Maternal age (years)					
≥40	63.50	(63.00-65.50)	67.00	(64.50-69.50)	
35-39	62.00	(57.00-65.50)	61.00	(57.75-67.50)	
30-34	62.00	(55.50-66.00)	62.00	(57.00-65.00)	
25-29	58.50	(55.00-61.50)	55.50	(54.00-61.25)	
<25	63.00	(58.50-64.00)	54.50	(51.75-58.75)	
Anthropometric measures (n=186)					
Body mass index (kg/m^2)					
Normal weight (< 25)	62.00	(56.00-66.00)	61.00	(55.00-65.25)	
Overweight (25-29.99)	59.00	(54.00-62.00)	62.50	(56.25-64.00)	
Obese (>30)	64.50	(61.25-66.50)	63.50	(59.75-65.75)	
Weeks of gestation (week)					
no premature	62.00	(56.00-65.00)	61.00	(55.00-65.00)	
premature (≤ 37 weeks)	63.00	-	63.00	(59.00-67.00)	
Parity, nulliparous	59.50	(55.00-64.25)	61.00	(54.50-65.00)	
Gestational diabetes, yes	64.00	(63.00-65.00)	55.50	(51.50-59.25)	**
Area					
City	62.00	(55.50-66.00)	61.00	(55.00-65.00)	
Residential area	61.00	(56.50-62.75)	61.00	(57.00-65.75)	
Rural	59.50	(56.75-63.50)	62.50	(56.25-64.75)	
Maternal education					
Incomplete secondary	58.00	(54.75-63.50)	57.00	(54.00-63.50)	
complete secondary and superior	59.00	(5.00-62.50)	61.00	(54.50-65.50)	
University	62.00	(58.00-66.00)	62.00	(57.00-66.00)	
Use of probiotics, yes (n=182)†	62.00	(56.00-64.75)	59.50	(54.00-62.25)	
Maternal Smoking, yes†	58.00	(56.00-65.00)	61.00	(54.00-64.00)	
Maternal alcohol consumption, yes†	60.00	(56.50-64.50)	62.00	(61.25-62.75)	
Use of antibiotics (n=182)	60.00	(56.00-63.75)	61.00	(54.25-64.75)	
1st trimester	62.00	(57.00-64.00)	61.00	(54.50-64.50)	
2nd trimester	58.00	(55.75-62.50)	61.00	(55.50-64.50)	
3rd trimester	59.00	(56.50-63.50)	62.00	(53.75-63.50)	
Use of intra partum antibiotics (IPA), yes (n=167)	62.00	(53.50-63.50)	61.00	(55.00-65.00)	
Mode of delivery					
Vaginal	62.00	(56.00-66.00)	62.00	(55.00-66.00)	
Caesarean	60.00	(58.00-63.00)	60.00	(55.00-63.00)	

Table 10 (continuation)

	Controls		Cases		
	Median	IQR	Median	IQR	*P*
Type of lactation					
Exclusive breastfeeding	62.00	(55.25-65.25)	61.00	(56.75-64.25)	
Mixed	60.00	(56.00-65.00)	63.00	(54.25-66.00)	
Exclusive artificial	59.00	(56.00-65.00)	55.00	(54.00-60.00)	

P: p-value; *p-value<0.05; **p-value<0.01; ***p-value< 0.001.
Numbers were expressed as median and interquartile range (IQR) for the quantitative variables.
U-Mann Whitney test for continuous variables to compare two individual groups.
Number of individuals belonging to this category (n); Percentage of total individuals belonging to this category (%).
†: Information obtained at 20 weeks of gestation.

As previously mentioned, the AHEI-2010 index allows us to have a vision of the level of adherence to a healthy diet associated with lower mortality and lower risk of suffering diseases. **Table 10,** shows the adherence levels of the mothers according to various sociodemographic and lifestyle factors, comparing between control and cases groups. Except for gestational diabetes factor, no significant differences were observed. And we could conclude that control mothers who had developed gestational diabetes had a statistically higher level of adherence than mother belonged to cases group.

4.2.3 Women's adherence to a Mediterranean diet during the pregnancy period and their association with a higher risk of EOAPS in early life: Controls vs. cases.

Table 11 presents the associations between the risk of EOAPS in the first three months of life and MD score of their mothers during pregnancy, after adjusting for several confounders.

Table 11. Multivariate logistic regression model adjusted to determine the possible association between adherence to the Mediterranean diet (MD) in pregnancy and the early onset of asthma precursor symptoms in infants at 3 months of age (n= 126) belonging to the NELA cohort.

	Wheeze, atopy, bronchitis and bronchiolitis*		
	OR	(95% CI)	*P*
Diet Index			
aMED (continuous variable)	1.08	0.88 1.33	
Low	0.33	0.09 1.10	
Medium	0.57	0.18 1.71	
High	Ref		
rMED (continuous variable)	1.05	0.91 1.21	
Low	1.12	0.16 8.75	
Medium	1.54	0.27 10.13	
High	Ref		
AHEI-2010 (continuous variable)	1.01	0.96 1.06	
Low	0.85	0.32 2.21	
Medium	0.90	0.34 2.35	
High	Ref		

Ref., Reference; CI: confidence interval.
aMED (Alternative Mediterranean Diet index score). aMED score: low (0-3); medium (4-5); high (6-9); rMED (Relative Mediterranean Diet index score). rMED score: low (0-6); medium (7-10), high (11-18). AHEI-2010 (Alternative Healthy Eating index 2010); AHEI-2010 score: low (42-58); medium (59-63); high (64-77).
* Multivariate logistic regression analyses were adjusted for maternal age, education, parity, used of antibiotics, mode of delivery, used of intra partum antibiotic (IPA), type of lactation and infant's sex. *P*: p-value; *p-value<0.05; **p-value<0.01; ***p-value< 0.001.

No association was observed between the degree of adherence to the 3 types of indices studied and an increased risk of EOAPS in infants.

In general, while previous studies have investigated a single index of adherence to the Mediterranean diet in pregnant mothers and its relationship with the appearance of wheezing, eczema, atopy, and other symptoms in the offspring, it is the first study in which two different scores have been applied to pregnant women and are compared between them, along with a third index which represent a healthy diet pattern. Furthermore, to our knowledge, this is also the first study performed to assess the impact of diet during pregnancy using 3 different indices on wheezing, eczema, atopy, and bronchiolitis or bronchitis in

the offspring at 3 months of age because most of previous published studies explore the appearance of these symptoms after the first year of life or later during childhood.

Only in five longitudinal birth cohorts the association between MD adherence and various symptoms indicative of asthma, mainly wheezing, atopy and eczema have been studied [152,277,278,289,290] and only in one, conducted in Spain, a significant association between high adherence to the MD in pregnant mothers and a lower risk of wheezing in offspring at 6.5 years of age was observed [152]. It should be noted that, Bédard *et al*., [289] in a recent study, suggested that adherence to MD during pregnancy may be associated with small improvement of airway function in childhood. None of these works are comparable regarding the age at which symptoms are defined and established in the offspring, ranging from the first year of age to 7.5 years. Neither that exists a single criterion in the questions to be asked at the questionnaires and their answers, to establish a single definition in each of the mentioned symptoms. Some studies consider a single episode of wheezing, while others consider periodic wheezing as a symptom when trying to stablish a relationship between diet and pulmonary health.

Finally, it should be borne in mind that the MD is based on the high consumption of certain food groups such as vegetables, legumes or whole grains and a moderate consumption of others such as meat. In addition, it is also characterized by a low consumption of saturated fats and a high consumption of fibre, mono and polyunsaturated fatty acids and antioxidants. Perhaps it is the consumption of certain food groups individually or of certain nutrients that explains the different adherence patterns observed between the different studies. Therefore, more studies are needed to investigate the possible association between pregnant women’s consumption of certain food groups, (including those foods considered allergenic) as well as specific nutrients (vitamin/mineral complexes included) and its relation with the appearance of precursor symptoms of asthma in the infant during the first year of life.

4.3. Study III: Characterization of the diet of pregnant women of the NELA cohort: food groups or specific foods and their association with the EOAPS in infants.

4.3.1 Characterization of the median daily intake of the different food groups that make up the diet in pregnant women belonging to the NELA cohort.

Figure 13, shows the median consumption of the different food groups (g/ day) described on the FFQ, by pregnant women in the 20th week of pregnancy selected to carried out the controls *vs.* cases pilot study.

The median consumption of dairy products was 329.50 g /day (IQR: 243.00-547.00), eggs 27.00 g/ day, white meats 75.00 g/ day (IQR: 37.00-75.00), red meat 21.00 g/ day (IQR: 10.00-64.00) and meat derivatives 27.50 g/ day (IQR: 16.00-49.00). It was observed that pregnant women in the NELA cohort consumed a total median of 63.00 g/ day (IQR: 43.00-92.75) of fish, seafood and derivatives. Regarding the consumption of vegetables, fresh fruits, potatoes and pulses, the median was 280.00 g/ day (IQR:197.00-318.20), 288.00 g/ day (IQR: 180.00-416.00), 78.00 g/ day (IQR: 32.00-88.00) and 43.00 g/ day (IQR: 23.00-60.00) respectively. The median daily intake of nuts was 3.00 g/ day (IQR: 1.00-9.00), for cereals and derivatives was 139.00 g /day (IQR:92.00-196.00) and for whole grains and derivatives 9.00 g/ day (IQR: 0.00-47.00). With respect to oils and fats, the median daily intake was 23.00 g/ day (IQR: 11.00-28.00). Finally, about unhealthy food groups such as pastries and sweets, and sauces the median daily intake was 41.00 g/ day (IQR: 23.00-65.00) and 6.00 g /day (IQR: 4.00-11.00) respectively. About drinks, the median daily intake of alcoholic drinks by pregnant was 0.00 g /day of alcohol but the average alcohol consumption was 22.15 g /day. With respect to sugary drinks the median daily intake was 29.00 g/ day (IQR: 0.00-97.00) and for sweetened drinks 0.00 g/ day (IQR: 0.00-32.00). The pregnant women in the cohort consumed a median of 45.00 g /day (IQR: 3.00-75.00) of coffee, tea or herbal teas.

The results observed regarding the daily intake of each of the food groups in our cohort were compared with those described by Jardi *et al*., [300]. The authors also studied the median dietary intake of foods by pregnant women that

constituted the ECLIPSES study, also a Spanish population in the Mediterranean area. The median daily intake of women belonging to our cohort regarding the food groups fruits, vegetables, legumes and nuts were higher than those described in the ECLIPSES study [300]. Regarding meats, despite of the fact that the groups were formed differently, when we jointed together red meat and meat derivatives, a lower consumption of meats and meat derivatives was observed in the pregnant women of the NELA cohort.

There is also another study carried out by Chatzi *et al*., [301] where the median daily intakes of different groups of foods by Spanish pregnant women from two cohorts were determined, one of them in the Mediterranean area, and it has been observed that in general our data are similar to those described by these authors for the Mediterranean cohort.

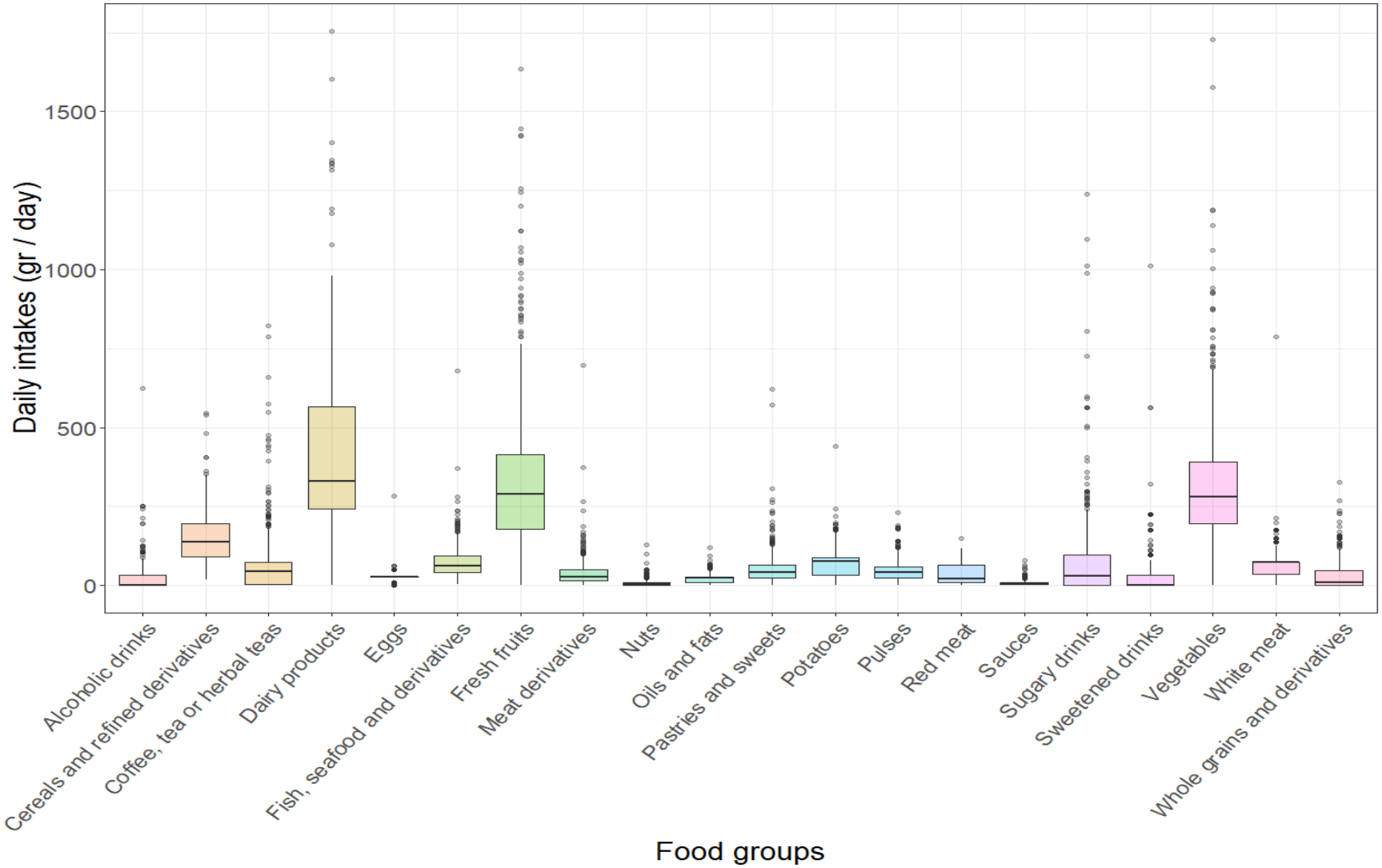


Figure 13. The median daily intake (g/ day) of food groups by pregnant women in the 20th week of pregnancy.

4.3.2 Comparative of the median daily intakes of different food groups that make up the diet among pregnant women: Controls vs. cases

In order to adequately represent the daily intakes of the different food groups comparing cases and controls, on the one hand the 3 groups with the highest median daily intakes had been represented in **figure 14** and on the other, the groups with the lowest daily intakes had been represented in **figure 15**.

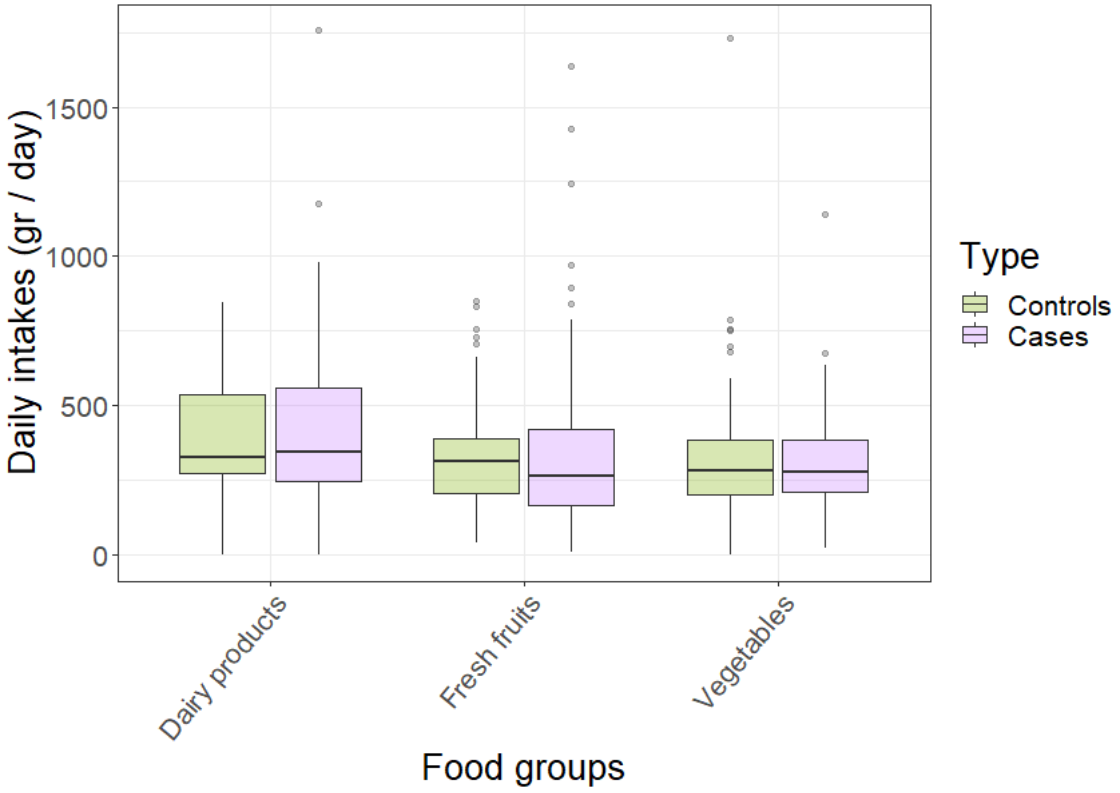


Figure 14. The median daily intake (g/ day) of major food groups by pregnant women comparing controls *vs.* cases.

Regarding the comparison of the median daily intakes between controls and cases for the vegetables, fresh fruits and dairy groups, no statistically significant differences were observed between both groups.

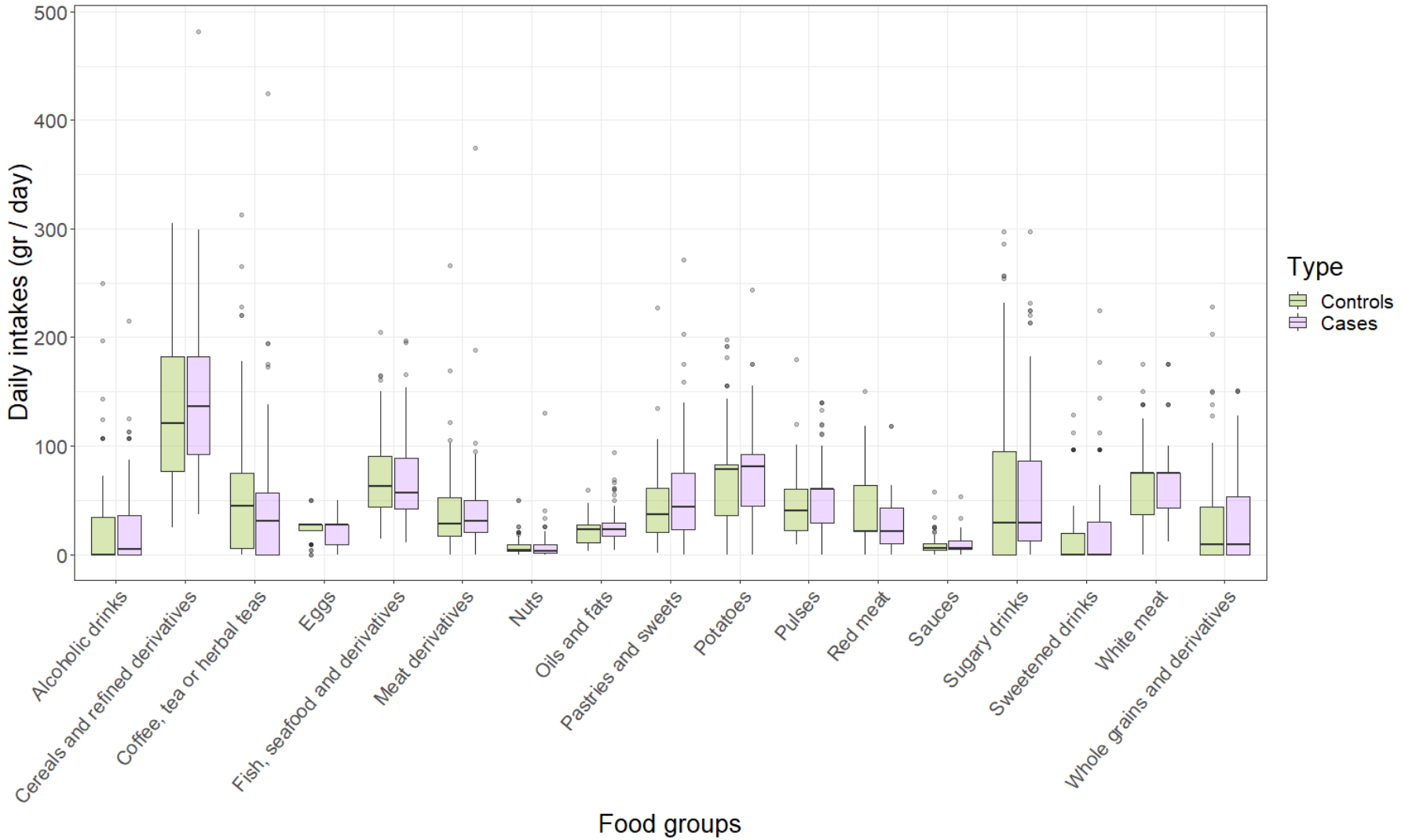


Figure 15. The median daily intake (g/ day) of minority food groups by pregnant women comparing controls *vs.* cases.

Nor statistically significant differences were observed between controls and cases with respect to the food groups with a lower median daily intake, such as nuts, legumes, fish and seafood, meats and meat derivatives, etc., represented in **figure 15**.

4.3.3 Association between median daily intakes of different food groups and the early onset of asthma precursor symptoms.

A multivariate logistic regression analysis was performed to study the possible associations between the different daily intakes of the 20 food groups studied and the EOAPS in the offspring **(Table 12)**.

In the multivariate logistic regression analysis, a statistically significant and positive association was observed between the consumption of pastries and sweets by pregnant women and a higher probability of early onset of wheezing, eczema and / or episodes of bronchiolitis or bronchitis in infants at 3 months of age. These results agree with that recently observed by Zallo *et al*., [302]. The authors observed that the consumption of industrial pastries one or twice a week (OR: 1.59; 95 % CI: 1.13-2.24) and more than three times a week during pregnancy, increases the risk of wheezing at 12 months of age (OR: 1.47; 95 % CI: 1.01-2.13).

On the other hand, an inverse and statistically significant association was observed between the consumption of coffee, tea and infusions by women of NELA cohort, and the appearance of the symptoms mentioned above in the offspring. This fact may explain by the antioxidant activity of coffee, tea and infusions. In this regard, in a recent study carry out by Huang *et al.,* [303] in 2021, the authors assessed the prospective associations between maternal metabolites, including those related to coffee intake, at first and third trimester and the risk of child asthma or recurrent wheeze at 3 age of life. The authors noted that caffeine, theophylline, trigonelline, quinate, and 3-hydroxypyridine sulphate (metabolites related to coffee, chocolate or tea) appeared to be important as they were inversely associated with asthma risk. The authors attributed these protective effects to the anti-inflammatory and immunomodulatory properties of caffeine. On the contrary, other authors who have studied the intake of caffeine during pregnancy and the risk of food allergies

in children, concluded that maternal caffeine intake during pregnancy may be positively associated with the risk of food allergy (studied in Japanese children) [304]. Finally, in a study of the effect of caffeine and their contribution to asthma susceptibility, carried out in a mice model, the authors concluded that prenatal caffeine exposure could be a risk factor for postnatal asthma [305].

Another reason that may justify the inverse association between this intake of infusions or teas and the EOAPS is the polyphenols content of this beverages, which are one of the most bioactive compounds in green tea, have antibacterial, antiviral, antioxidative, anticancer and chemo-preventive activities. In addition, within this food group were also herbal teas, being ginger possibly one of the ingredients that compose them, since our study population is pregnant women, most of whom during the first months of pregnancy have nausea and ginger is widely known for its antiemetic ability. Ginger, and more specifically bioactive compounds such as gingerols, have anti-inflammatory and antioxidant properties, as well as some antimicrobial potential that can help in the treatment of infectious as well as inflammatory diseases [306].

In reference to the rest of the food groups, the European Academy of Allergy and Clinical Immunology, in a recent systematic review published in 2020 about dietary factors during pregnancy and the development of atopy in childhood [307], observed contradictory results related to dietary intake of food groups during pregnancy and its association with allergic outcome and concluded that it was not possible to establish a clear relationship between the consumption of certain food groups and the increase or decrease in the development of allergic outcome during childhood. For example, in reference to the group of vegetables, contradictory results have been found in the literature, since various authors have observed a protective effect in the consumption of vegetables during pregnancy and the appearance of wheeze [308], atopic dermatitis [185], or asthma and allergic rhinitis [186] in childhood, but other authors have observed the opposite effect [194].

With respect to other food groups such as dairy products, Miyake *et al.,* in two different studies observed a reduction in the probability of developing wheezing or atopic dermatitis due to their consumption by the mother during pregnancy [309,310]. Also, Chatzi *et al.,*[278] observed that a high maternal dairy

products intake during pregnancy were associated with lower risk of wheeze in the first year of life. These results did not agree with what was observed in our study, where no type of association was found. The same disagreement occurs with regard to intake of meat. Regarding meat consumption, in a study carried out by Bäiz *et al*., in 2019 [186], the authors reported a significant positive association between the consumption of meat during pregnancy and the risk of wheezing in the childhood and Chatzi *et al*., observed the same results [278]. But on the other hand, Miyake *et al*., [311] in another work carried out in 2009, did not reported any type of association between the consumption of meat and the development of asthma or wheezing in the offspring. Regarding the fruit group, Erkkola *et al.*, [194] concluded that a high consumption of fruits during the pregnancy period was positively associated with an increased risk of allergic rhinitis in children but Alvarez-Zallo *et al.,* [302] found that the high consumption of fruits by the mother during the pregnancy generated a protective effect against wheezing in the offspring at one year of age.

The differences observed between the results obtained in our study and those found in the literature may be due to the fact that in our case the analyses were not carried out based on each of the outcomes separately, but rather together, which can lead to a different result. On the other hand, in our case the study population were 3-month-old infants, whereas in most of the studies mentioned above, the symptoms described have been established during childhood (a period from the first year of life to 7 years of age).

Table 12. The median daily intake of food groups by pregnant women during pregnancy (n=187) and their association with the early onset of asthma precursor symptoms in the offspring at 3 months of age.

	Wheeze, eczema, atopy, bronchitis and bronchiolitis*			
Food groups (g/ day)	OR	95 % CI		*P*
Dairy products	1.00	1.00	1.00	
Eggs	0.98	0.95	1.02	
White meat	1.01	0.99	1.02	
Red meat	1.00	0.98	1.01	
Meat derivatives	0.99	0.98	1.01	
Fish, seafood and derivatives	1.00	0.98	1.01	
Vegetables	1.00	1.00	1.00	
Potatoes	1.00	0.99	1.01	
Pulses	1.01	0.99	1.03	
Fresh fruits	1.00	1.00	1.00	
Nuts	1.02	0.99	1.06	
Cereals and refined derivatives	0.99	0.99	1.00	
Whole grains and derivatives	1.00	0.99	1.01	
Pastries and sweets	1.01	1.00	1.03	**
Oils and fats	1.03	0.99	1.06	
Sauces	1.03	0.97	1.11	
Coffee, tea or herbal teas	0.98	0.97	0.99	*
Alcoholic drinks	1.01	0.99	1.02	
Sugary drinks	1.00	1.00	1.01	
Sweetened drinks	1.01	1.00	1.02	

OR: Odds Ratios; CI: confidence interval.
* Multiple logistic regression analyses were adjusted for maternal age, maternal pre-pregnancy BMI, maternal smoking, parental clinic history, mother's education, parity, type of lactation, infant's sex and newborn birth weight.
P: p-value; *p-value<0.05; **p-value<0.01; ***p-value< 0.001.

4.3.4 Characterization of the median daily intake of the different foods that make up the diet in pregnant women belonging to the NELA cohort.

A characterization, and a subsequent comparison between control and cases, of the median daily intakes of specific foods such as kiwi, strawberry, chocolate, lettuce, was made. The reason of the election of these particular foods was that, according to the bibliography consulted, an allergenic character or, on the contrary, protective against cutaneous or respiratory allergies and / or atopic dermatitis have been stated. On the other hand, a great variability has been observed among the different publications when it comes to forming the different

food groups. This is other reason why we consider interesting to study the effect of these specific foods on the health of the infant, as well as to characterize its daily intake in pregnant women of the NELA cohort.

Firstly, a characterization of the median daily intakes of the consumption of specific foods such as, hamburgers or processed meat, white fish and oily fish, seafood, chard or spinach, cruciferous (broccoli, cauliflower and brussels), leafy vegetables (lettuce, endive and escarole) citrus juices, kiwi, apples and pears, strawberries, olive oil or chocolate and bonbons, was carried out to the entire cohort (**figure 16**).

The median consumption of hamburgers was 6.00 g (6.00-13.00) (g/ day (IQR)) and the consumption of processed meat was 21.00 g (7.00-36.00). Foods such as lettuce, endive and escarole (all together) were the foods with the highest median daily consumption of 26.00 g (9.00-47.00), followed by olive oil with a median of 23.00 g (9.00-23.00). The food with the lowest median daily intake, of all the foods studied, was chocolate and chocolates (3.00 g (1.00-6.00)). Regarding fish and seafood, a higher consumption of white fish, was observed compared to the consumption of oily fish, being the medians for both foods 21.00 g (10.00-21.00) and 10.00 g (0.00-21.00), respectively. In relation to seafood, a median daily intake of 11.00 g (5.00-16.00) was observed. With respect to foods from the vegetables group, other than those mentioned above, fairly low daily intakes of chard and spinach, and of cruciferous vegetables were observed. In both cases, a median daily intake of 8.00 g (0.00-18.00) was observed. Within the fruit group, citrus fruits were the most consumed (21.00 g (0.00-62.00)), followed by apples and pear (19.00 g (9.00-57.00) and strawberries (9.00 g (0.00-58.00)). Kiwi was the less consumed fruit, with a median daily intake of 6.00 g (0.00-13.00).

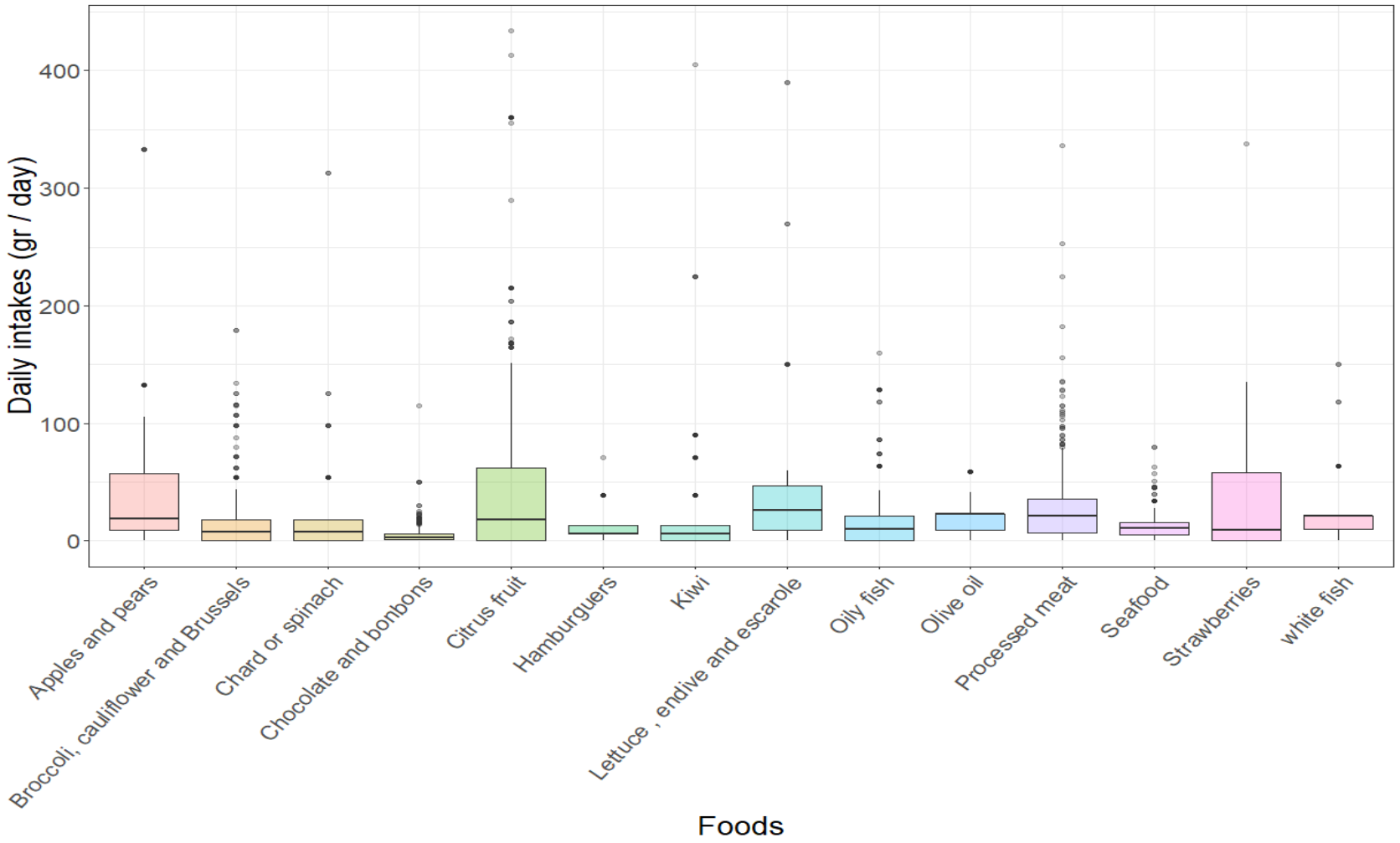


Figure 16. The median daily intake (g/ day) of specific foods by pregnant women belonging to NELA cohort in a controls vs cases pilot study.

4.3.5 Comparative of the median daily intakes of different specific foods that make up the diet among pregnant women: Controls vs. cases

Once the median consumption of these specific foods was characterized for the entire cohort, a comparison of the median daily intakes was made, between control group and the cases group. Foods were represented in **figure 17**.

No significant differences were observed between the median daily intakes of the controls group and the cases group. A significantly lower consumption of strawberries was observed by the control group (9 g (0.00-19.00)) compared to the cases group (19.00 g (4.50-58.00)), although these differences were not statistically significant (p-value =0.065).

Despite not observing significant differences between the two groups for any of the specific foods, it is necessary to perform a multivariate statistical analysis, taking into account other confounding variables such as type of delivery, age of the mother, sex of the newborn, etc. in order to observe if there is any type of association between the consumption of these foods and the appearance of asthma precursor symptoms in the offspring.

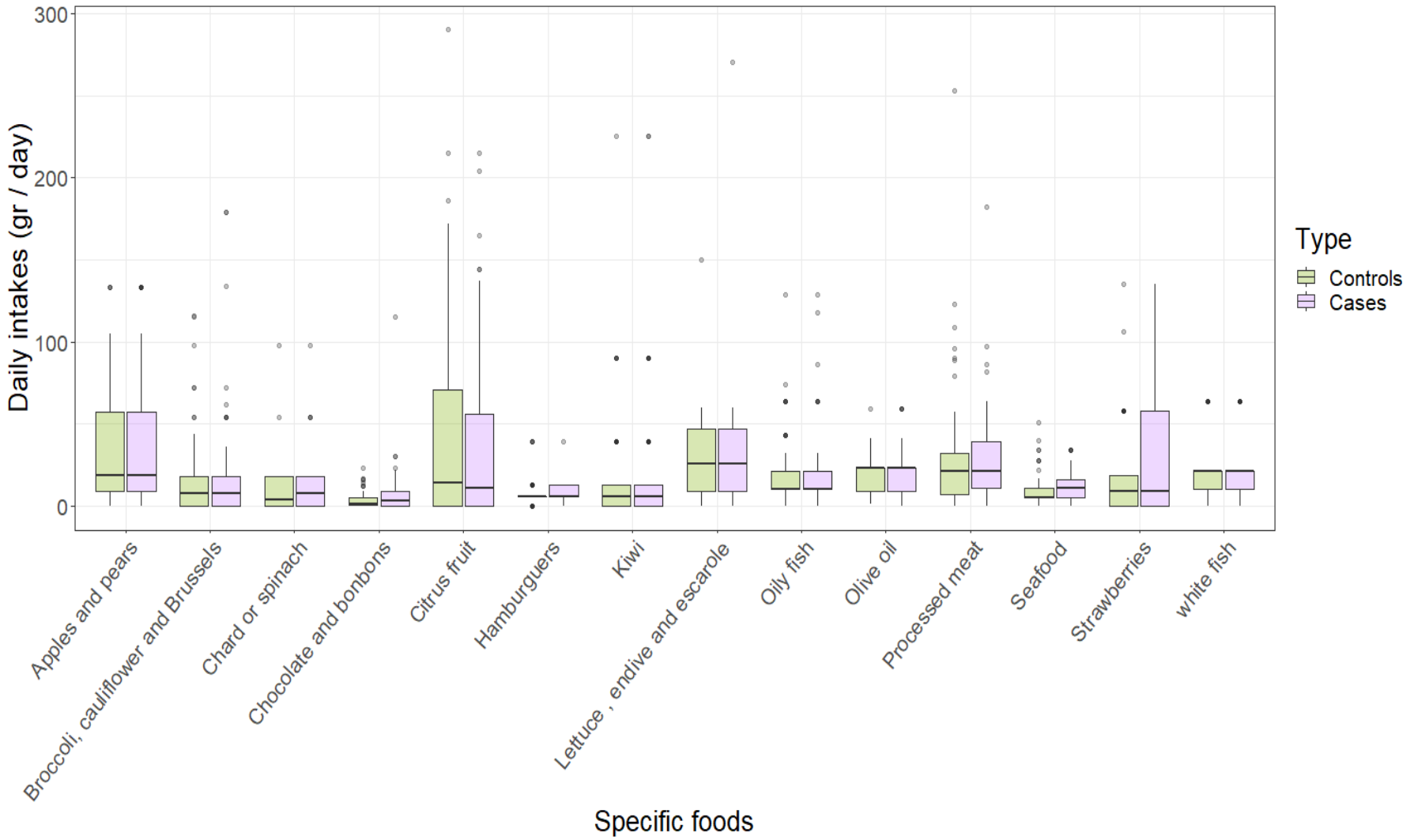


Figure 17. The median daily intake (g/ day) of specific foods by pregnant women comparing controls *vs.* cases.

4.3.6 Association between median daily intakes of different foods and the early onset of asthma precursor symptoms.

A multivariate logistic adjusted regression analysis was performed to study the possible associations between the different daily intake of the 15 specific foods studied and the EOAPS in the offspring **(Table 13)**.

Table 13. The median daily intake of specific foods (g/ day) by pregnant women (n=187) and their association with the early onset of asthma precursor symptoms in the offspring at 3 months of age.

	Wheeze, atopy, bronchitis and bronchiolitis*		
Foods	OR	95 % CI	*P*
Hamburgers	1.00	0.94 1.05	
Processed meat	1.00	0.99 1.01	
White fish	0.99	0.97 1.01	
Oily fish	1.00	0.98 1.02	
Seafood	1.03	0.99 1.07	
Chard or spinach	1.00	0.98 1.03	
Broccoli, cauliflower and Brussels	1.00	0.99 1.02	
Lettuce, endive and escarole	1.00	0.98 1.01	
Citrus fruit	1.00	0.99 1.01	
Apples and pears	1.00	0.99 1.01	
Strawberries	1.01	0.99 1.02	
Kiwi	1.01	1.00 1.02	
Olive oil	1.02	0.99 1.06	
Chocolate and bonbons	1.07	0.99 1.14	
Water	1.00	0.99 1.00	

OR: Odds Ratios; CI: confidence interval.
* Multiple logistic regression analyses were adjusted for maternal age, maternal pre-pregnancy BMI, maternal smoking, parental clinic history, mother's education, parity, type of lactation, infant's sex and newborn birth weight. *P*: p-value; *p-value<0.05; **p-value<0.01; ***p-value< 0.001.

No type of association was observed between the median daily intake of the different foods studied by the pregnant woman and the appearance of wheezing, atopic dermatitis, bronchitis and / or bronchiolitis in the offspring at 3 months of age. The results obtained in our pilot study do not agree with some studies that have observed an association, either positive or inverse, between the consumption during pregnancy of certain foods such as nuts, fast food, leafy vegetables, citrus fruits or olive oil and the development of wheezing, allergic

rhinitis or atopic dermatitis during childhood. Specifically, Castro-Rodriguez *et al.,* [277] reported that a high fast food consumption by pregnant women was associated with a higher prevalence of dermatitis in children and von Ehrestein *et al.,* [312] also found that a maternal fast food consumption during pregnancy was related to a higher risk of severe and current asthma symptoms in their children in a dose-dependent manner. With respect to olive oil, Castro-Rodriguez *et al.,* [313], observed an association between the maternal olive oil consumption during pregnancy and less wheezing during the first year of life in the offspring.

In other studies, such as the one developed by Erkkola *et al.,* [49] a low maternal consumption of leafy vegetables, malaceous fruits such as peach, and chocolate were positively associated with the risk of wheeze in children. In addition, Bäiz *et al*., [48] also observed a significant inverse association between the consumption of cooked green vegetables and raw vegetables and the risk of allergic rhinitis in young children.

A high consumption of fish during pregnancy and more specifically oily fish rich in omega-3 have been widely studied and related to better health in the offspring. In the case of oily fish consumption and its association with the prevalence of asthma in offspring, Fitzsimon *et al.,* [55] observed that a higher maternal oily fish consumption was associated with lower risk of children developing asthma at 3 years, and Salam *et al.,* [56] in their research about maternal fish consumption during pregnancy and risk of early childhood asthma, also concluded that maternal oily fish intake during pregnancy may protect offspring from asthma. These results do not coincide with what was observed in our work where we did not obtain any type of association with the development of wheezing, atopy, bronchitis and / or bronchiolitis in the offspring at 3 months of age.

Our results do not either coincide with what is reported in the bibliography regarding the consumption of apples and pears, since Willers *et al*., [314] in 2007 observed that the consumption of apples during pregnancy had a protective effect against wheezing and asthma in children.

5. Conclusions

5.1 Study I: Characterization of the level of adherence to MD in pregnant women belonging to the NELA cohort and the lifestyle and sociodemographic factors associated with a low MDA.

The level of adherence to the Mediterranean diet of the pregnant women belonging to the NELA cohort corresponded to a Medium level of adherence for both scores, aMED and rMED. Also, these women presented a Medium level of adherence to a healthy dietary pattern (AHEI-2010 index). In addition, pregnant women with younger age, previous deliveries, low educational level, and who practice unhealthy lifestyles, such as lack of physical activity, are associated with a higher risk of low adherence to these three indices. This fact should be taking in mind to design successful educational interventions in the future because a healthy diet has a protective effect on the health of the mother and also of the offspring. The pre-pregnancy and pregnancy periods are ideal time window to introduce some stimulus that helps to modify dietary and lifestyle habits in a positive way, since there is also a very large motivational factor.

5.2 Study II: The possible association between adherence to MD of gestational women of the NELA cohort and the risk of wheezing, atopy and eczema in their offspring at 3 months of age.

In this study the principal conclusion is that no statistically significant differences were observed, with respect to the level of adherence to the three dietary patterns studied, between healthy infant's mothers and mothers whose offspring had early symptoms of asthma. In addition, it is not observed an association between the degree of adherence to the Mediterranean diet of mothers during pregnancy and the appearance of asthma precursor symptoms in the offspring at 3 months of age.

5.3 Study III: The possible association between the consumption of food groups or specific foods by pregnant women of the NELA cohort and the risk of wheezing, atopy and eczema in their offspring at 3 months of age.

As a first conclusion regarding food groups, a higher consumption of pastries and sweets by the mother during pregnancy is associated with a greater probability of early onset of asthma precursor symptoms in the offspring at 3 months of age. Besides, a higher consumption of coffee, tea and / or herbal tea by the mother during pregnancy is associated with a lower probability of appearance of these symptoms.

In relation to the consumption of specific foods, which according to scientific evidence have an allergenic or protective character against allergies, no association was observed between the median daily intake of the foods studied during pregnancy and the early onset of asthma precursor symptoms in infants at 3 months of age.

Finally, we believe that it is necessary to establish a single criterion to create the different food groups and also a consensus in relation to the confounding variables used to adjust the regression models. Both actions would reduce the heterogenicity of future studies and thus facilitate the comparison between them.

CHAPTER 2. Study of the gut microbiota and their metabolites in infants of 3 months of age from NELA Cohort. Factors that influence colonization and their association with the early onset of asthma precursor symptoms

1. Introduction

The human body harbours trillions of microorganisms whose coordinated actions are believed to be important for human life. Such microbial cell populations reach their highest density in the gut compartment, where they collectively form a complex microbial community known as the gut microbiota, which develops over the first three years of life until it reaches its adult form [7]. The microbiota is composed of a significant number of different bacteria, approximately 160 species per person per faecal sample, and this ecosystem plays an important role in human health [17]. Gut microbiota members may belong to any of the three domains of life, i.e., Archea, Bacteria, and Eucarya, and also include viruses. The gut microbiota is dominated by two phyla, Bacteroidetes and Firmicutes, followed by Actinobacteria, Proteobacteria, Verrucomicrobia, Fusobacteria, and Cyanobacteria in lower proportions [49]. Bacteroidetes, which account for 90% of the gut microbiota, include bacteria belonging to the genus *Bacteroides* and *Prevotella*. Firmicutes include a large number of genera, the most important being *Lactobacillus* and *Clostridium,* and Proteobacteria include the genera *Enterobacteriaceae.* The main genus belonging to the Actinobacteria phylum in the human intestine is *Bifidobacterium* [315]. These microorganism are known to establish complex trophic relationships with each other and their human host, ranging from symbiosis to parasitism [7].

The abundant and diverse members of the human gut microbiota exert critical roles in the maintenance of human health by assisting in the breakdown of food substances so as to liberate nutrients that would otherwise be inaccessible to the host, e.g., enzymatic digestion of complex carbohydrates. Also, promoting host cell differentiation and protecting the host from colonisation of pathogens such as *Escherichia coli* and *Salmonella spp.* through the production of short-chain fatty acids (SCFAs) which acidifies the gastrointestinal tract, or also through competitive colonisation of commensal bacteria [7,140]. The interactions with human host play an important role in the functionality of immune response, as well as in the regulation of gastrointestinal motility, gut barrier homoeostasis and development, nutrient metabolism or fat distribution [49]. SCFAs are the main metabolic end products from bacterial fermentation of HMOs during breastfeeding and dietary fibre or resistant starches later, which rage in

concentration from 50 to 100 mM in the gut, and are key energy sour for many host tissues and gut bacterial species [158,316]. SCFAs are the major metabolite produced by the gut bacteria from MACs and are involved in immune modulation, inflammation, epigenetic changes and energy production being a relevant source of energy for the human host, supplying approximately 10% of total energy requirement [317]. The three most common SCFAs produced in the gut are acetic, propionic, and butyric acid, which are present in a molar ratio of 60:20:20, respectively [316]. SCFAs are also precursors for gluconeogenesis, liponeogenesis and protein and cholesterol synthesis. The types of carbohydrates consumed determine the quantities and types of SCFAs produced, as well as the prevalence of gut microbial species that produce them [39].

Until now, it was believed that during the first year of life, the infant gastrointestinal microbiota should evolve from being almost sterile, known as the “sterile uterus paradigm”, to a complex and varied community, with its composition and structure subjected to great variability. However, recent studies suggest that infants host an initial microbiome from the mother through the amniotic fluid, umbilical cord blood and foetal membranes and receive a maternal microbe supplementation through birth and breastfeeding. In general, during childhood, various behaviours of the infant such as skin-to-skin contact with the mother, the introduction of objects, as well as parts of the body in the mouth like hands and foots, especially during the stage of crawling, are actions that promote the exposure to microbes. In addition to these actions, there are some windows of opportunity for microbiota modulation, from pregnancy to childhood, where the microbiota is more affected to environmental factors such as lifestyle, mode of delivery, the use of antibiotics or the type of feeding among others. All these factors can alter the microbiota and therefore promote a disruption in a way that may favour the development of disease later in life [39].

There is a wide inter- and intra-individual diversity for the gut microbiota; however, there is some consensus among the scientific community that a healthy gut microbiota in adult is mainly composed of bacteria belonging to the phylum Firmicutes and Bacteroidetes [318]. A large number of articles associate the appearance of various diseases with the imbalances of the gut microbiota in the

early life, this imbalance is also called gut microbial dysbiosis. One of the main causes of a poor exposure to microbes or a pattern of dysbiosis is the lack of proper development of the immune system and therefore the appearance of different hypersensitivity diseases such as atopy and asthma. If we focus on asthma, the prevalence of this disease has suffered a continuous increase in industrialized countries and developing countries because of higher sanitation standards, increased use of antibiotics and other factors that are been studied. Asthma is a disease with many clinical phenotypes and whose clinical symptoms are very variable and not specific during the first years of life but there are several cohorts and other longitudinal studies in which a different pattern of colonization has been observed between healthy infants and those who develop asthma [33,168,169].

Considering the importance of the pioneer infant microbiota for human development and health, it is essential to elucidate the exact mechanisms by which this community is acquired, endogenous and exogenous factors that influence these events and which of these prenatal, perinatal and postnatal factors may pose a greater risk of early development of precursor symptoms of this disease.

2. Objectives

2.1 General Objectives

To study the prenatal, perinatal and postnatal factors that can influence the infant's microbiota and its metabolites, as well as their association with the EOAPS at 3 months of age.

2.2 Specific Objectives

- Characterisation of infant microbiota and short-chain fatty acid profile according to different factors, and their possible relationship with EOAPS at 3 months of age.
- To study whether there is any type of association between the gut microbiota of infants and their SCFAs profile and the EOAPS at 3 months of age, taking into account other confounding variables such as the type of childbirth, gestational age, type of lactation, etc.

3. Material and methods

3.1 Study design

The objectives of this chapter were achieved through a single study. The experimental design is presented in **figure 18**.

The gut microbiota and metabolites (SCFAs) of 100 healthy infants and 100 infants classified as cases at 3 months of age were characterized by qPCR and FID-coupled gas chromatography. Subsequently, an analysis was carried out of how prenatal factors (use of antibiotics during pregnancy, maternal diet during pregnancy, consumption of probiotics, gestational diabetes), perinatal factors (intrapartum antibiotic, type of delivery and gestational age) and postnatal factors (type of lactation) affect the intestinal microbiota of infants and their metabolites through a bivariate analysis with the data collected in different questionnaires provided to mothers at different times (20 weeks of gestation, delivery and 3 months after delivery).

Finally, a multivariate analysis of the data was carried out to study the possible associations between the infant's gut microbiota and their faecal SCFAs profile with the EOAPS at 3 months of age.

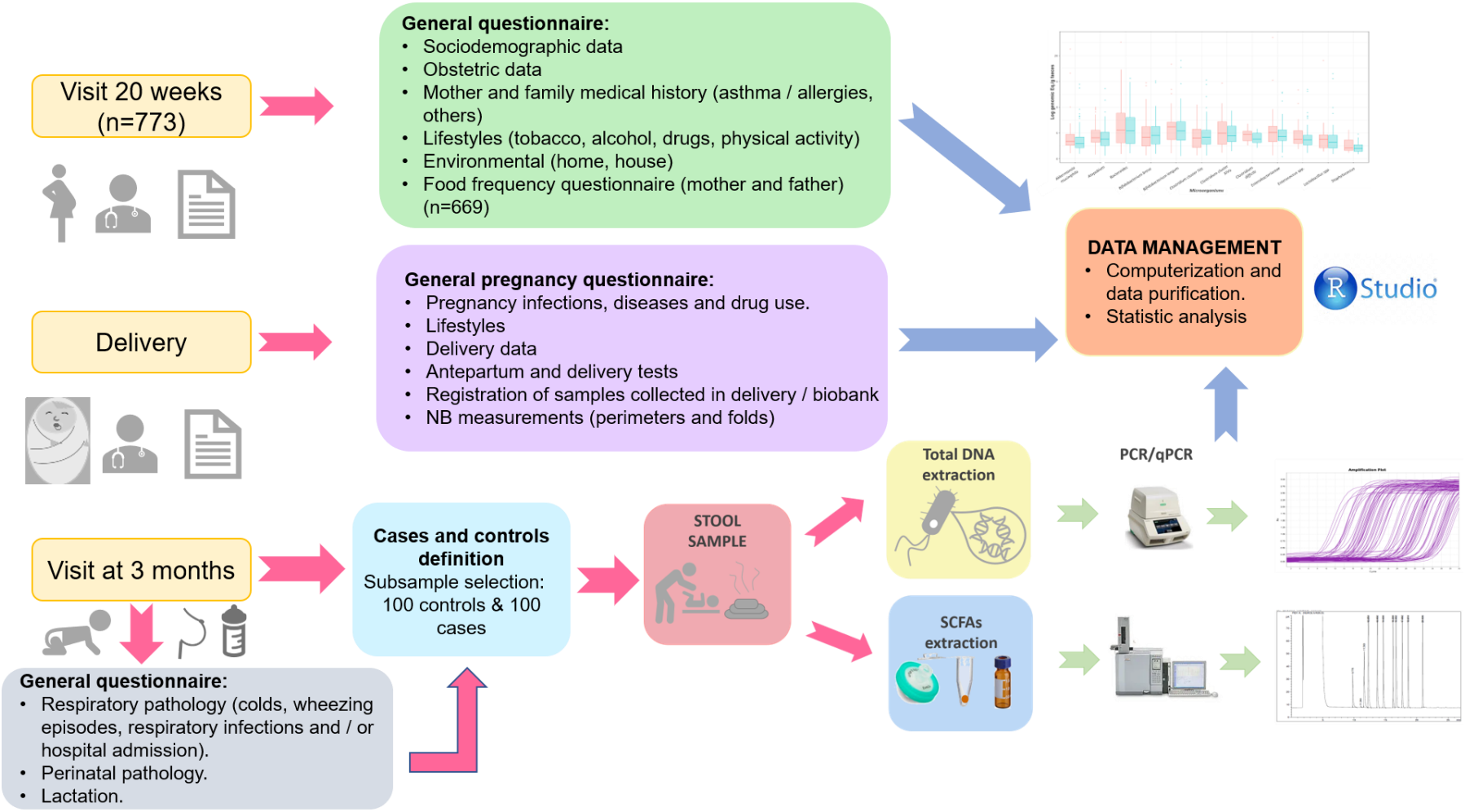


Figure 18. Experimental design from chapter 2**.**

3.2 Sample collection

Two stool samples were collected from infants belonging to NELA cohort, at the third month of life (n =216). For the collection, parents received two sterilized tubes with a sterile depressor and an instruction form. Samples were collected directly from the diaper, and then were placed at -20 ºC. Parents delivered the faecal samples at the three-months-visit at the Virgen de la Arrixaca Hospital. The same day stool samples were aliquoted and stored in the Biobank Platform of the Research Institute Biosanitary Virgen de la Arrixaca Clinical Hospital (IMIB-Arrixaca, Murcia) at -40 ºC until later use.

3.3 Quantification of SCFAs by gas chromatography

3.3.1 SCFAs extraction and processing.

For the SCFAs extraction, samples were thawed at room temperature and 0.1 g were transferred into a fresh 2 mL tube containing 5 sterile zirconia beads (1.5 mm). 0.5 mL of milliQ water were added and homogenised by vortexing during 5 minutes. After that, the suspension was centrifuged for 30 min at 4500 x g at 5 ºC, giving a clear supernatant. 100 µL of the cell free supernatants were transferred into a 1.5 mL tube, and 650 µL of a mixture of extra pure formic acid (Scharlau, Spain) (20%), methanol of HPLC gradient grade (J.T. Baker, The Netherlands) and 2-ethyl butyric acid (Merck, Germany) (internal standard, 2 mg/mL in methanol) at a ratio of 1:4.5:1 was added. The mixtures were homogenized by vortexing and filtered through a 13 mm (diameter), 0.22 µm (pore size) PTFE filter (VWR International, USA). Then, 1 µL of samples were analysed using a gas chromatography (Agilent 7890A) equipped with a flame ionization detector and a Nukol™ GCcolumn (30 m × 0.25 × 0.25 um) following the method proposed by Zhao et al., in 2006 [319]. The SCFAs were identified on chromatograms by their specific retention times (**table 14**) under the conditions described in **table 15**.

Table 14. Retention times of the 9 short chain fatty acids analysed.

SCFAs	Retention time (min)
Acetic	9,763
Propionic	11,602
iso-Butyric	12,259
n-Butyric	13,699
iso-Valeric	14,644
n-Valeric	16,626
iso-Caproic	17,706
n-Caproic	18,646
Heptanoic	20.97
2-Ethylbutyric	16.69

The calibration curves were obtained using a volatile acid standard mix (Supelco, Bellefonte, PA, USA) of acetic, propionic, butyric, iso-butyric, valeric, iso-valeric, caproic, iso-caproic and heptanoic acid, and preparing a concentration of 10, 5, 2, 1, 0.5 and 0.25 mM. The calibration curve for acetic acid (acetic acid glacial. AppliChem Panreac. Spain) was performed at concentrations of 200, 100, 50, 10, 5 and 2 mM.

Table 15. Chromatographic conditions.

Equipment Parameters	Conditions
Elution Method	Constant pressure injection
Gas flux	He^2 25 mL/min (carrier)
	Air 400 mL/min
	H^2 30 mL/ min
Injection method	Splitless
Volume of injection	2µl
Oven temperature	80 °C
Temperature ramp	80°C for 5 min
	5°C/ min until 185 °C
Injector temperature	220 °C
Detector temperature	220 °C
Injector Pressure	58,99 kPa

3.3.2 Quantification of SCFAs concentration. Standard curves.

Curves for acetic, propionic and butyric acids can be observed in **figure 19**. The area of each peak of SCFAs was integrated using Agilent Chemstation Operation software (Santa Clara, USA), and concentrations were calculated by comparing their peak areas with those of the standard and were expressed in mM. Every sample was run in duplicate. The concentrations of the different SCFAs were expressed as mmol/g or in proportion, grouped minority SCFAs into a single group.

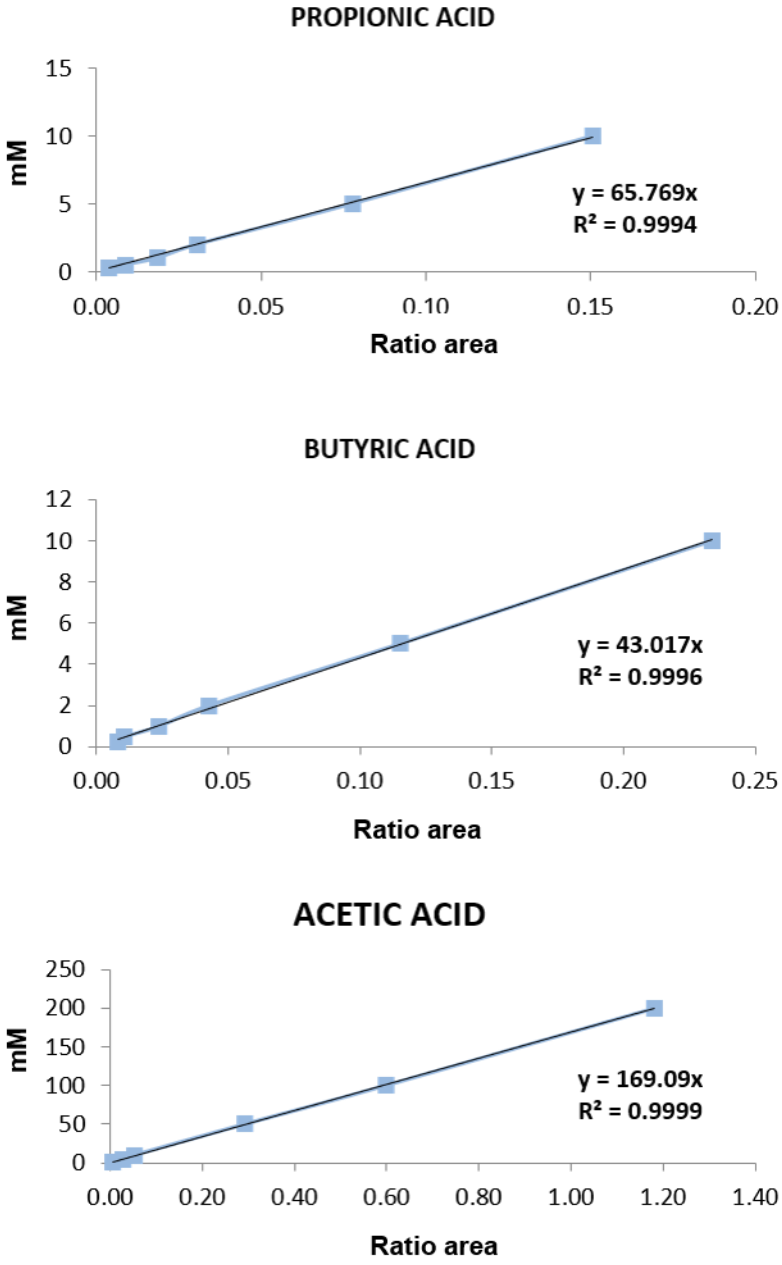


Figure 19. Acetic, propionic and butyric acid calibration curves

3.4 Faecal Microbial community analysis by qPCR.

3.4.1 DNA extraction

The entire procedure of manipulation and extraction of DNA from faecal samples was carried out in the biobank platform (IMIB.Arrixaca) by qualified personnel. All faecal samples were processed after defrosting. For DNA isolation, 1 mL of CTAB buffer from the Maxwell® RSC PureFood GMO and Authentication

Kit (Promega, Wisconsin, USA) was added to a 2 mL vial containing 0.25 g of faeces and then were homogenised during 30 seconds in a vortex. Samples were incubated at 95 ºC for 5 minutes to further lyse cells, allowing the samples to cool for 2 minutes at room temperature before another minute of homogenisation. In some cases, it was necessary to use the TyssueLyser (Qiagen, Hilden, Germany) for 1 min at 50 Hz to finish sample homogenisation. The samples were treated with 40 µL of Proteinase K and 20 µL of RNAse, vortexed and incubated at 70 ºC for 10 minutes. Samples were centrifuged during 5 minutes (16.000 g), 300µL of supernatant was transferred to a cartridge in position 1 to carry out the automatic extraction process using automated DNA Purification on the Maxwell® 16 Instrument (Promega, Wisconsin, USA). The DNA obtained at the end of this process was stored at -20 ºC until microbiota analyses.

3.3.2 Quantitative PCR (qPCR) primer design and qPCR conditions.

For faecal microbiota characterization the following groups were included: *Atopobium* cluster (including *Atopobium* and *Collinsella*), *Bacteroides (Bacteriodes-Prevotella-Porphiromonas*), *Enterobacteriaceae*, *Enterococaceae*, *Lactobacillus*, *Clostridia cluster IVa* (*Clostridium leptum - Faecalibacterium praustnitzii*), *Clostridia cluster XIVa* (*Clostridium coccoides – Eubacterium rectale*) and *Sthaphylococcus*. Also, some certain species of great interest for the study was included such as *Akkermansia muciniphila*, *Bifidobacterium longum*, *Bifidobacterium breve* and *Clostridium difficile*. All primers, specific for the 16s ribosomal gene, were used and described in **table 16** (all of them were purchased from Sigma-Aldrich (Barcelona, Spain)).

Table 16. Specific primers used in the study to target different bacterial groups.

Bacterial group	Primer sequence (5'-3')	Amplicon size NCBI	Annealing Temp.	Ref.
Atopobium cluster	GGGTTGAGAGACCGACC CGGRGCTTCTTCTGCAGG	190pb	55 ºC	[320]
Bacteroides-Prevotella	GAGAGGAAGGTCCCCCAC CGCKACTTGGCTGGTTCAG	108pb	60 ºC	[321]
Clostridia XIVa	CGGTACCTGACTAAGAAGC AGTTTYATTCTTGCGAACG	429 pb	55 ºC	[322]
Clostridia IVa	TTAACACAATAAGTWATCCACCTGG ACCTTCCTCCGTTTTGTCAAC	314 pb	60 ºC	[321]
Enterococaceae	CCCATCAGAAGGGGATAACACTT ACCGCGGGTCCATCCATC	115pb	60 ºC	[323]
Lactobacillus	AGCAGTAGGGAATCTTCCA CATGGAGTTCCACTGTCCTC	331pb	60 ºC	[322]
Enterobacteriaceae	TGCCGTAACTTCGGGAGAAGGCA TCAAGGACCAGTGTTCAGTGTC	428pb	60 ºC	[323]
Akkermansia muciniphila	CAGCACGTGAAGGTGGGGAC CCTTGCGGTTGGCTTCAGAT	327pb	50 ºC	[324]
Staphylococcus	GCGATTGATGGTGATACGGTT AGCCAAGCCTTGACGAACTAAAGC	267pb	55 ºC	[325]
Bifidobacterium breve	AATGCCGGATGCTCCATCACAC GCCTTGCTCCCTAACAAAAGAGG	235pb	62 ºC	[326]
Bifidobacterium longum	TTCCAGTTGATCGCATGGTCTTCT GGCTACCCGTCGAAGCCACG	110pb	65 ºC	[326]
Clostridium difficile	TTGAGCGATTTACTTCGGTAAAGA TGTACTGGCTCACCTTTGATATTYA	114pb	62 ºC	[327]

The qPCR protocol was performed in a 96-well Applied Biosystems QuantStudio™ 5 Real-Time PCR thermocycler and detection system (Applied Biosystems, California, USA). The temperature cycling protocol has been detailed in **figure 20** and consisted in a DNA initial denaturation and enzyme activation step at 95 ºC for 10 min, followed by 40 cycles of denaturation at 95 ºC for 15 seconds, annealing at 55 ºC/60 ºC depending on the target group (see **table 16**) for 30 seconds and extension at 72 ºC for 45 seconds. After the completion of 40 cycles, a melting-curve analysis was performed to distinguish targeted from nontargeted PCR products and was from 65 ºC to 95 ºC, with an increase of 0.5 ºC every 0.5 seconds. The fluorescent products were detected in the last step of each cycle. Reactions were conducted in a total volume of 25 µL. For detection of *Lactobacillus*, *Bifidobacterium breve* and *Akkermansia muciniphila* amplifications were conducted containing 3 µL of purified target DNA, 12.5 µL SensiMix™ SYBR® Low-ROX Kit (1x) (Bioline, London, UK), 1 µL of both primers (0.2 µM) and 7.5 µL of nuclease-free water (Sigma-Aldrich, Misuri, USA). For the rest of the species and groups, amplifications were conducted in a total volume of 25 µL, containing 1 µL of purified target DNA, 12.5 µL SensiMix™ SYBR® Low-ROX Kit (1x) (Bioline, London, UK), 0.5 µL of both primers (0.2 µM) and 10.5 µL of nuclease-free water (Sigma-Aldrich, Misuri, USA). All samples were run in triplicate and normalized according to the cycle threshold (Δ CT) method using total 16s rDNA bacteria as the reference gene.

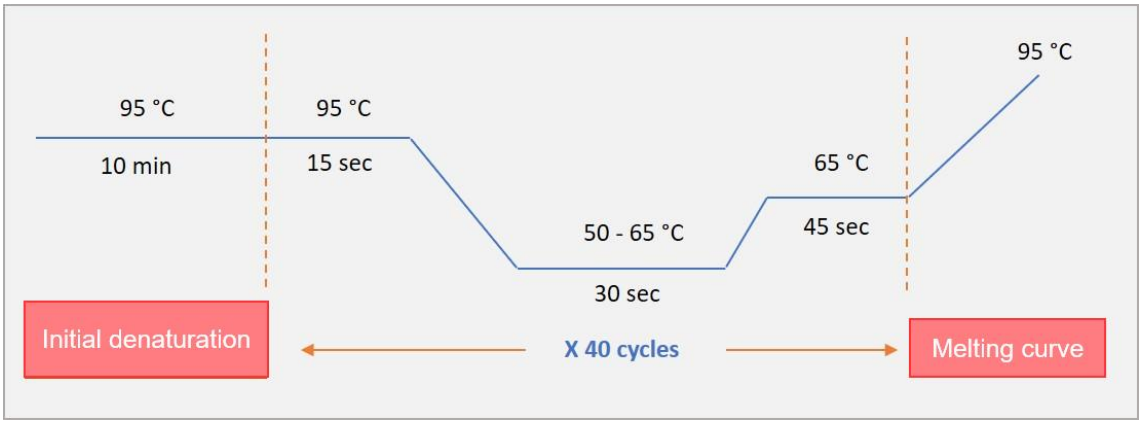


Figure 20. qPCR. Temperature cycling protoco

3.3.3 Quantification of DNA concentration. Standard curves.

Collection bacterial strains, whose reference numbers are provided in **table 17**, were obtained from the German Collection of microorganism and cell cultures (DSMZ) or from the Spanish Type Culture Collection (CECT). All strains were reconstituted and cultured following the instructions provided by the supplier. From each pure culture, DNA was extracted using the QIAamp DNA Stool Minikit (Qiagen, Germany). After extraction, DNA was amplified according to the previously described PCR protocol (**see figure 20**). Following amplification, DNA was cleaned up using the QIAquick PCR Purification Kit (Qiagen, Germany), to remove any remaining enzyme and nucleotides. Then, DNA concentration and integrity were analysed immediately. Concentration was measured using a spectrophotometer (NanoDrop-1000, Thermo Scientific, Villebonsur Yvette, France) at 260/280 nm ratio. Samples were maintained at -80 ºC until use.

Bacterial DNA concentration per sample, expressed as Logarithm of Equivalent genomic/ g faeces, was calculated using linear regression ($R \geq 0.99$) from a seven points (CT-values) standard curve. Standard curves were established using serial 10-fold dilution (10^8 - 10^2 Logarithm of Equivalents genomic /mL) of DNA extracted and purified from pure bacterial cultures. The theoretical genome equivalents were calculated assuming that the size of the amplicon is similar to the size of the 16S ribosomal RNA gene copy for each microbial group and that the average weight of a single DNA base pair (bp) is 650 g/mol [328,329].

$$\text{Genome} - \text{equivalentes} = \frac{\text{Amplicon} - \text{size(bp)} \times 1\text{mol}}{(6.023 \times 10^{23}\text{molecules}) \times (650\text{g/mol})}$$

For each target gene, amplicon length/size (pb) was obtained combining the results extracted from the bibliography and the data provided by the Primer-Blast tool from the National Centre for Biotechnology Information (NCBI). (Available: http://www.ncbi.nlm.nih.gov).

Table 17. Reference bacterial strains used for DNA quantification.

Strain	Reference number (CECT/DSMZ)
Bifidobacterium longum	CECT 4503
Collinsella gutis	DSMZ 13280
Bacteroides thetaiotaomicron	DMSZ 2079
Clostridium coccoides	DSMZ 7935
Clostridium leptum	DSMZ753
Enterococcus faecalis	DSMZ 2478
Lactobacillus gasseri	DSMZ 20077
Escherichia coli	CECT 434
Akkermansia muciniphila	DSMZ 22959
Staphylococcus aureus	CECT 435
Bifidobacterium breve	DSMZ 4839
Clostridium difficile	CECT 531

CECT: Spanish Type Culture Collection; DSMZ: German Collection of microorganism and cell cultures.

For each bacterial group included in the study, standard curves have been presented in **figure 21**.

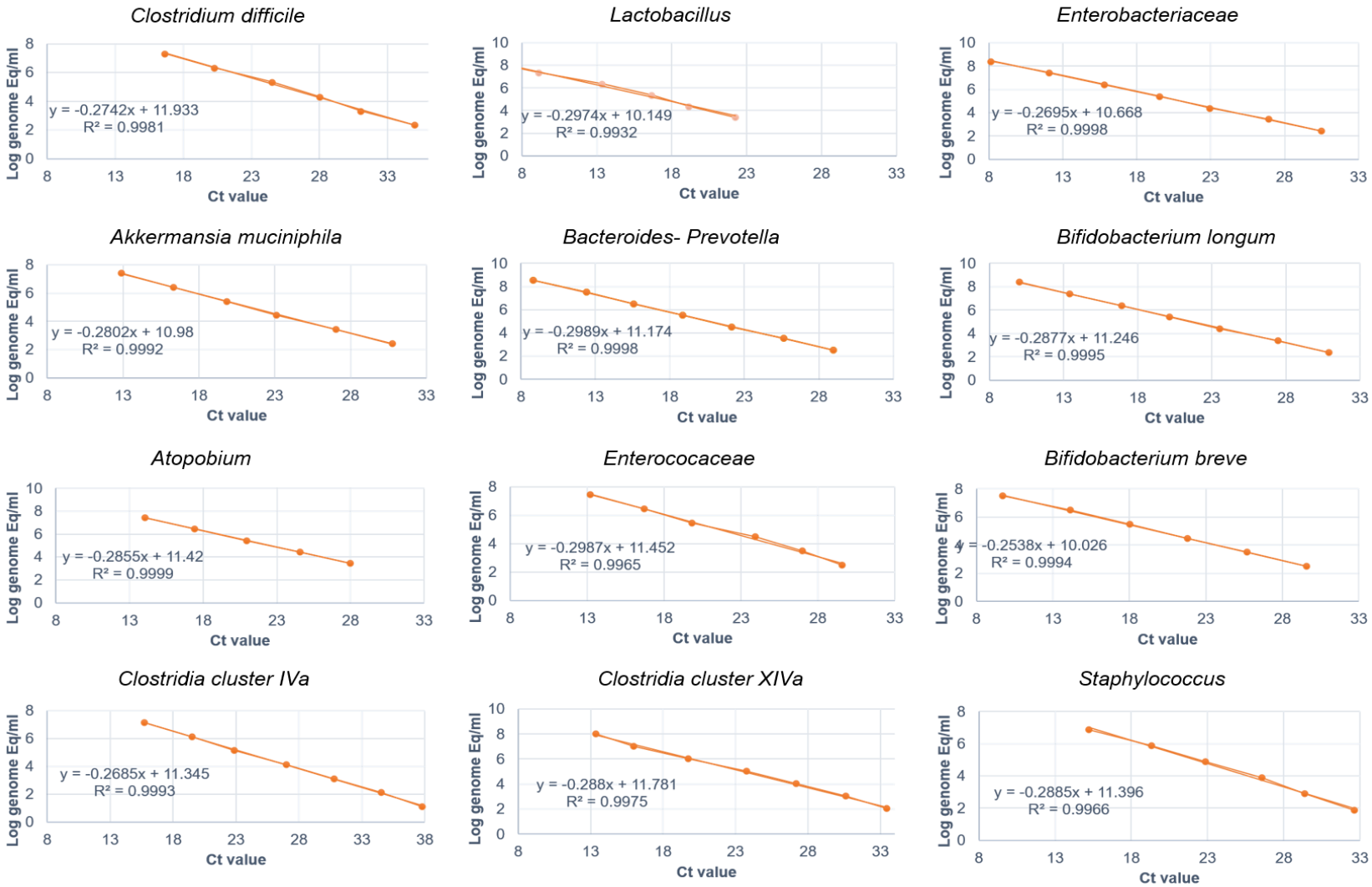


Figure 21. qPCR. Standard curves for bacterial DNA concentration quantification. Ct value: Cycle threshold value

3.4 Potential determinants of gut microbiota and SCFAs profile.

Information about sociodemographic characteristics and parental lifestyles patterns, which have an established or a potential association with a higher risk of early development of precursor symptoms of asthma and potential modulators of the pattern of the intestinal microbiota and its metabolites in the infant, was collected through questionnaires administered in person during pregnancy: maternal age; parity (0, nulliparous; *vs.*. 1 or more, no nulliparous); maternal education level (incomplete secondary or less, complete secondary, and university); maternal social class (defined as maternal occupation during pregnancy by using a widely used Spanish adaptation of the international ISCO88 coding system: I–II, managers/technicians; III, skilled; IV–V, semiskilled/unskilled; and unemployed) [298]; gestational diabetes (yes/no); maternal body mass index (BMI Kg/m^2) based on height and pre-pregnancy self-reported weight (kg/m2), maternal smoking and consumption of alcohol during the first 20 weeks of gestation (yes/no).

The degree of adherence to a healthy diet pattern such as the Mediterranean diet during the pregnancy period was measured using one of the indices analysed in chapter 1, the Alternative Mediterranean Diet (aMED) (scale: ≤ 3, Low; 4-5, Medium and 6-8, High). Of the 3 indices analysed in chapter 1, the aMED index was finally selected for its better distribution of the population and better definition and score of the food groups that compose it, representing in a more faithful way the diet of pregnant women during pregnancy. Also, use of probiotic supplements (yes/no); antibiotics (yes/no) during the pregnancy period; use of IPA (yes/no); infant's gestational age; mode of delivery (vaginal/ caesarean); and type of lactation during the infant's first 3 months of life (categorized as exclusive breastfeeding, mixed feeding or exclusive infant formula). Finally, information was also collected on the medical history of the parents, specifically the history of asthma and atopic dermatitis of both the father and the mother (yes/no).

3.5 Statistical analysis

Data analysis were performed using RStudio version 1.2.5001 (RStudio Team (2019), Boston, MA) [299]. The packages and libraries used for all the analyses were *tidyverse*, *ISLR, normtest, corrplot, PerformanceAnalytics, wrs2, sfsmisc, FactoMineR, factoextra, rgl* and *vegan.* Prior to analysis, normality and homoscedasticity were analysed using Kolmogorov-Smirnov test, with Lilliefors correction, and Levene test, respectively, setting the level of significance to $p<0.05$. In the absence of normality and homoscedasticity results were represented as medians and IQR for the quantitative variables of the study, and relative frequency distribution were estimated for qualitative variables.

3.5.1 Study population characteristics: Control vs. cases

The non-parametric Kruskal-Wallis test or U-Mann Whitney test was performed for statistical comparisons between controls and cases according to the sociodemographic and lifestyle factors of the population.

3.5.2. Correlation study

The Spearman correlation test was used to study the correlation between the different bacterial groups and species that determine the profile of the gut microbiota of infants and their metabolites (SCFAs), for the total population, cases and controls.

3.5.3 qPCR and SCFAs vs. wheeze and eczema at 3 months of age

The U Mann-Whitney test was applied to make comparisons between bacteria concentration or SCFAs in healthy infants and infants with early development of asthma precursor symptoms, for each of the analyses performed. Differences in the prevalence of bacterial groups were established by applying the Chi-squared (X^2) test. P <0.05 was considered statistically significant for all tests.

3.5.4 Factor Analysis of Mixed Data (FAMD).

In order to examine how multiple factors affect infants' gut microbiota and their SCFAs profile, a multivariate analysis was performed. The statistical tool used was factorial analysis of mixed data (FAMD). It is a factorial method that

analyse quantitative variables as a principal components analysis, and as a multiple correspondence analysis for qualitative variables, hence the term mixed. FAMD was performed to highlight the separation of infants based on the phenotype (control and cases), mode of delivery, exposure to antibiotics during pregnancy, used of IPA, clinical history of parents (asthma and/or atopy), and mode of lactation. Differences between groups were analysed using permutational multivariate analysis of variance (PerMANOVA).

3.5.5 Multivariate logistic regression models

The two phenotypes were modelled as categorical variables and a multivariate logistic regression analysis (controls *vs.* cases) was carried out to identify the possible prenatal, perinatal and postnatal factors associated with a greater probability of the appearance of asthma precursor symptoms. Multivariate logistic regression models were also performed to determine the possible association between the different bacterial species or groups and SCFAs with the appearance of these symptoms. Model were adjusted for gestational age, BMI, parental history of asthma and atopic dermatitis, sex, mode of delivery, type of lactation, use of antibiotics during pregnancy and use of IPA. The reference category was healthy infants. The significance for all tests performed was set at p-value <0.05.

4. Results and discussion

4.1 Study population: mothers-infant pairs baseline characteristics.

Using a nested case-control design, we selected 197 of 216 mother-infant pairs from the NELA Study for gut microbiota analysis (see Material and Methods for the inclusion and exclusion criteria) and their metabolites as SCFAs.

The study population characteristics are presented in **table 18** (median (IQR))**.** The median age of the mothers were 33 years of age in both groups (controls: 33.00 (30.00-36.00); cases: 33.00 (30.75-35.00)). 52.48 % of the control's mothers were primiparous compared to 44.79 % of the mothers in the case group and 6.93 % *vs.* 6.38 % of the mothers (control and cases respectively) had gestational diabetes. The median weight gain (kg) during gestation was also very similar in both groups (control: 12.90 (10.00-16.00); cases: 12.00 (9.20-15.50)). No significant differences were found for any of the characteristics described above. None of the parents whose infants belonged to the control group had asthma or atopic dermatitis. However, in the case group, 13.54 % of the mothers and 10.42 % of the fathers had a history of asthma and 9.38 % of both parents presented atopic dermatitis. Regarding anthropometric measures, no significant differences were found between the control group and the cases group in terms of the mothers' height (cm) (controls: 1.64 (1.60-1.68); cases: 1.65 (1.61-1.68)), weight (kg) (controls: 62.25 (64.00-75.60); cases: 62.00 (56.00-69.00)) and BMI pre-pregnancy (controls: 23.20 (21.38-25.23); cases (23.30 (20.80-25.85)). Nor significant differences were found in the degree of MDA by the mothers between the two groups (measured using aMED index); (controls: 4.00 (3.00-5.00); cases: 4.00 (2.00-5.00)).

Table 18. The comparison of the distribution of main characteristics between healthy Spanish mother- infant pairs (n=197) recruited in the NELA cohort whose infants were classified into cases and controls.

	Controls (n= 101)				Cases (n= 96)				
	n	**%**	**M**	**IQR**	**n**	**%**	**M**	**IQR**	***P***
Maternal age (years)			33.00	(30.00-36.00)			33.00	(30.75-35.00)	
Parity, nulliparous	53	55.21			43	44.79			
Gestational diabetes, yes (n=192)	7	53.85			6	46.15			
Paternal history									
Asthmatic mother, yes	0	0.00			13	13.54			**
Atopic dermatitis mother, yes	0	0.00			9	9.38			**
Asthmatic father, yes	0	0.00			10	10.42			**
Atopic dermatitis father, yes	0	0.00			9	9.38			**
Anthropometric measures (20 weeks) (n=191)									
Height (m)			1.64	(1.60-1.68)			1.65	(1.61-1.68)	
Weight (kg)			69.00	(64.00-75.60)			68.15	(61.65-76.00)	
BMI (kg/m^2)			25.91	(23.84-27.85)			25.59	(23.03-28.00)	
Acceptable thinness (<18.5)	74	54.01			63	45.99			
Normal weight (18.5-24.9)	18	40.91			26	59.09			
Overweight (25-30)	8	57.14			6	42.86			
Obese (>30)									
aMED (n=188)	97		4.00	(3.00-5.00)	91		4.00	(2.00-5.00)	
Low (0-3)	40	53.33	3.00	(2.00-3.00)	35	46.67	2.00	(1.00-3.00)	
Medium (4-5)	43	52.44	4.00	(4.00-5.00)	39	47.56	5.00	(4.00-5.00)	
High (6-9)	14	45.16	6.00	(6.00-7.00)	17	54.84	6.00	(6.00-7.00)	
Weight gain (Kg) (n=182)			12.90	(10.00-16.00)			12.00	(9.20-15.50)	
Gestational age (weeks)			40.00	(39.00-41.00)			40.00	(39.00-40.25)	
Full-term (≥37 weeks)	99	52.38	40.00	(39.00-41.00)	90	47.62	40.00	(39.00-41.00)	*
Preterm (<37 weeks)	2	25.00	34.00	(33.50-34.50)	6	75.00	36.00	(36.00-36.00)	
Weight of the newborn (Kg)			3.25	(2.99-3.59)			3.29	(2.99-3.54)	
Area									
Urban area	69	53.49			60	46.51			
Residential area	15	41.67			21	58.33			
Rural	17	53.13			15	46.88			
Maternal education									
Incomplete secondary or less	17	47.22			19	52.78			
complete secondary and superior	24	50.00			24	50.00			
University	60	53.10			53	46.90			
Use of probiotics (20 weeks), yes	24	66.67			12	33.33			*
Maternal Smoking (20 weeks), yes	13	41.94			18	58.06			
Maternal alcohol consumption (20 weeks), yes	8	57.14			6	42.86			

Table 18. (continuation)

	Controls (n= 101)				Cases (n= 96)				
	n	**%**	**M**	**IQR**	**n**	**%**	**M**	**IQR**	***P***
Use of antibiotics (n=190), yes									
1st trimester	12	38.71			19	61.29			
2nd trimester	16	48.48			17	51.52			
3rd trimester	11	45.83			13	54.17			
Use of antibiotics during pregnancy period, yes	35	49.30			36	50.70			
Use of (IPA), yes (n=174)	25	49.02			26	50.98			
Mode of delivery									
Vaginal	86	56.58			66	43.42			
Caesarean	15	33.33			30	66.67			*
Sex of the newborn									
Girl	48	53.93			41	46.07			
Boy	53	49.07			55	50.93			
Type of lactation									
Maternal lactation	48	51.61			45	48.39			
Mixed	43	51.19			41	48.81			
Exclusive artificial	10	50.00			10	50.00			

Number of individuals belonging the category (n). Of the total number of individuals with the same characteristic, the percentage of individuals belonging to the control group or the case group was expressed as percentage (%). aMED (Alternative Mediterranean Diet index score); *p-value<0.05; **p-value<0.01; ***p-value<0.001. Numbers were expressed as median (M) and interquartile range (IQR) for the quantitative variables and relative frequency distribution were estimated for qualitative variables. U-Mann Whitney test for continuous variables or Person's X^2 test for categorical ones.

In the control group, 59.41 % of the mothers had a high educational level and 68.32 % lived in urban area and in the case group this percentages were very similar (55.21 % and 62.50 % respectively). Neither, significant differences were observed in the relative frequency distributions for the variables “tobacco consumption” and “alcohol consumption” at 20 weeks of gestation between control and case mothers. However, significant differences were observed in the percentage of mothers who consumed probiotics during pregnancy between both groups: 25.00 % of control mothers *vs.* 13.04 % of cases (p-value < 0.05). If we focus on the consumption of antibiotics by mothers throughout pregnancy, globally or in each of the three trimesters of pregnancy, no significant differences were observed in the percentages of mothers who consumed them between the control and cases group. Neither, significant differences were observed with respect to the variables “weeks of gestation” and “use of IPA” between the two groups. Concerning the type of delivery, significant differences were observed between the two groups (controls *vs.* cases) in the percentage of mothers who

gave birth by CS (14.85 % *vs.* 31.25 % respectively; p-value <0.05). Regarding the characteristics that concern newborns, no significant differences were observed in relation to the weight of the newborn (controls: 3.25 Kg (2.99-3.59) *vs.* cases: 3.29 Kg (2.99-3.54)) or sex (52.48 % *vs.* 57.29 % boys in controls and cases groups respectively). The type of lactation received by infants up to 3 months of age was similar between both groups: 47.52 % and 46.88 % of infants (controls and cases respectively) received breastmilk as food.

4.2 Infant gut microbiota and SCFAs. Comparative controls *vs.* cases

The counts of 12 microorganisms of interest (median (IQR)), quantified in the faeces of infants at 3 months of age by q-PCR, and their prevalence comparing cases (n = 96) and controls (n = 101) were represented in **figure 22** and **table 19** respectively.

In general, a trend towards lower counts was observed in faecal samples of the case group compared to the control group for all the microorganisms studied, including bacteria considered beneficial for health [39]. Specifically, significantly lower counts (Log genomic Eq./g faeces) were observed for the microorganisms *Atopobium* (controls: 4.48 (3.17-5.69); cases: 3.41 (2.59-4.89); p-value <0.05), *Bacteroides-Prevotella* group (controls: 7.07 (4.46-9.97); cases: 3.32 (2.10-6.22); p-value <0.01), *B. breve* (controls: 5.15 (3.28-7.01); cases: 3.97 (2.46-5.77); p-value <0.05), *B. longum* (controls: 6.32 (4.86-7.73); cases: 4.76 (2.71-6.58); p-value <0.01), *Clostridium* cluster IVa (controls: 5.28 (4.07-6.69); cases: 3.07 (1.24-4.67); p-value <0.01), *Clostridium* cluster XIVa (controls: 5.24 (4.09-7.21); cases: 2.88 (2.37-4.24); p-value <0.01) and *C. difficile* (controls: 4.99 (4.38-5.46); cases: 3.51 (3.13-3.75); p-value <0.01). In contrast, the counts of *A. muciniphila* were significantly higher in the cases group (controls: 2.98 (2.23-3.62); cases: 3.49 (2.43-4.90); p-value <0.05).

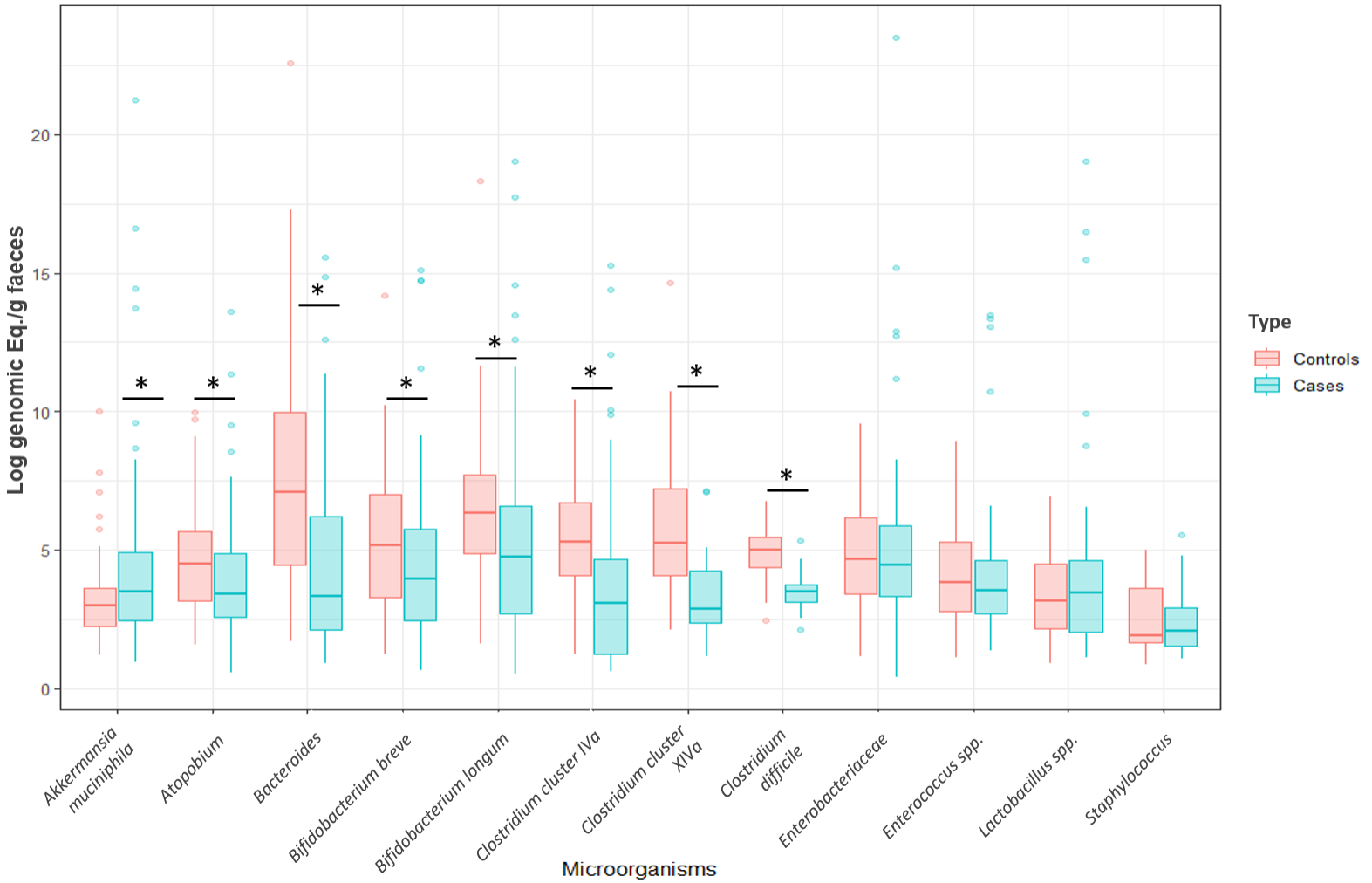


Figure 22. Gut microbiota profile of infants at 3 months of age, representing the counts of 12 microorganisms of interest quantified in faeces by q-PCR and comparing cases (n = 96) and controls (n = 101).

These results, with respect to the counts of *B. breve* and *B. longum*, as well as *Bacteroides-Prevotella* group, were similar to the results observed by several authors [330–332] and show higher counts of bacteria in faeces of infants classified as controls compared to allergic infants (those with atopic and/ or a positive skin prick test between the age of 1 and 2 years of age; those with dermatitis or allergic manifested allergic symptoms and a skin prick test at 5 years of age) It is interesting because these bacteria are considered beneficial for health for their probiotic effect. *Lactobacillus* and *Bifidobacterium* species, which produce lactic acid during their metabolic reactions, has been observed to have antimicrobial activity, preventing the colonization of pathogens and other unwanted flora [333,334]. In addition, various authors have reported that the bacteria *B. breve* and *B. longum* [335,336], as well as *Bacteroides* [337] induce the production of Immunoglobulins A (IgA) and the cytokine response helping to a correct immune development. The high counts of *Atopobium* cluster in the control group compared to the case group may be due to the type of delivery. Since, as indicated in **table 18**, a higher percentage of caesarean births was observed in the latter group and *Atopobium*, specifically *Atopobium vaginae* is a commensal specie of the vaginal flora of women. Therefore, caesarean deliveries, in which there is no transmission of the vaginal microbiota from the mother to the infant, could be the reason for less colonization by this bacterial species in the case group. There are other studies in which the authors had observed a higher count of *Atopobium* cluster in infants born by vaginal delivery compared to those born by caesarean section [338].

It is also important to highlight the lower counts of groups *Clostridium* cluster XIVa and *Clostridium* cluster IVa in infants classified as cases since these bacteria have been shown to play an important role in the induction of T-reg cells, which downregulate proinflammatory adaptative responses, including those associated with allergic asthma in mice [339]. In addition, these bacteria are producers of SCFAs such as propionic acid and butyric acid, which, have been shown to have anti-inflammatory and immunomodulatory properties [157,158,161]. Candela *et al.,* in 2015 also showed a depletion of *Clostridium* cluster IVa in stool samples of atopic children at 4-14 years of age [340].

Besides the counts, it is also interesting to observe the bacteria prevalence, since it reflects the number of positive amplifications from total samples analysed by q-PCR and therefore provides an idea of the greater or lesser colonization of the microorganisms (**table 19**). In this regard, significant differences were observed between the prevalence of some microorganisms when controls and cases were compared. The prevalence of *Lactobacillus spp.* (86.14 % *vs.* 56.25 %), *Enterobacteriaceae* (99.01 % *vs.* 87.50 %) and *Clostridium* cluster XIVa (70.30 % *vs.* 33.33 %) (controls *vs.* cases respectively) were higher in the control group (p-value <0.01). While infants with a high risk of asthma-related symptoms were more often colonised by *Akkermansia muciniphila* (70.30 % *vs.* 92.71 %), *Bifidobacterium breve* (45.54 % *vs.* 78.13 %), *Staphylococcus* (6.93 % *vs.* 29.17 %), considered as possible pathogen, and *Clostridium* cluster IVa (47.52 % *vs.* 73.96 %) (p-value <0.01). Our results agree with that described by Sjögren et al., (2009) [332] who observed that a lower colonisation with species such as *Lactobacillus, Bifidobacterium* and higher of *C. difficile* were more common in faecal samples from allergic compare with non-allergic infants. As discussed earlier during the study of correlations, it has been show that *Lactobacillus* and other LABs can prevent the colonization of *C. difficile* [328,334], and other studies have observed associations between low prevalence of Bifidobacteria species in early life and later allergy development [330,331].

Respect to the counts of *C. difficile,* several authors have observed a higher ratio of C. *difficile* in faeces of infants who developed allergy outcomes later in life, and it had also been associated as a risk factor for allergy development during childhood [173,330]. Specifically, Penders and colleagues (2007) founded that infants with higher rates of *C. difficile* were at higher risk of developing eczema, recurrent wheeze and allergic sensitisation, but this association was not observed when the authors analysed the association between *C. difficile* counts and the development of eczema, atopic dermatitis, recurrent wheeze and atopic sensitisation [341].

Table 19. Bacterial numbers in faecal samples (Log genome equivalents/ per g faeces) analysed by qPCR according to controls or cases group.

	Controls (n= 101)				Cases (n= 96)					
	Prevalence*		Bacterial numbers (Log genome Eq./g faeces)		Prevalence*		Bacterial numbers (Log genome Eq./g faeces)			
Microbial groups	n	%	Median (IQR)		n	%	Median (IQR)		*P*†	*P*‡
Bifidobacterium longum	94	93.07	6.32	(4.86-7.73)	87	90.63	4.76	(2.71-6.58)		***
Bifidobacterium breve	46	45.54	5.15	(3.28-7.01)	75	78.13	3.97	(2.46-5.77)	***	*
Staphylococcus	7	6.93	1.92	(1.66-3.63)	28	29.17	2.08	(1.51-2.90)	***	
Lactobacillus spp.	87	86.14	3.15	(2.18-4.49)	54	56.25	3.45	(2.02-4.60)	***	
Akkermansia muciniphila	71	70.30	2.98	(2.23-3.62)	89	92.71	3.49	(2.43-4.90)	***	*
Enterobacteriaceae	100	99.01	4.66	(3.42-6.15)	84	87.50	4.46	(3.35-5.88)	**	
Bacteroidetes	93	92.08	7.07	(4.46-9.97)	89	92.71	3.32	(2.10-6.22)		***
Enterococcus spp.	93	92.08	3.83	(2.77-5.27)	80	83.33	3.54	(2.71-4.63)		
Clostridium cluster XIVa	71	70.30	5.24	(4.09-7.21)	32	33.33	2.88	(2.37-4.24)	***	***
Clostridium cluster IVa	48	47.52	5.28	(4.07-6.69)	71	73.96	3.07	(1.24-4.67)	***	***
Clostridium difficile	15	14.85	4.99	(4.38-5.46)	14	14.58	3.51	(3.13-3.75)		**
Atopobium	88	87.13	4.48	(3.17-5.69)	85	88.54	3.41	(2.59-4.89)		*

† Difference in prevalence between the case group and the control group.
‡ Difference in concentrations between the case group and the control group.
*p-value<0.05; **p-value<0.01; ***p-value<0.001.
Number were expressed as median and interquartile range (IQR) for the quantitative variables.
U-Mann Whitney test for continuous variables and Person's X2 test for categorical ones
* Prevalence reflects the number of positive amplifications from total samples analysed by q-PCR (n= number of samples analysed).

The functional implications of the gut microbiota in infant with risk of atopy or allergy disease were further investigated by measuring SCFAs levels in faeces since, as previously mentioned, they have an immunomodulatory effect. Also, faecal SCFAs during infancy are associated with reduced risk of asthma with allergic sensitization later in life [33,342,343]. We compared the levels of SCFAs between the cases and control group, both referring to concentration of the 3 main (acetic, butyric and propionic) and total SCFAs (µM / g of wet faeces) (**figure**

23), and to molar proportion (%) (acetic, propionic, butyric and minority SCFAs (the sum of caproic, iso-caproic, iso-valeric, valeric, iso-butyric and heptanoic acids)) with respect to the total SCFAs quantified (**figure 24).**

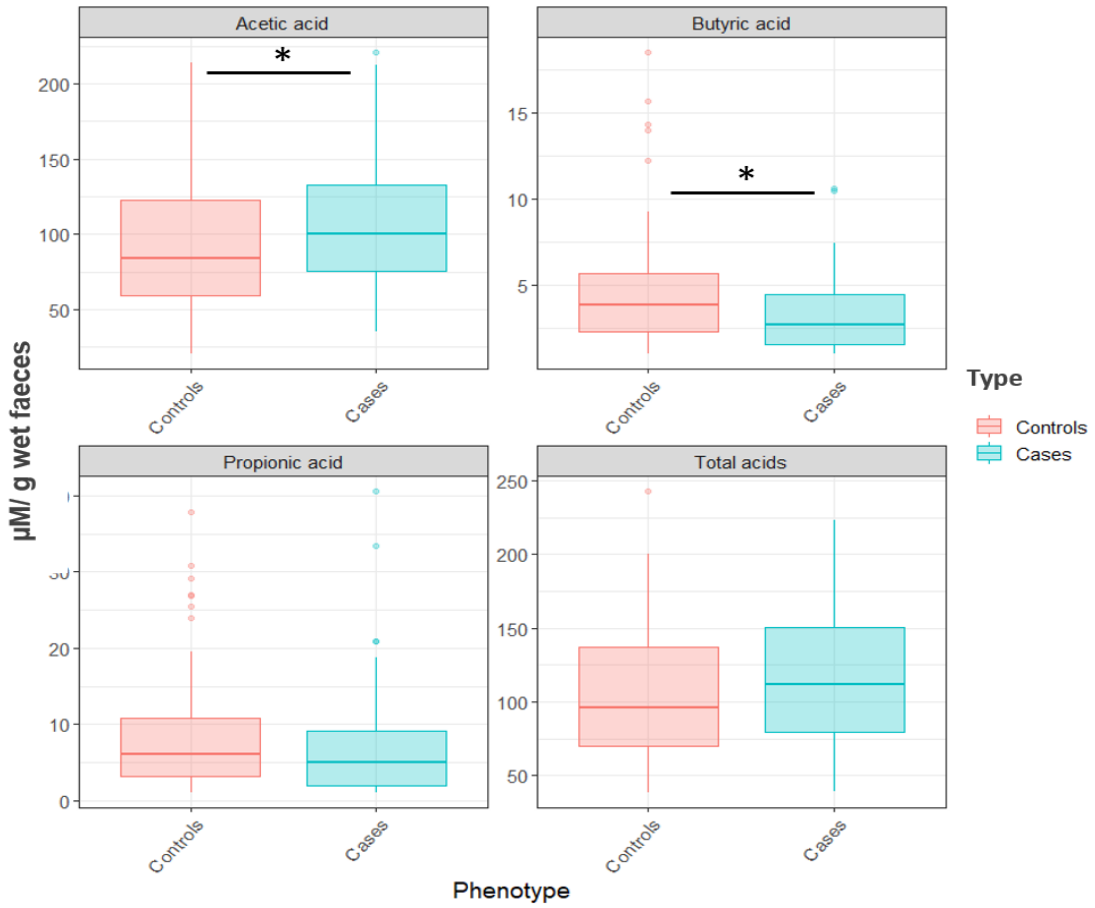


Figure 23. SCFAs profile of infants at 3 months of age, representing the concentrations of 3 principal fatty acids and the total amount, quantified in faeces and comparing cases (n = 96) and controls (n = 101).

A significantly lower concentration of acetic acid (83.95 (58.95-122.46) *vs.* 100.14 (75.06-132.91); p-value <0.007)) and total acids (95.75 (69.88-137.42) *vs.* 112.20 (79.36-150.88); p-value <0.05) were observed in controls group compared with cases group, but regarding to butyric (3.84 (2.33-5.70) *vs.* 2.72 (1.59-4.44); p-value <0.05) and iso-valeric (1.86 (1.47-2.25) *vs.* 1.33 (1.10-1.62); p-value <0.01) SCFAs, higher concentrations were observed in the controls group *vs.* cases. Regarding the molar proportions of the fatty acids, in the controls group lower proportions of acetic acid (86.51 % *vs.* 92.81 %) but higher

proportions of propionic acid (5.62 % *vs.* 4.09 %), butyric acid (1.06 % *vs.* 0.50 %) and minority acids (2.84 % *vs.* 1.48 % *vs.*) were obtained with respect to cases (p-value < 0.01).

In the case of acetic acid our results do not agree with those observed by

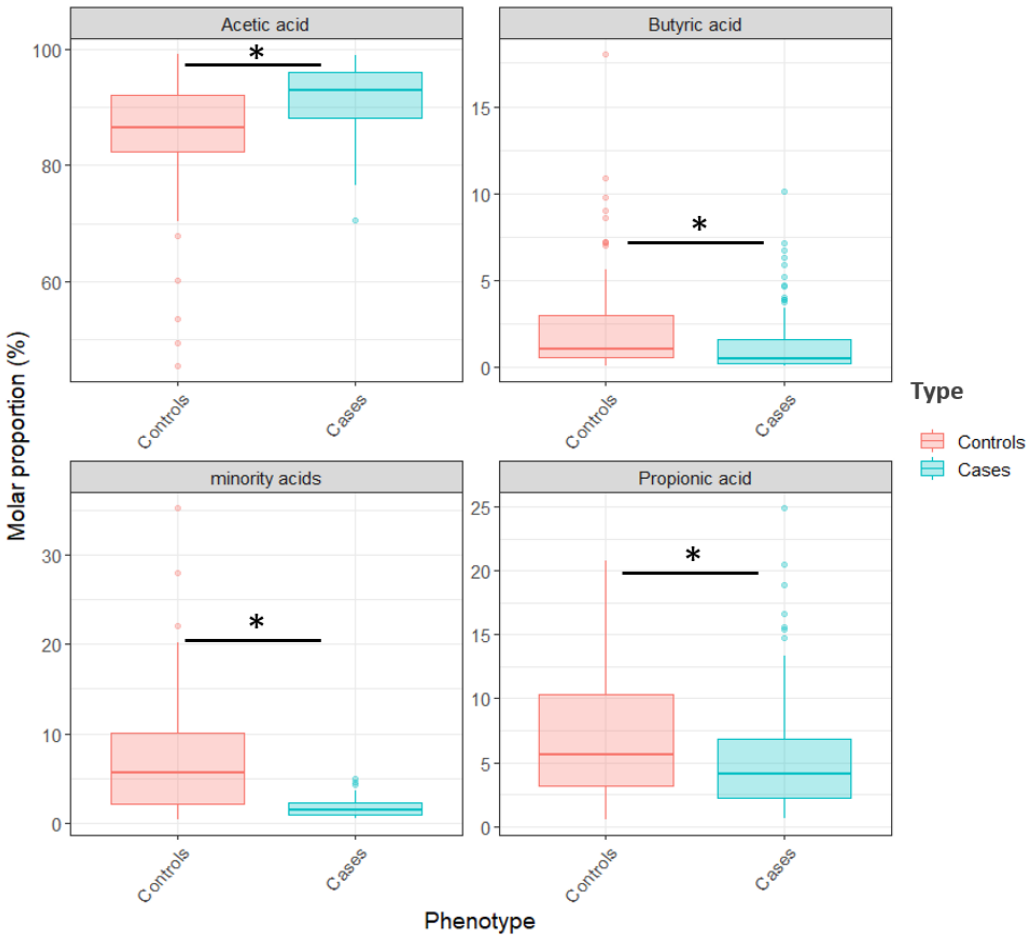


Figure 24. SCFAs profile of infants at 3 months of age, representing the Molar proportions (%) of 3 principal fatty acids and the minority fatty acids group in faeces and comparing cases and controls.

Arrieta *et al.,* (2015) [33]. The authors observed a higher concentration of acetic acid in the stool of the 3-month-old infants classified as controls compared to infants who had atopy or wheezing at one year of age. In our study there was lower concentration and proportion of acetic acid in the controls group, which may be due to the higher counts of Lactic Acids Bacteria (LABs). These LABs synthesize lactic acid and later propionic acid from pyruvic acid, the same substrate that is needed for the formation of acetic acid. As there are higher LABs counts in the controls, perhaps the reaction is more directed towards the production of lactic acid rather than acetic acid. In addition, *Bifidobacterium*,

which are found in greater counts in the control group, can also transform acetic acid to butyric acid, helping to reduce it. Another possibility may be the presence of other acetic-forming bacteria that have not been studied and that may be intervening in the acetic production.

Our results do coincide with the data published by Böttcher *et al.,* (2000) [344] for butyric, propionic and iso-valeric acids, finding lower concentrations in allergic infants and a higher molar proportion of acetic acid and lower proportion of propionic acid as in our cases group. They also coincide with the study of Roduit *et al*., (2018) [342]. Also, it is important to point out the higher concentrations and molar proportions of propionic and butyric acids in the group of control infants, as various authors have studied their influence on the adequate immune system development. It has been observed that propionic acid has an anti-inflammatory effect, decreasing the susceptibility to allergic air-way inflammation, as well as increasing the haematopoiesis of dendritic cell precursors, resulting in lung dendritic cells less effective to reactivate Th2 responses [345]. With regard to butyric acid, it is known that this acid is the main source of energy for the growth and differentiation of intestinal cells, specifically enterocytes, and performs other actions such as stimulating mucin production, helping to improve the integrity of the epithelium intestinal and maintaining the barrier function. In addition, it is also an activator of G pair receptors, an inhibitor of histone deacetylases and aids the intestinal immune system, thus contributing to the prevention of inflammatory disorders [346]. For all these reasons, as well as Böttcher *et al.,*[344] commented in their article, the lower levels of some of the SCFAs and the higher relative proportion of acetic acid among infants with allergy may disrupt the proper development of the immune system, through a disruption of the Th1/Th2 balance, by decreasing or delaying the development of the gut microbiota.

4.3 Correlation between infant gut microbiota and SCFAs profile

A correlation study was carried out to observe the possible interactions between the different bacterial species, between the metabolites produced by them (SCFAs in molar proportion) and finally, to study the relationships between the bacterial community and their metabolites (**figure 25)**.

The aim was to elucidate the intra-species relationship, which microorganisms may be the main producers of metabolites (such as acetic, propionic and butyric acid), which are particularly interesting because of their relationship with beneficial effects on infant health, and finally, the interactions between the metabolites themselves. These analyses have been performed for the whole sample and also subdivided according to the phenotype of the infants (cases or controls).

4.3.1 Correlation between the different microbial species studied.

Regarding the correlation between the different microbial species studied in the infants (**figure 25**), in general it was observed that most of them had a medium and positive correlation with each other with the exception of *C. difficile* and *Staphylococcus*. With regard to *C. difficile*, only a negative and low correlation was observed with *Lactobacillus,* being statistically significant only in the total population and in the control group. This negative correlation may be due to the probiotic effect of LABs, especially *Lactobacillus* and *Bifidobacterium* species, since these bacteria have the capability of, on the one hand inhibit the growth of pathogens such as *C. difficile* and on the other hand by the production of inhibitory substances, such as bacteriocins [334]. It was also observed that the genus *Staphylococcus* has a low, positive relationship only with the species *A. muciniphila* in the total population and in the case group.

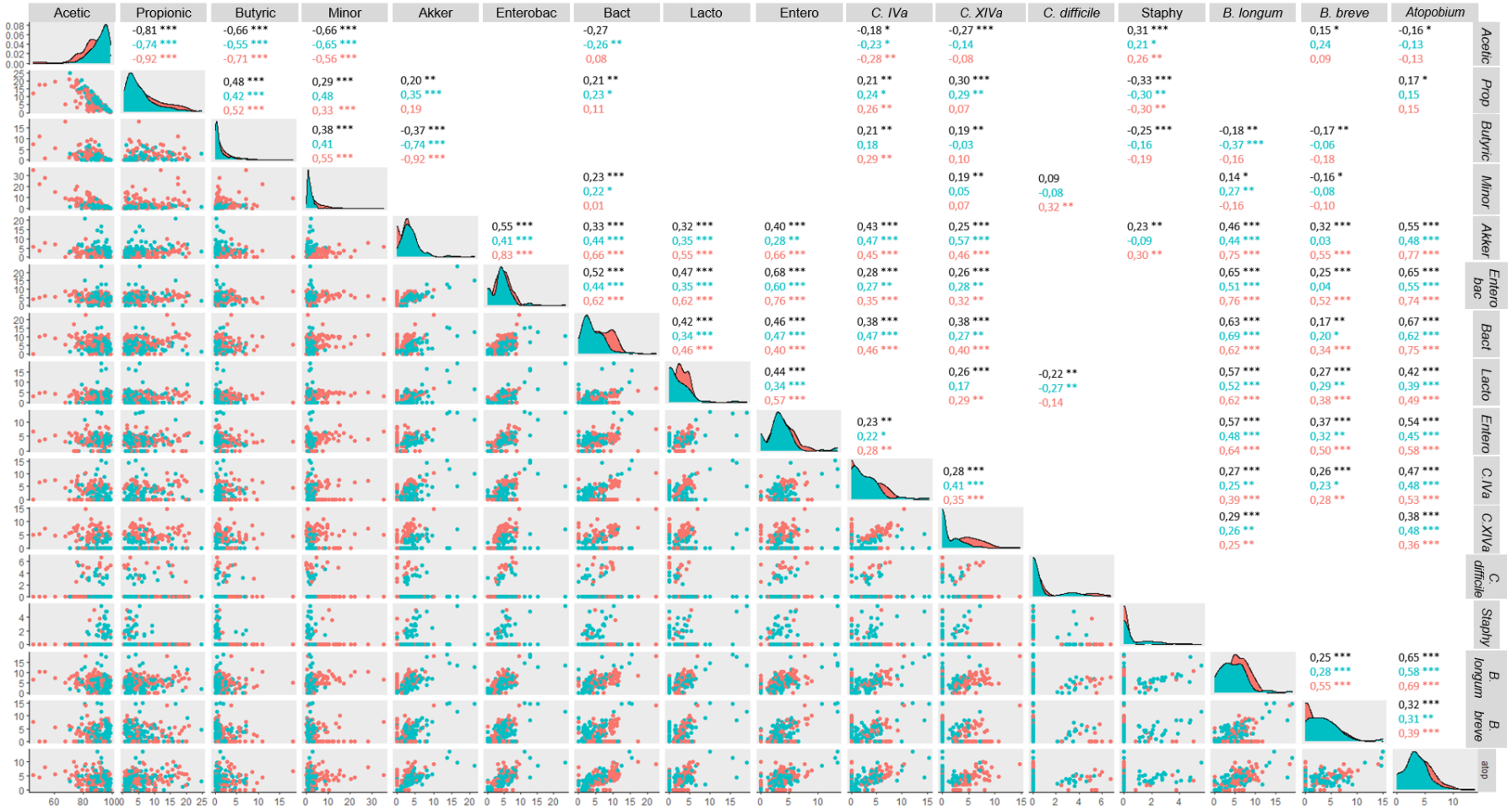


Figure 25. Correlation study between the different bacterial genera and species studied and the SCFAs in molar proportion. Prop: propionic; Akker: *A. muciniphila*; Enterobac: *Enterobacteriaceae*; Bact: *Bacteroides*; Lacto: *Lactobacillus*; Entero: *Enterococcaceae*; Staphy: *Staphylococcus*. Colours: Grey= Total population; Blue= Controls; Red= Cases. *p-value<0.05; **p-value<0.01; ***p-value<0.001.

High positive correlations were found between: *Enterococcus* and *Enterobacteriaceae; B. longum* and *Enterobacteriaceae, Bacteroides, Lactobacillus, Atopobium and Enterococcus; and Atopobium* with *A. muciniphila, Enterobacteriaceae, Enterococcus* and *Bacteroides.* Finally, positive mean correlations were also observed between *Enterobacteriaceae* and *A. muciniphila* and *Bacteroides*. In all of them, the correlation was statistically significant for all 3 groups (overall population, cases and controls).

4.3.2 SCFAs correlation.

In the study of the correlations between the SCFAs in the total population (**figure 25**), it was observed that acetic acid is the fatty acid found in the highest proportion, which has a negative average correlation with the rest of the SCFAs and is therefore highly and positively correlated with the total concentration of SCFAs. On the other hand, a positive mean correlation was observed between butyric acid and propionic acid, and a low positive correlation between these two fatty acids and the minority fatty acids (sum of caproic, iso-caproic, iso-butyric, valeric, iso-valeric and heptanoic).

When the population was subdivided into case and control groups, the same high and negative correlation was observed for acetic acid and the other fatty acids. In addition, a medium, positive correlation was also observed between butyric and propionic acid. Regarding the minority fatty acids and their correlation with butyric and propionic acid, the positive correlation observed in the overall population was also observed when the population was subdivided into cases and controls, however, in the latter group this relationship was not statistically significant.

4.3.3 Correlation between microbial species and their metabolites.

Finally, the relationship between bacterial species and their metabolites was also studied (**figure 25**), and, in general, very low correlations were obtained. The correlation observed between *C. difficile* and the sum of the minority fatty acids should be highlighted. This correlation was positive and statistically significant only in the case group. When we studied this correlation in more detail, to see which acid of the group of minority fatty acids was most correlated with the

bacteria, we observed that iso-caproic acid was the fatty acid that was positively correlated with *C. difficile* (rho: 0.25; $p<0.05$). This correlation has also been observed in other studies in which *C. difficile* has been associated with higher iso-caproic acid levels in allergic infants [344] and also higher *C. difficile* colonisation and higher *C. difficile* specific IgG levels have been reported in infants with a higher predisposition to wheezing, eczema, atopy and allergies [173,344,347].

On the other hand, in the case of LABs such as *Bifidobacterium* genus, when the general population was studied, a significant correlation was only observed between *B. breve* specie and a higher proportion of acetic acid and lower proportion of butyric acid. A negative correlation was also detected between *B. longum* species and the proportion of butyric acid in infant faeces. This negative correlation of both species with respect to butyric acid and/or propionic acid was also observed by Pham *et al.,* [348]. This fact may be due to the capability of these bacteria to produce both acetic and butyric acid, with lactic acid as intermediate metabolite, and in this case, they perhaps were directing their metabolic pathways mainly to the production of acetic acid and not to the production of lactic acid and butyric acid as the final product. With respect to *Lactobacillus spp.* another LABs bacterium, no statistically significant correlations were observed with any of the fatty acids studied and this result also agree with that observed by other authors [348]. On the other hand, Bacteroides and *A. muciniphila* had a positive correlation with the production of propionic acid as other author observed for both microorganisms [346]. A negative correlation was also observed between *A. muciniphila* and the molar ratio of butyric acid in the faeces of infants. This negative correlation is not in agreement with the literature [346,349] which indicates that *A. muciniphila* has the ability to convert acetic acid to butyric acid through fermentation of gut mucins. The bacteria that obtained the higher positive correlation with acetic acid was the genus *Staphylococcus,* in all 3 groups. This may be because it is a bacterium which acetate and CO_2 are the products of aerobic growth conditions and lactic acid is the end product of anaerobic glucose metabolism.

Finally, *Clostridium XIVa* was the bacterial group with the highest positive correlation with propionic acid, although this correlation was only observed for the total population and the control group, and it also had a positive correlation with butyric acid production, only in the overall population. *Clostridium cluster IVa* was the only bacterial group that had a positive correlation with both propionic and butyric acid, although this correlation was low. The role of *Clostridium* cluster XIVa and IVa needs to be highlighted as other author observed [350], *Clostridium* species are the main forces to generate SCFAs from carbohydrate fermentation, particularly butyrate.

4.4 Prenatal factors: their effect on infant gut microbiota and faecal SCFAs profile.

4.4.1 Mother's consumption of antibiotics during pregnancy.

Antibiotics exposition (AE) & infant's gut microbiota

There were no significant differences in the counts and prevalence for any of the microorganisms studied between the group of infants whose mothers were treated with antibiotics during pregnancy (IEA) and those who did not (INEA) (**figure 26**).

Our results agree with those observed by Penders *et al.,* (2006) [87] who found no association between the use of antibiotics, in their case during the last month of pregnancy, and changes in the infant's gut microbiota. But other authors such as Fouhy *et al.,* (2012) [351], have observed lower proportions of *Bifidobacterium* and *Lactobacillus,* and more proportion of *Proteobacteria* in infants whose mothers underwent a short course of antibiotics during pregnancy. Also, Arboleya *et al.,* (2015) [66] observed that infants whose mother were exposed to antibiotics (including intrapartum antimicrobial prophylaxis) during pregnancy had higher percentages of *Enterobacteriaceae* and lower of *Bifidobacteriacea* and *Lactobacillus* at 30 days of age.

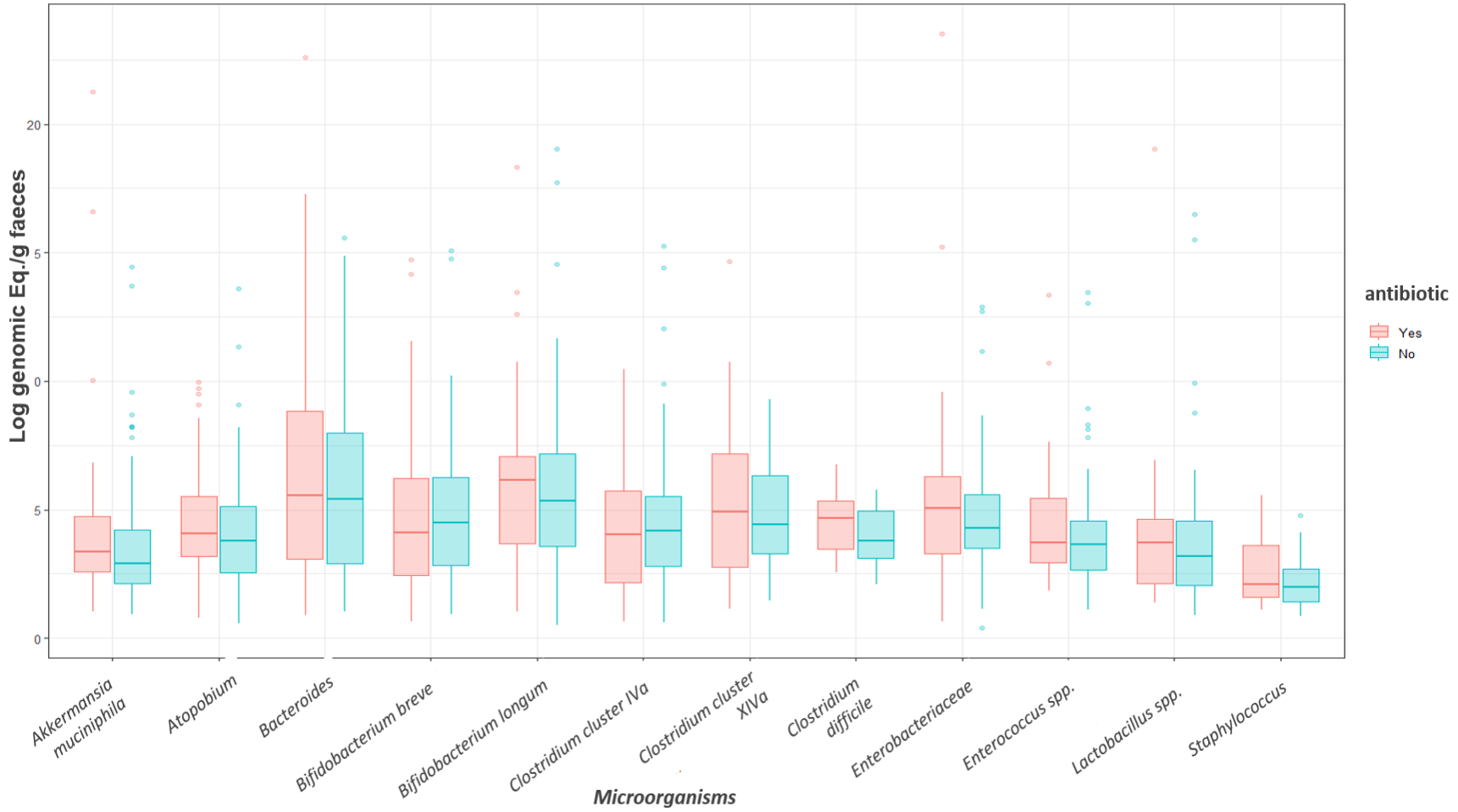


Figure 26. Gut microbiota profile of infants at 3 months of age, representing the counts of 12 microorganisms of interest quantified in faeces by q-PCR and comparing between those who have been exposed to antibiotics during pregnancy (n=70) and those who have not (n=119).

Finally, within the control group or the cases group, significant differences were also observed between INEA or IEA during pregnancy (f**igure 27**). For example, lower counts of *B. breve* (2.95 (1.83-4.62) *vs.* 4.52 (3.02-6.13)) were observed in INEA compared to IEA only for cases group. However, in control group, only significant differences were observed in *Lactobacillus spp*. (2.91 (2.07-4.38) *vs.* 4.02 (2.64-4.96) and *Staphylococcus* (3.62 (3.03-4.06) *vs.* 1.72 (1.29-1.82)) counts among INEA *vs.* IEA. Our results do not agree with those observed by Fallani *et al*., [89] where infants (6 weeks of age) from mothers treated with antibiotics perinatally had lower proportions of Bacteroides and members of the *Atopobium* cluster.

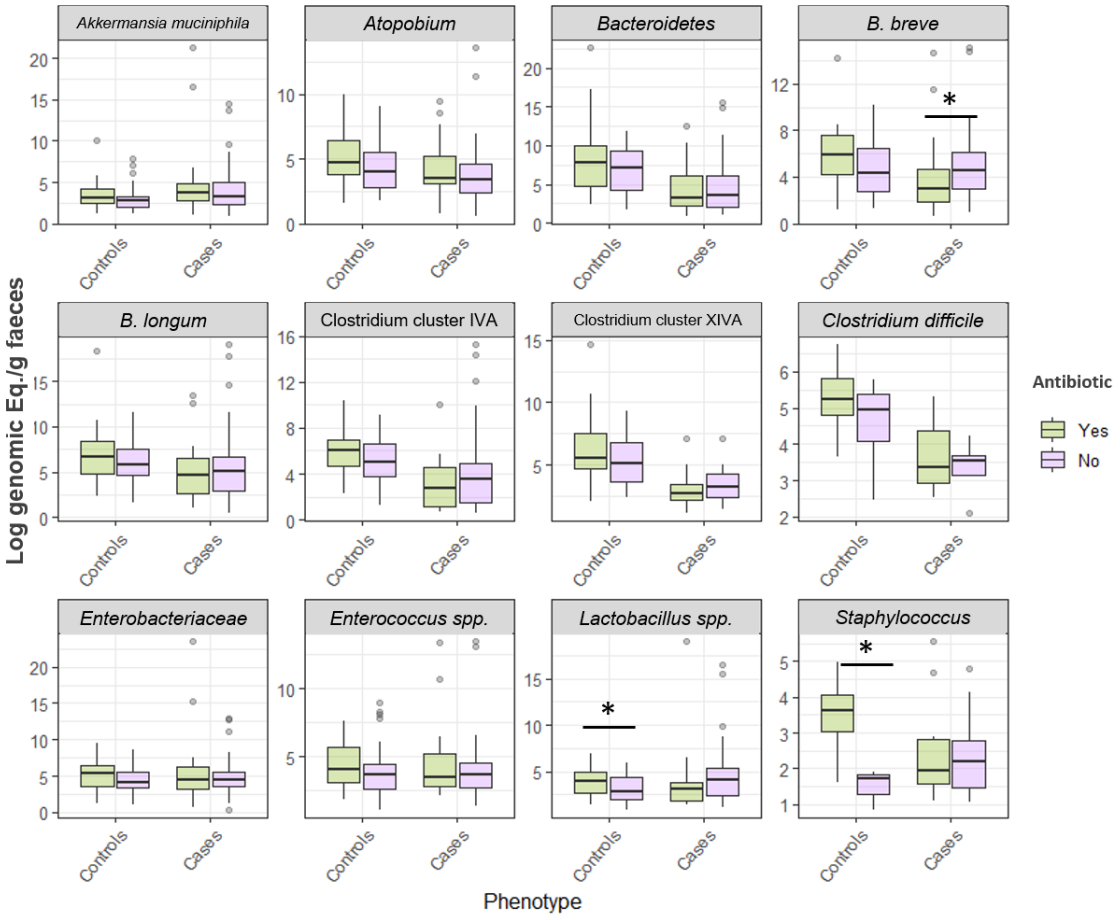


Figure 27. Gut microbiota profile of infants at 3 months of age, comparing within the control group or the case group between infant exposed to antibiotics and infants no exposed.

The absence of differences between IEA and INEA groups (**figure 26)** may be due to the period chosen for faecal sampling, since in authors observed that the effect of antibiotics on the infant's gut microbiota lasts between 10 days and 6 weeks [65] and the infants in our study are 3 months old. On the other hand, other factors, such as the mode of delivery or the type of lactation, perhaps have most effectively influenced the microbiota of infants, overshadowing or offsetting the effects that exposure to antibiotics may have caused in the perinatal stages. Also, the genetic background should be taken into account, since it has been observed that the microbiota of the infants of mothers with a history of atopy is less affected than that of the infants of healthy mothers [147]. It is also necessary to take into account that some authors have observed different effects on the microbiota of infants depending on the type of antibiotic administered, the dose and the duration of treatment [147].

Antibiotics exposition & infant's SCFAs

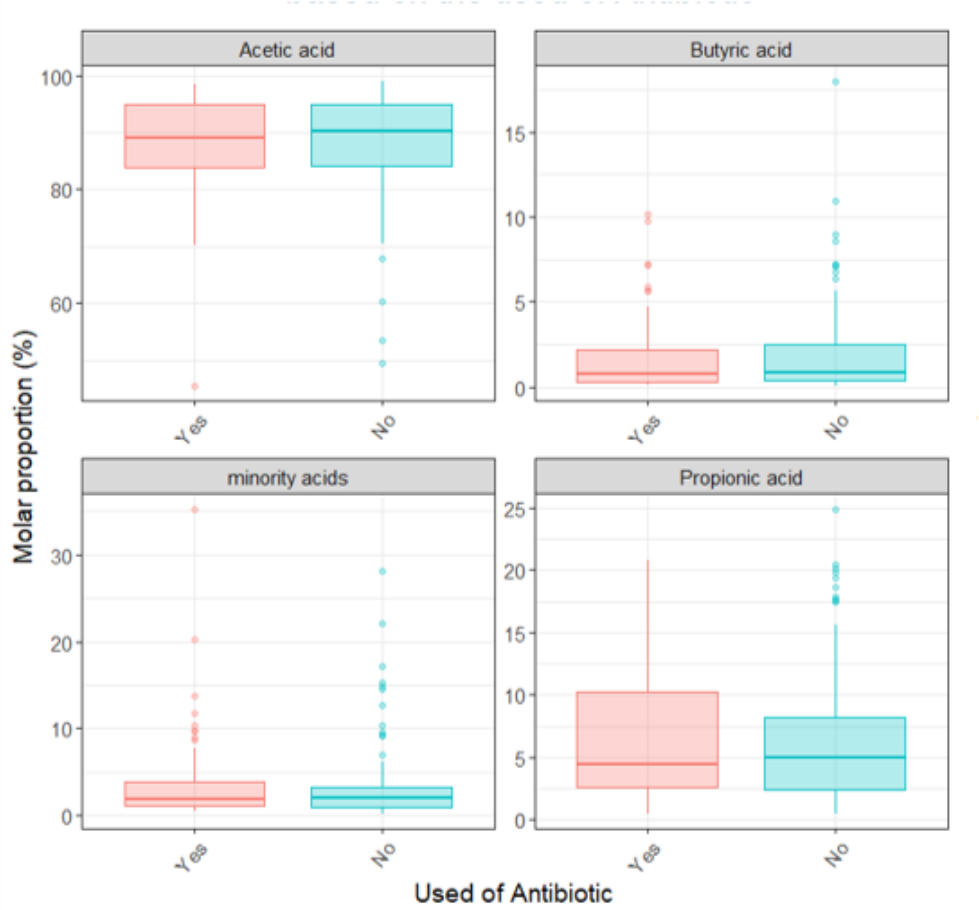


Figure 28. SCFAs profile of infants at 3 months of age, representing the molar proportion and comparing between those who have been exposed to the use of antibiotics by the mother during pregnancy n=71) and those who have not (n=119).

Just as no significant differences were observed in the gut microbiota of INEA compared to IEA, no significant differences were observed between the proportions of the main SCFAs between these groups (**figure 28**). No significant differences were found between the controls and the cases within the INEA group and neither within the IEA group (**figure 29**).

No studies have been found in the literature that have analyzed how exposure to antibiotics during the prenatal stage affects the fatty acid profile of the infant. Only in a study carried out in mice, the authors studied how exposure to low doses of antibiotics during pregnancy affected the offspring's SCFAs profile.

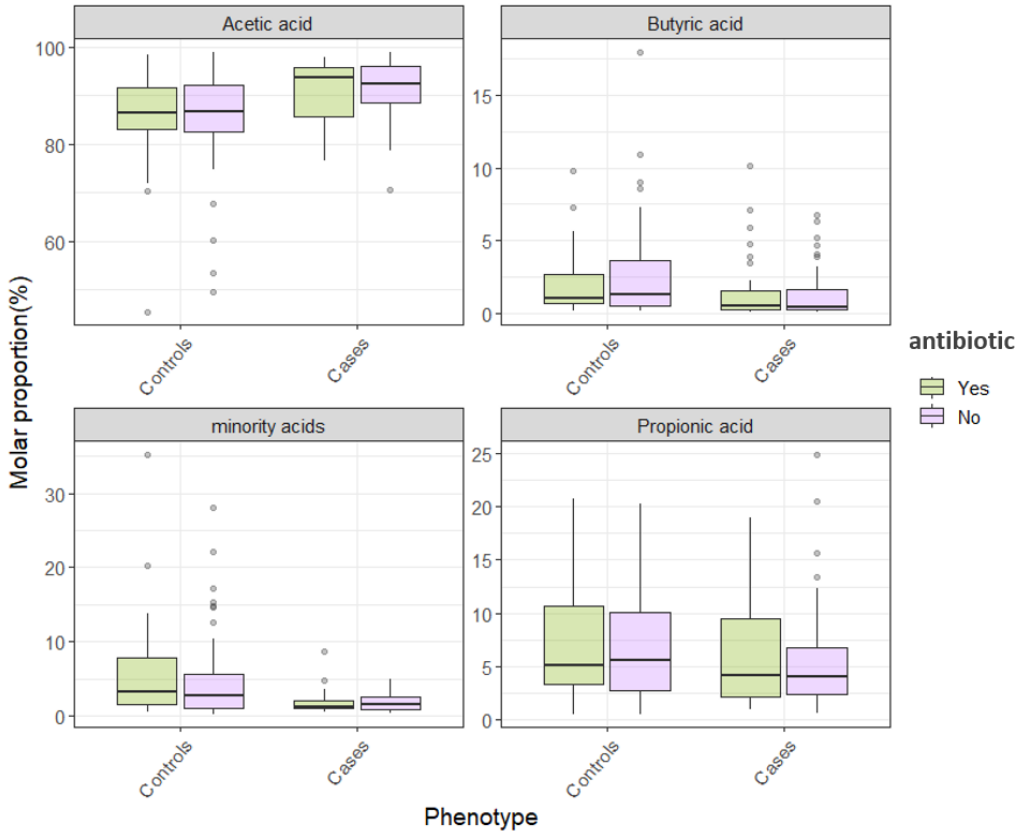


Figure 29. SCFAs profile of infants at 3 months of age expressed as molar proportion, comparing between infant exposed to antibiotics and infants who have not been exposed within the control group or the case group.

The authors found no significant differences in the main SCFAs when compared between mice whose mothers were exposed to antibiotics during pregnancy and controls [352].

4.4.2 Mother's consumption of probiotics during pregnancy.

Probiotic exposition & infant's gut microbiota

There were no significant differences in the counts of the microorganisms studied between the group of infants whose mothers consumed probiotics during pregnancy (IEP) and those who did not (INEP) (**figure 30)**. But significant

differences were observed comparing the prevalence between IEP and INEP for *Bacteroidetes* (83.33 *vs.* 94.77 %) and *Clostridium cluster XIVa* (72.22 % *vs.* 48.37 %) (p-value <0.05) respectively (data not showed).

No significant differences were observed when the bacterial counts of the INEP and IEP groups were compared, within the group of infants classified as controls. Nor were differences observed when comparing within the cases group (**figure 31**).

Despite the existence of a wide range of research conducted on the use of probiotics during pregnancy, the data on how probiotics influence the offspring's gut microbiota are very limited. The study carried out by Lahtinen *et al*., (2009) [52] is the only study found to date that employed an exclusively prenatal supplementation strategy. Unlike what was observed in our study, the author observed an increase in the prevalence of *B. longum* among the infants (at 90 days of life) whose mothers consumed probiotics during late pregnancy and a trend towards a higher increased prevalence of *B. breve*. The contradictory nature of our data may be due to, as on previous occasions, a synergistic effect on the modulation of the gut microbiota of infants by other factors such as the type of delivery, lactation, etc.

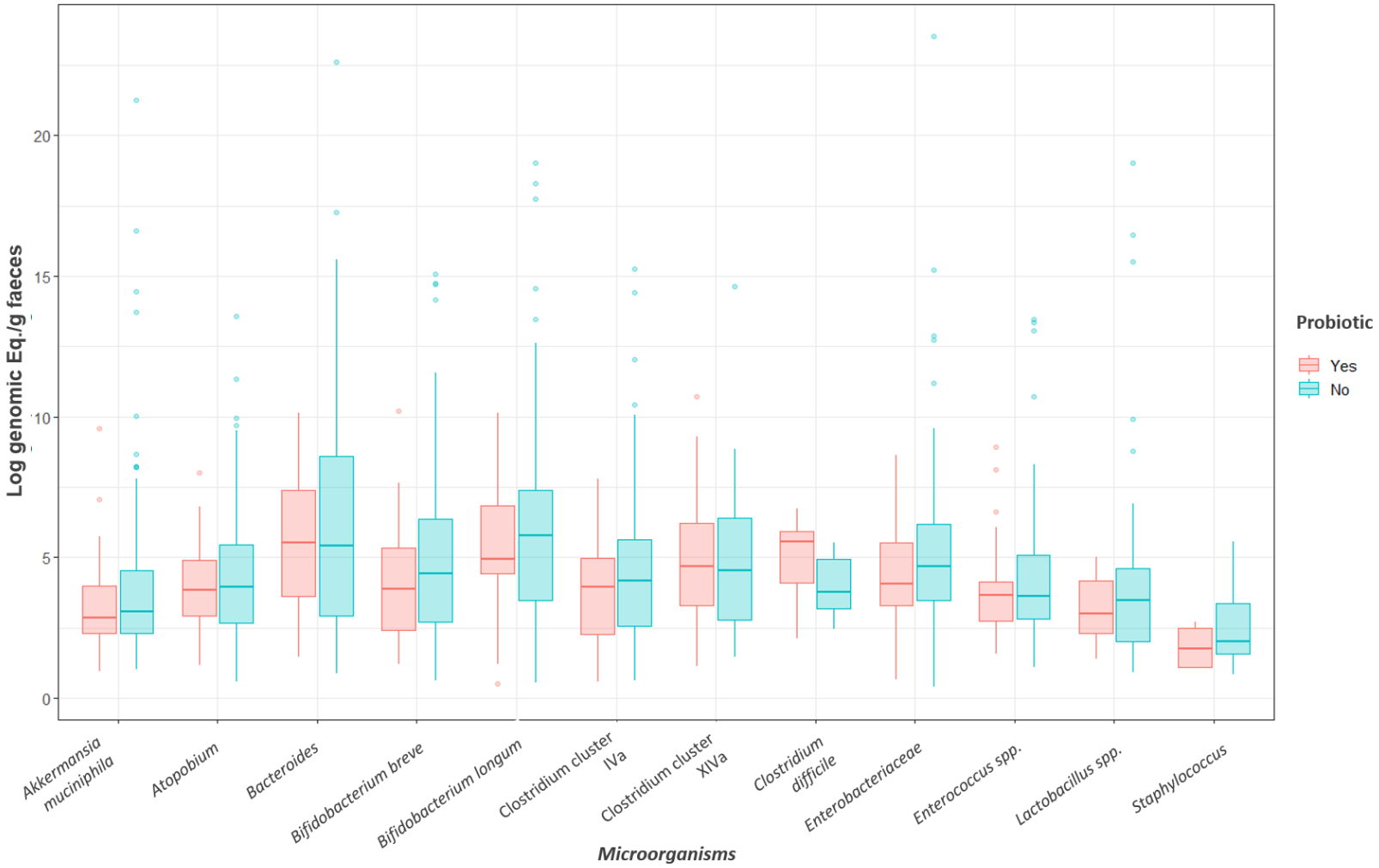


Figure 30. Gut microbiota profile of infants at 3 months of age, representing the counts of 12 microorganisms of interest and comparing between those who have been exposed to the use of probiotics by the mother during pregnancy (n=36) and those who have not (n=153).

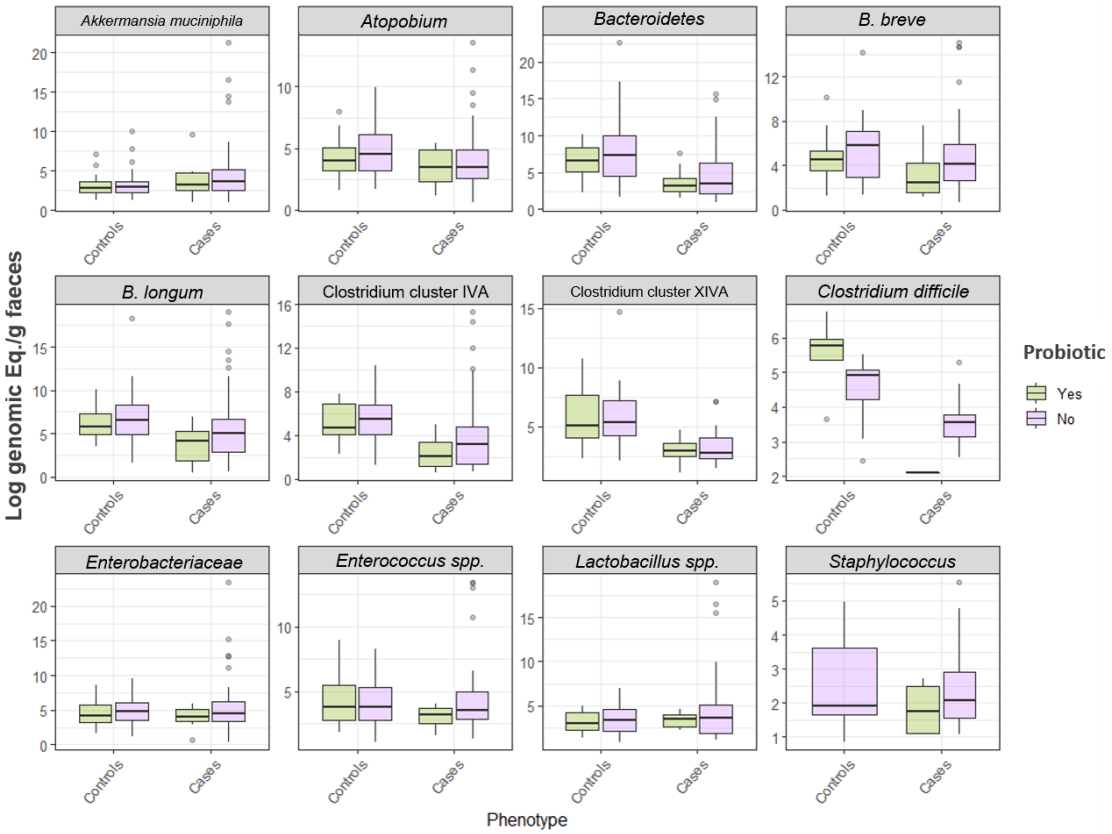


Figure 31. Gut microbiota profile of infants at 3 months of age, comparing within the control group or the case group between infant exposed to probiotics and infants who have not been exposed.

Probiotic exposition & infant's SCFAs

Regarding the SCFAs, no significant differences have been observed either between INEP *vs.* IEP, nor comparing INEP *vs.* IPE within controls or cases groups (**figure 32** and **33**).

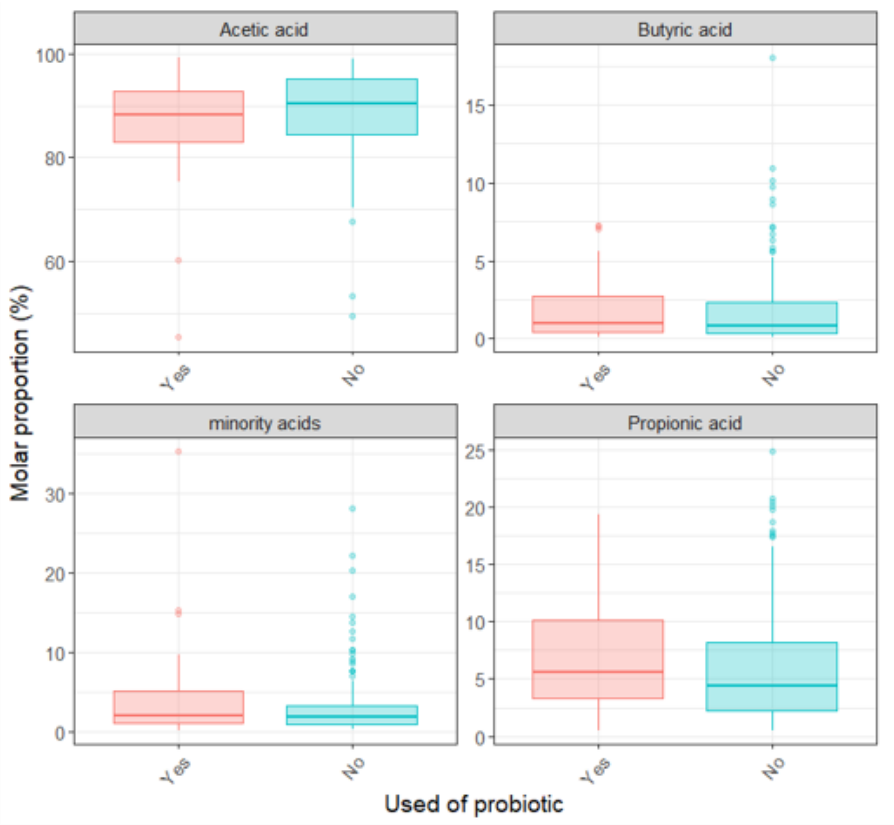


Figure 32. SCFAs profile of infants at 3 months of age, representing the molar proportion and comparing between those who have been exposed to the use of probiotics by the mother during pregnancy (n=36) and those who have not (n=154).

Such absence of differences observed in the molar proportions of the main SCFAs is consistent with what was observed regarding the infant's gut microbiota, where no differences were observed either.

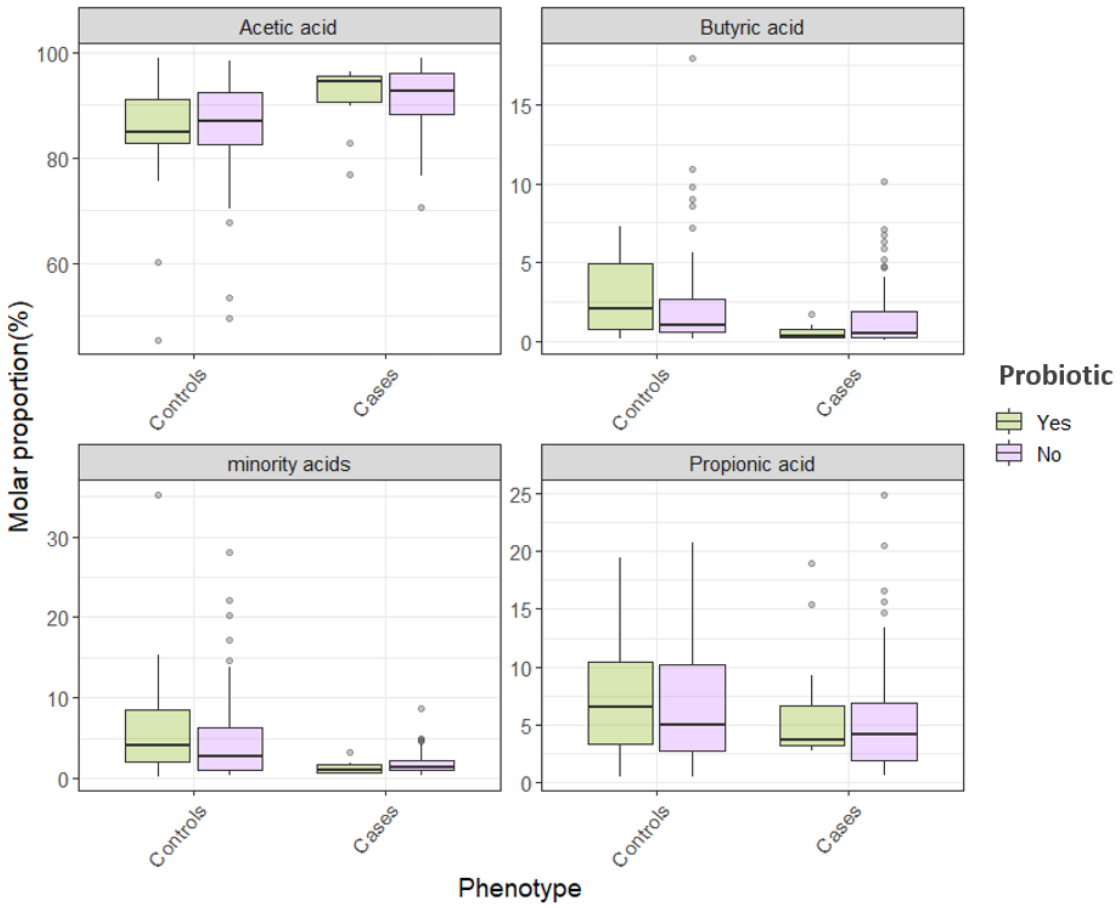


Figure 33. SCFAs profile of infants at 3 months of age in molar proportion, comparing within the control group or the case group between infant exposed to probiotics and infants who have not been exposed.

To our knowledge, no other studies have been found comparing the SCFAs profile of infants as a function of probiotic consumption by the mother during pregnancy. Neither, animal studies have been found in this regard.

4.4.3 Mother's Mediterranean diet adherence (MMDA)) during pregnancy.

MMDA & Infant's gut microbiota.

The counts of the bacteria studied were analysed comparing among infants whose mothers had a low (IMDAL), medium (IMDAM) or high (IMDAH) level of MDA during pregnancy (**figure 34**).

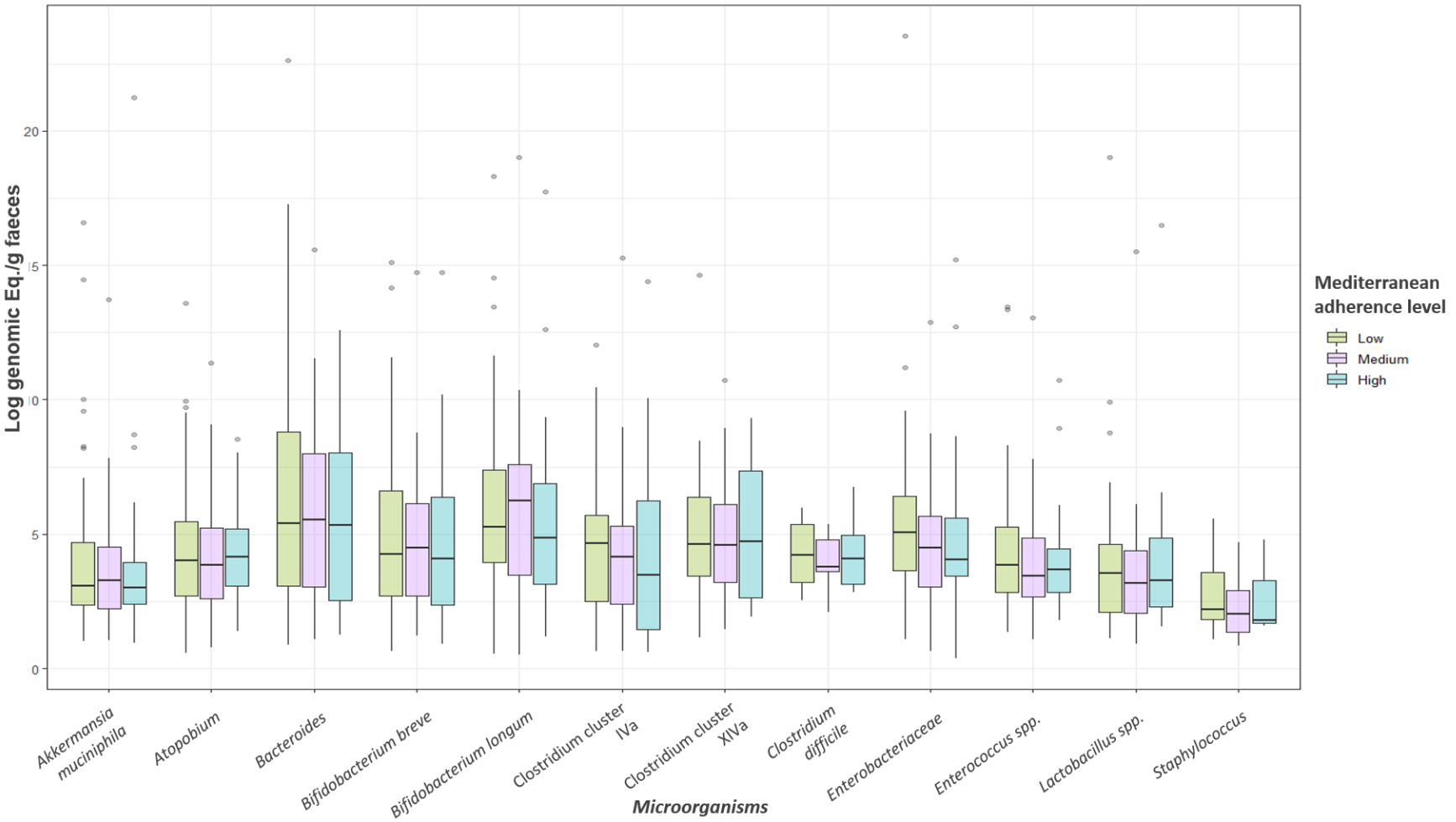


Figure 34. Gut microbiota profile of infants at 3 months of age, representing the counts of 12 microorganisms of interest and comparing between those whose mothers had low (n=75), medium (n=81) or high (n=31) level of adherence to a Mediterranean diet during the pregnancy period.

On the other hand, the comparison of the counts of each of the adherence levels within the control group or the case group were analysed and represented in **figure 35**.

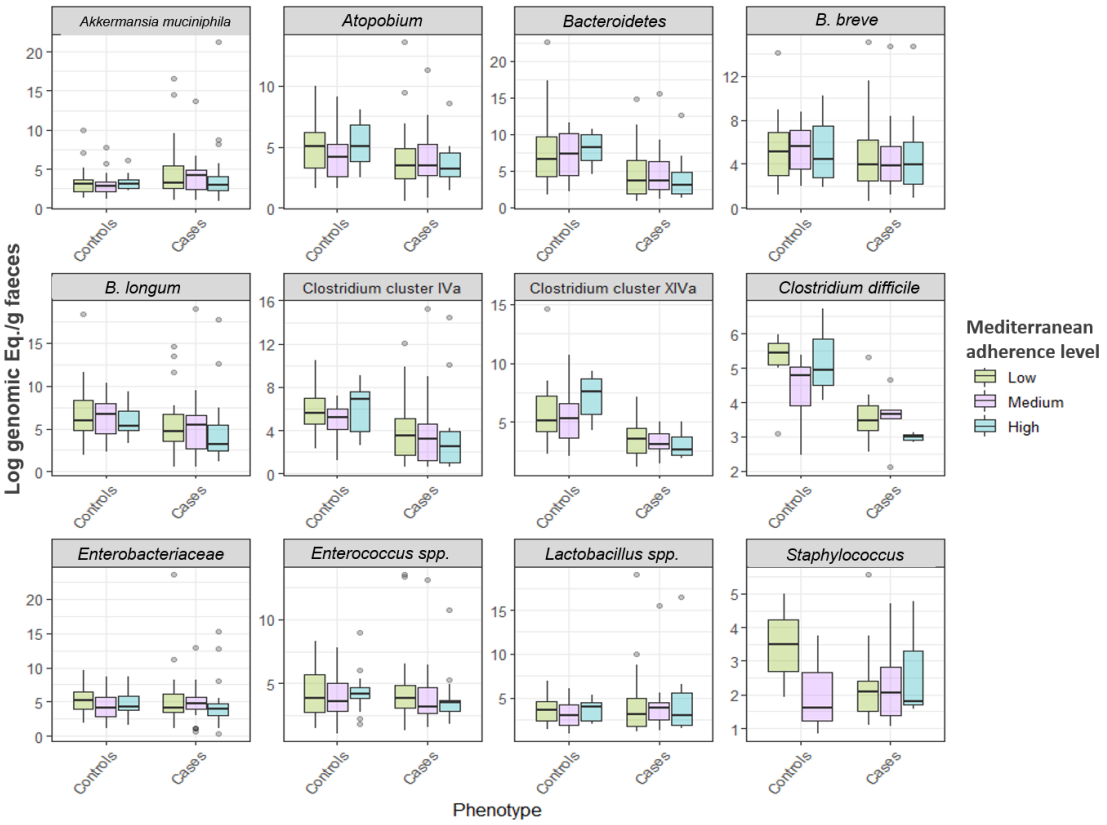


Figure 35. Gut microbiota profile of infants at 3 months of age, comparing within the control group or the case group between infant whose mothers had low, medium or high level of adherence to a Mediterranean diet during the pregnancy period.

No significant differences were observed between the counts and the prevalence (**data not shown**) of any of the bacteria when they were compared between the 3 groups of infants according to the level of adherence to the MD by the mother (**figure 34**). Neither significant differences were observed in the counts for any of the bacteria among the 3 groups established according to the level of adherence of the mother (IMDAL, IMDAM and IMDAH) when comparing within the control group or the case group independently (**figure 35**).

In a recent review, written by Maher *et al*., (2020) [353], in which the association of the maternal diet during pregnancy and the microbiota of the mother and the newborn were studied, only two investigations described the effect of the maternal diet on the neonatal microbiota using next generation sequencing methods. Both publications reported that maternal diet in pregnancy is associated with distinct changes in the neonatal gut microbiome [74,76]. Specifically, Chu *et al*., (2016) [76] observed a notable decrease of *Bacteroides* in the newborns exposed to a maternal high-fat diet during gestation and Lundgren *et al.,* (2018) [74] observed a negatively association between maternal consumption of fruit and the presence of *Bifidobacterium* group.

Perhaps we have not observed any difference in the gut microbiota of infants based on the mother's diet for various reasons. On the one hand, it may be possible the existence of an effect on the infant's gut microbiota but that this effect was diluted or disappeared after a few weeks of delivery. On the other hand, in the studies that have observed an effect, the infant's gut microbiota has been analysed using massive sequencing techniques, which provides relative abundances of the whole microbiota compared to our analyses where only 12 microbial groups were studied by qPCR.

MMDA & Infant's SCFAs.

Figure 36 shows the SCFAs profiles of infants at 3 months of age as a function of the degree of adherence to the Mediterranean diet of the mother during pregnancy. The SCFAs between IMDAL, IMDAM and IMDAH were compared without significant differences among the 3 groups.

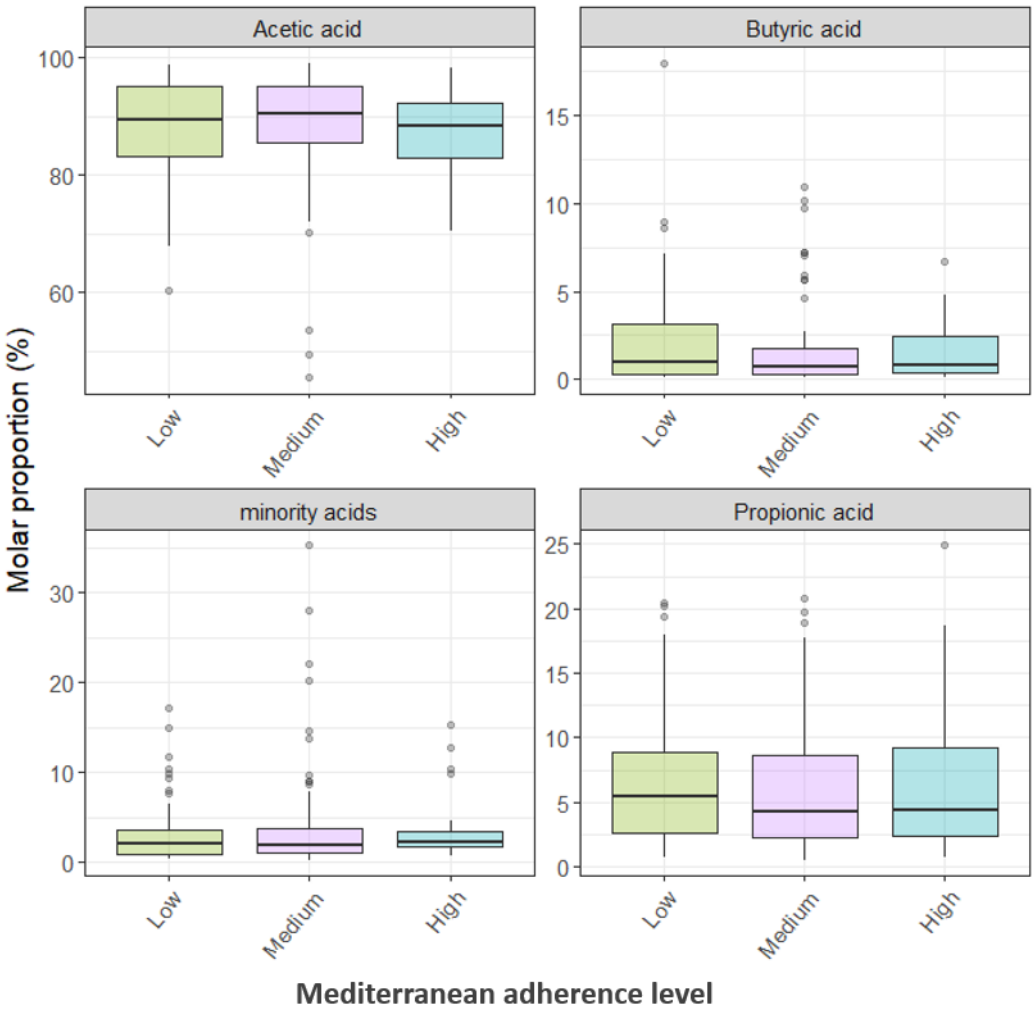


Figure 36. SCFAs profile of infants at 3 months of age, comparing between those whose mothers had low (n=75), medium (n=82) or high (n=31) level of adherence to a Mediterranean diet during the pregnancy period.

A statistical analysis of the SCFAs was also performed comparing the molar ratio between the 3 adherence groups within the control group and the case group independently (**figure 37**) and no significant differences were observed in any of the fatty acids studied.

There have not been other researches that have studied if the SCFAs profile of the infant changes at 3 months of age depending on the degree of MDA of the mother during gestation. Nor have there been studies in relation to other types of diets, for example diets rich in fiber or saturated fats, during pregnancy and the SCFAs profile of infants.

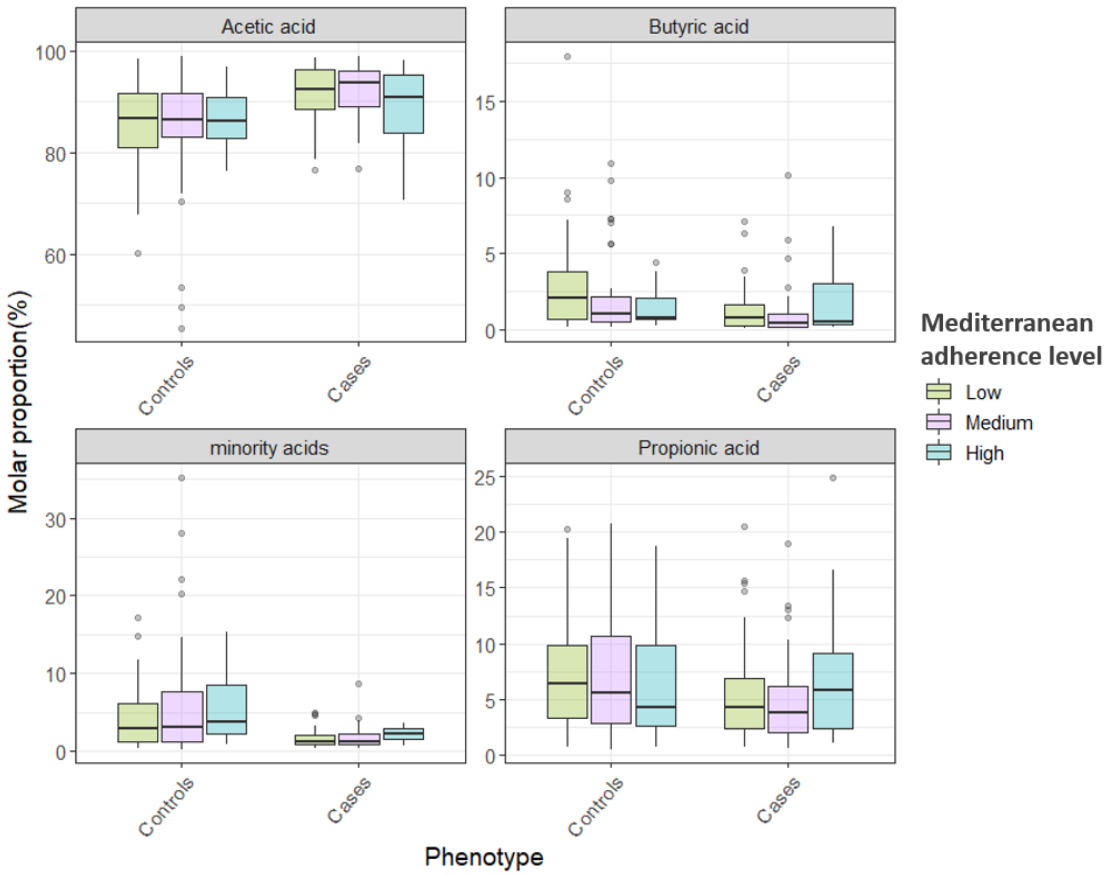


Figure 37. SCFAs profile of infants at 3 months of age, comparing within the control group or the case group between infant whose mothers had low, medium or high level of adherence to a Mediterranean diet during the pregnancy period.

4.4.4 Mother's gestational diabetes mellitus (MGDM) status during pregnancy.

MGDM & Infant's gut microbiota.

In the analysis of MGDM and their influence on the gut microbiota of infants at 3 months of age, on the one hand, the counts and prevalence of the bacteria studied were analysed comparing between infants whose mothers developed gestational diabetes (IMGDMY) and those who did not (IMGDMN) (**figure 38**). In this comparison, no significant differences were observed regarding the counts or the prevalence (data not shown) of any of the bacteria studied between both groups.

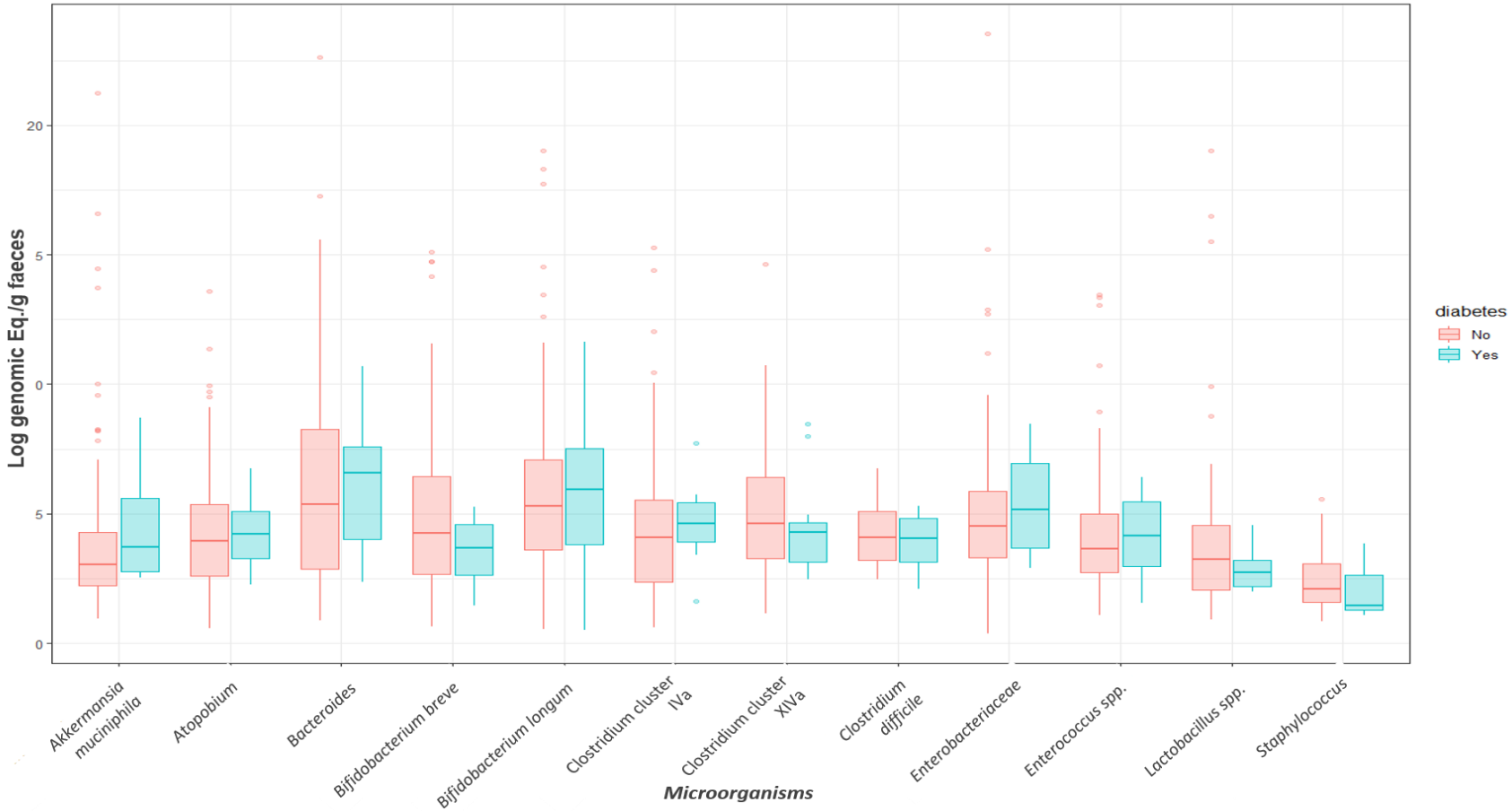


Figure 38. Gut microbiota profile of infants at 3 months of age, representing the counts of 12 microorganisms of interest and comparing between those whose mothers have developed gestational diabetes during pregnancy (n=15) and those who have not (n=180).

With the intention of delving in the analysis of how gestational diabetes can influence the gut microbiota of infants, taking into account their phenotype (cases or controls), we made a comparison of the counts between IMGDMY and IMGDMN for the same category controls or cases (**figure 39)**. Neither in this analysis significant differences were observed in the counts of the bacteria studied when IMGDMY and IMGDMN were compared within the same controls or cases group.

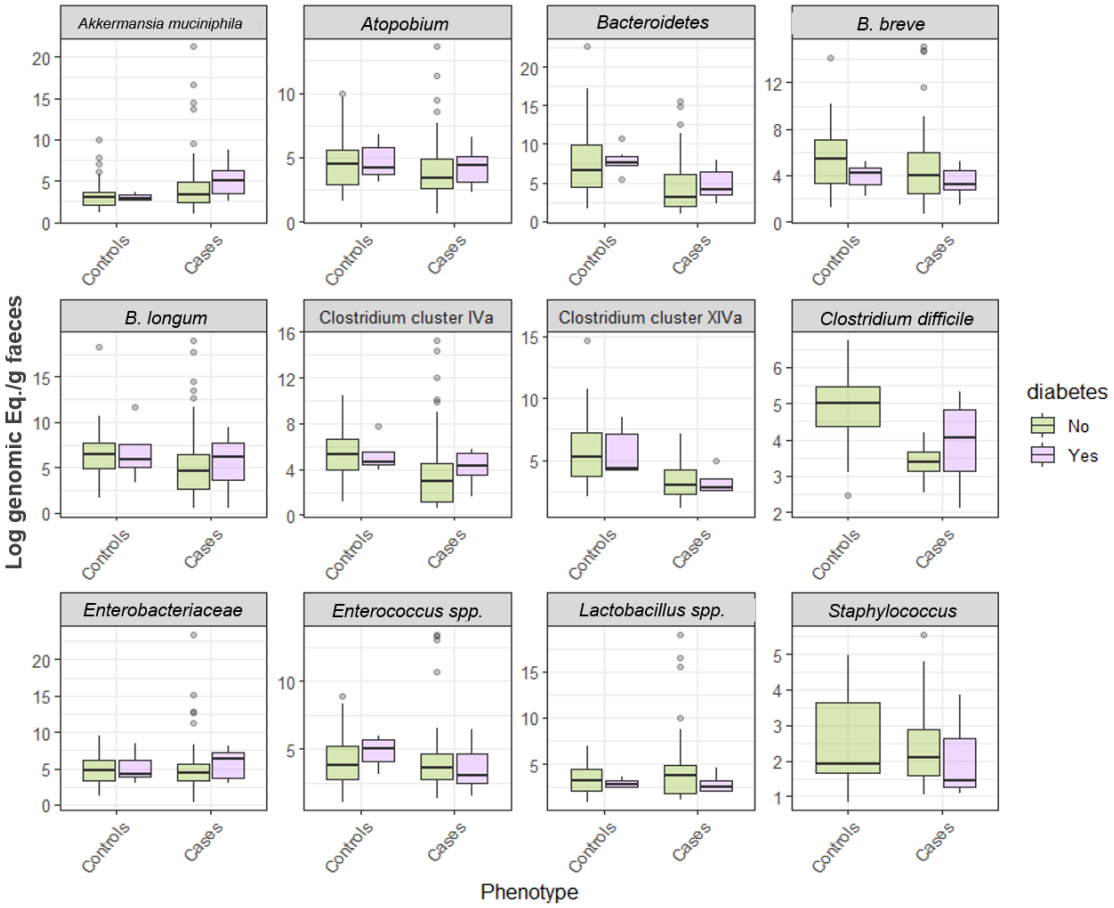


Figure 39. Gut microbiota profile of infants at 3 months of age, comparing within the control group or the case group between infant whose mothers have developed gestational diabetes during pregnancy (n=15) and those who have not.

However, other authors have observed differences. For example, Su *et al.,* (2018) [354], using sequencing and bioinformatics analysis of 16S rRNA, observed lower alpha-diversity in the group of newborn whose mother had gestational diabetes than that of control group. The phyla of Proteobacteria and

Actinobacteria in MGDM newborns were higher, while that of Bacteroidetes was significantly lower ($p < 0.05$). Also, Crusell *et al.,* (2020) [355], using the same methods, showed that the children of MGDM were characterized by having a different microbiological profile, both during the first week of life and at 9 months, at higher taxonomic and OTU levels. Specifically, these authors observed that the gut microbiota of the newborns of MGDM was enriched with bacterial genera and relatives within the phylum Firmicutes, while the genera *Veillonella* and *Megasphaera*, as well as the genera *Subdoligranulum* and *Ethanoligenes* were reduced. Similarly, the genus *Prevotella* and the parental family *Prevotellaceae* within *Bacteroidetes*, and the genus *Rothia* and the parental family *Micrococcaceae* within Actinobacteria were depleted in newborns of MGDM. However, in this study there was no difference in the Shannon diversity index (quantitative measure (in proportion) that reflects how many different types (such as species, genera or phyla) there are in a community) at any time. By last, in another study carried out by Hu *et al.,* in 2013, the authors concluded that there is experimental evidence signalling that the gut microbiota in meconium significantly differs depending on maternal diabetes status [18]. Specifically, the meconium of infants whose mothers had diabetes were enriched with the phylum *Bacteroidetes* and the genus *Parabacteroides* while in the meconium of newborns from nondiabetic mothers there was a higher abundance of Proteobacteria in the meconium of infants from nondiabetic mothers. It should be noted that this last study was carried out on meconium samples and not on faeces from infants at 3 months of age, as is our case.

MGD & Infant's SCFAs

Regarding the SCFAs, no significant differences have been observed either between IMGDMN *vs.* IMGDMY (**figure 40**).

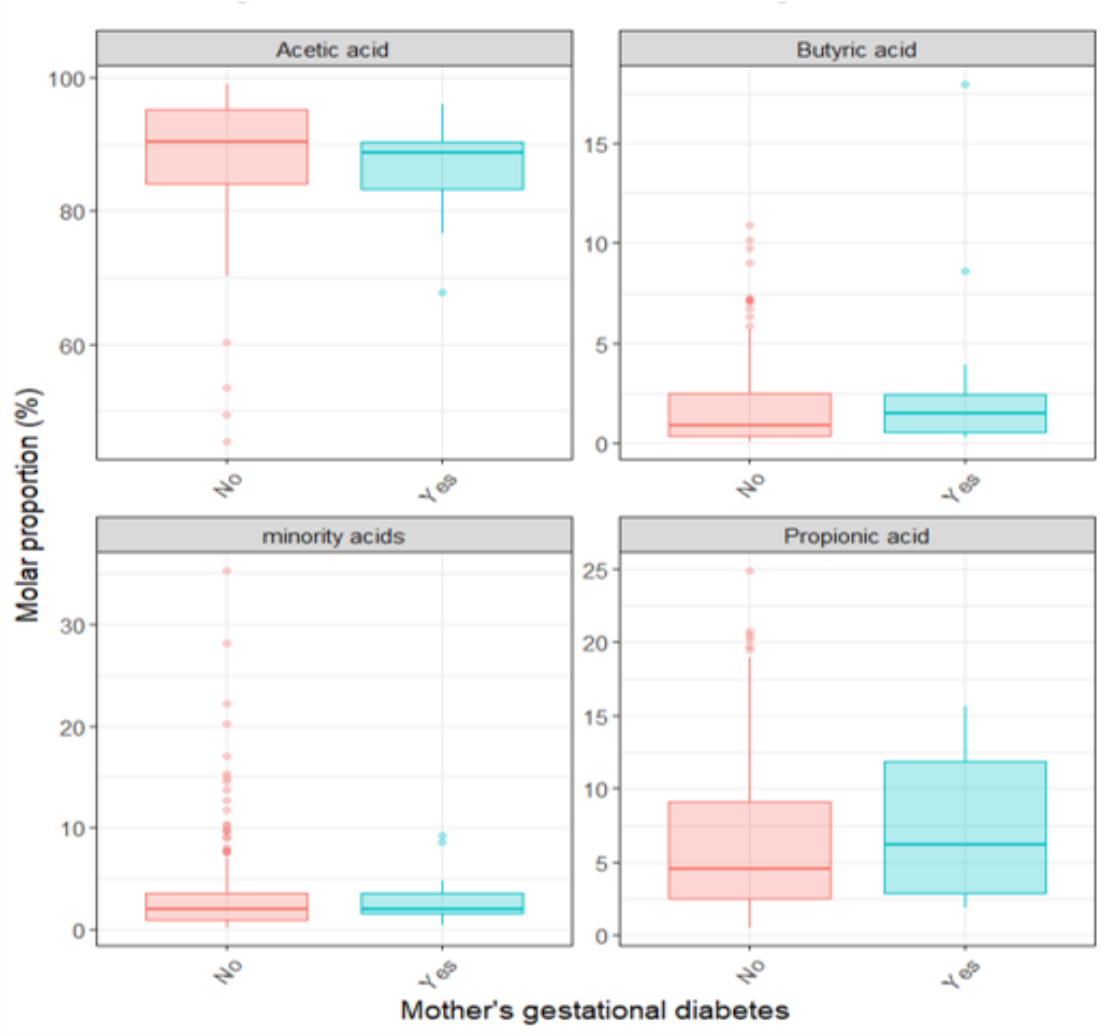


Figure 40. SCFAs profile of infants at 3 months of age, comparing between those whose mothers have developed gestational diabetes during pregnancy (n=14) and those who have not (n=181).

The same non-modulating effect by gestational diabetes over the gut microbiota of infants, was also clearly observed in their generated metabolites such as SCFAs. Our results regarding SCFAs coincide with what was observed by Soderborg *et al.,* (2020) [356]. Specifically, the authors observed no differences in infant faecal SCFAs levels between neonates born to MGDM and without MGDM.

To our knowledge, no other investigations have been carried out that study the profile of SCFAs in infants in function of whether the mother has developed gestational diabetes or not during pregnancy.

4.5 Perinatal factors: their effect on the gut microbiota and SCFAs profile of the infant

4.5.1 Mother's intrapartum antibiotic (IPA) exposition during delivery.

IPA & Infant's gut microbiota

Figure 41 shows the mean counts of the bacteria studied in the gut microbiota of infants, depending on whether or not the mother was exposed to IPA during delivery. A wide difference was observed in the counts (Log genomic Eq./ g faeces) (median (IQR)) of *Atopobium* although this was not significant, with higher counts being observed in infants not exposed to IPA (INEIPA) compared to those exposed (IEIPA) (4.31 (3.13-5.47) *vs.* 3.25 (2.29-4.80); p-value = 0.054). On the contrary, higher counts of *Staphylococcus* were observed in the IEIPA compared to the INEIPA, although these differences were not significant either (3.09 (2.13-4.47) *vs.* 1.92 (1.47-2.73); p-value= 0.056). On the other hand, when the prevalence of bacteria was studied (**table 20**), comparing again between INEIPA and IEIPA, significant differences were only observed for the *Bacteroidetes* phylum, being higher in the INEIPA group (95.12 % *vs.* 84.31 %; p-value <0.05).

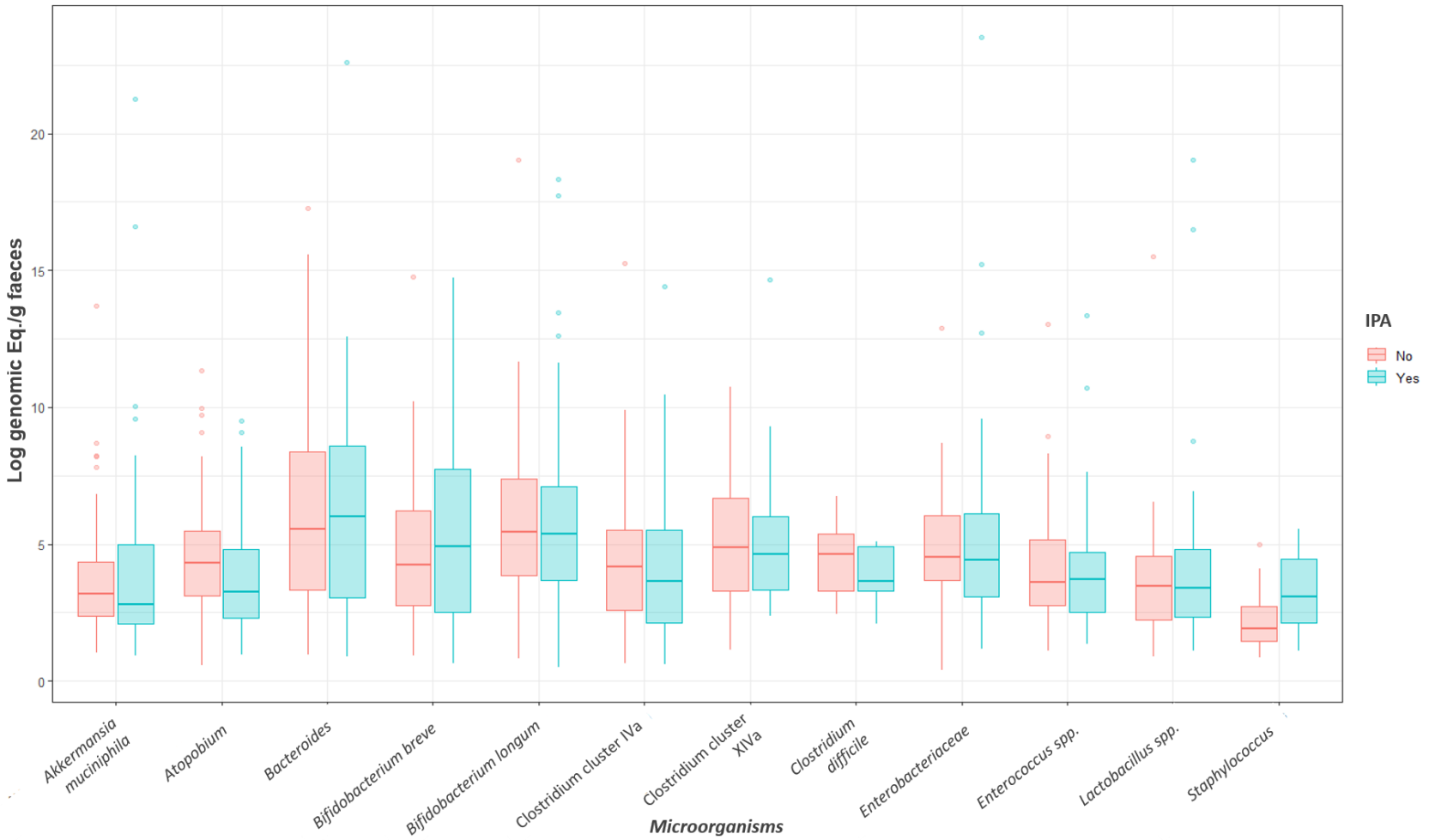


Figure 41. Gut microbiota profile of infants at 3 months of age, representing the counts of 12 microorganisms of interest and comparing between those whose mothers have been exposed to IPA during delivery (n=51) and those who have not (n=123).

Table 20. Faecal microbiota prevalence (qPCR) of infants at 3 months of age based on the use of intrapartum antibiotics by the mother (n=174).

	No IPA (n=123)		Yes IPA (n=51)		
Microorganisms	n	%	n	%	*P*
Bifidobacterium longum	115	93.50	45	88.24	0.393
Bifidobacterium breve	76	61.79	28	54.90	0.501
Staphylococcus aureus	23	18.70	10	19.61	1.000
Lactobacillus spp.	89	72.36	31	60.78	0.186
Akkermansia muciniphila	99	80.49	43	84.31	0.705
Enterobacteriaceae	115	93.50	47	92.16	1.000
Bacteroidetes	117	95.12	43	84.31	**0.038**
Enterococcus spp.	109	88.62	44	86.27	0.860
Clostridium cluster XIVa	66	53.66	26	50.98	0.877
Clostridium cluster IVa	73	59.35	37	72.55	0.141
Clostridium difficile	15	12.20	8	15.69	0.709
Atopobium	112	91.06	43	84.31	0.303

IPA: Intrapartum antibiotic
Bold values means "statistical significance" (P- value <0.05).
n: Number of samples with positive amplification. %: percentage of the number of positive samples with respect to the total samples in the category.
Chi-squared test was used for the statistical analysis.

Moreover, when the counts between INEIPA and IEIPA were compared within the control group or the case group, no significant differences were observed in the counts of any of the bacteria studied (**figure 42**).

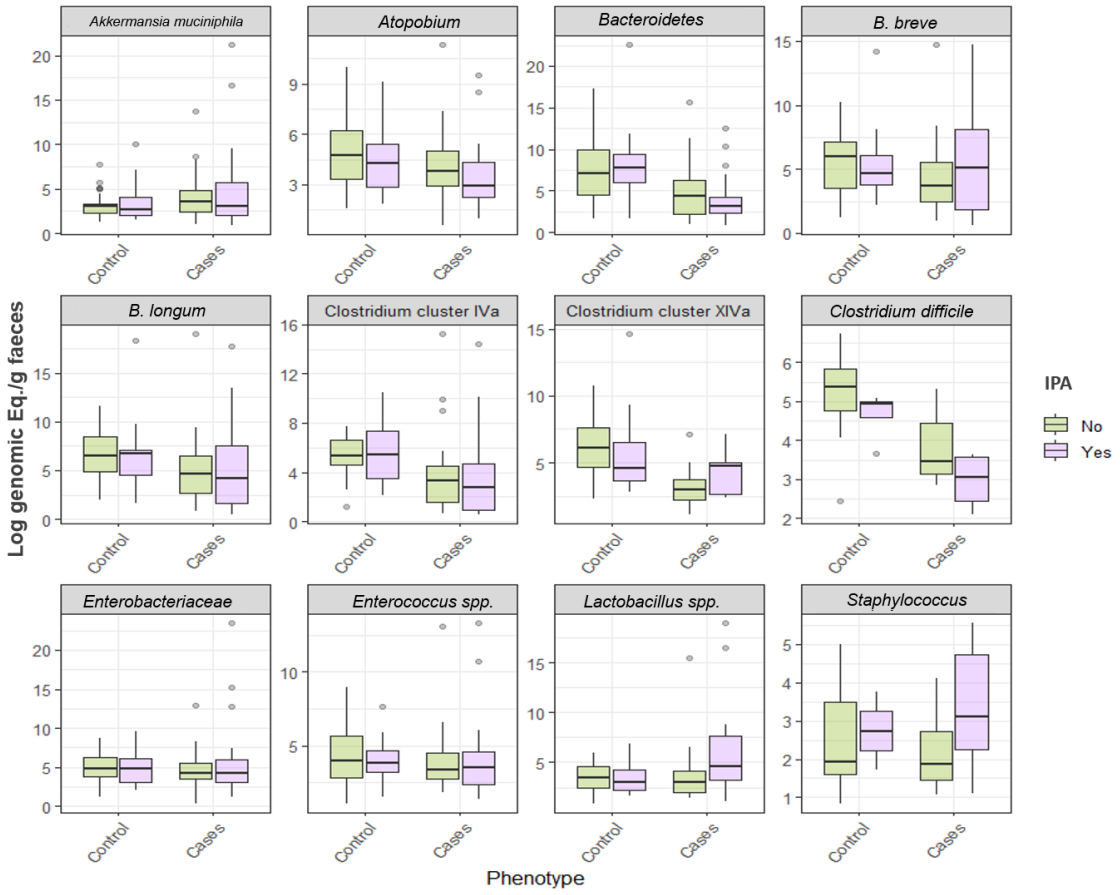


Figure 42. Gut microbiota profile of infants at 3 months of age, comparing within the control group or the case group between infant mothers have been exposed to IPA during delivery and those who have not.

The results obtained coincide with that described by other authors [71,89,357]. For example, Fallani *et al.,* [89] observed less proportion of *Atopobium* and *Bacteroides* in infants at 6 weeks of age when the mothers were exposed to IPA. Also, in a prospective longitudinal birth cohort of 83 mother-child pairs conducted by Steams *et al*., in 2017 [70] the authors studied the association of IPA on the infant's gut microbiome and observed that the gut microbiota of infants exposed to IPA differed from that of unexposed infants at 10 days and 6 weeks of age ($p < 0.05$), but these differences were not observed at 12 weeks. Regarding the time of exposure to antibiotics, in this same study the authors observed that for each hour of IPA administration during vaginal delivery, there was a 7.2% decrease in the abundance of *Bifidobacterium* and a positive effect on the abundance of *Clostridium* [70]. Aloisio *et al.,* (2016) [71] found a similar

pattern of lower abundance of *Actinobacteria* and *Bacteroidetes* as well as an overrepresentation of Proteobacteria in infants born to mothers who had received IPA and Nogacka *et al.,* (2017) [357], in a study evaluating the impact of IPA in a cohort of 40 full-term vaginally born infants, also observed the same results. Infants exposed to IPA had lower relative proportions of *Actinobacteria* and *Bacteroidetes* and increased levels of Proteobacteria and Firmicutes. Another study reported lower *Bifidobacteria* counts in infants exposed to IPA at 7 days of age, but this difference disappears when the infants reach 30 days of age. Nor did the authors observe differences at this age stage in the counts of *Lactobacilli* and *Bifidobacterium fragilis* [358]. On the contrary, Azad *et al.,* [67] found that, at 3 month of age, infants exposed to IPA differed significantly compared with infant no exposed to IPA, and these differences persisted to 12 months for infants delivered by emergency CS. Specifically, the genera *Bacteroides* and *Parabacteroides* were under-represented, and *Enterococcus* and *Clostridium were* over-represented at 3 months following maternal IPA. Some studies also point out that the differences observed in the microbial profile of infants exposed and not exposed to IPA were reduced when the infants were breastfed [67].

As we commented above, the genus *Atopobium*, and more specifically *Atopobium vaginae*, is a commensal bacterium present in the vaginal microbiota of women. Due to the use of IPA, this genus can be affected by reducing its presence in the vagina and therefore affecting the colonization of this bacterium in the offspring during vaginal delivery. Moreover, a higher *Staphylococcus* count in IEIPA may be due to the fact that the use of IPA is associated with caesarean deliveries where the offspring are exposed to bacteria from the environment and from attendants, including *S. aureus*. Another reason may be the decrease in the competing commensal flora due to the use of IPA, thus allowing the establishment and development of bacteria considered pathogenic, such as *S. aureus*.

IPA & Infant's SCFAs

Figure 43 shows the SCFAs profiles of infants at 3 months of age based on whether the mother received IPA during pregnancy or not without observing significant differences between the 2 groups.

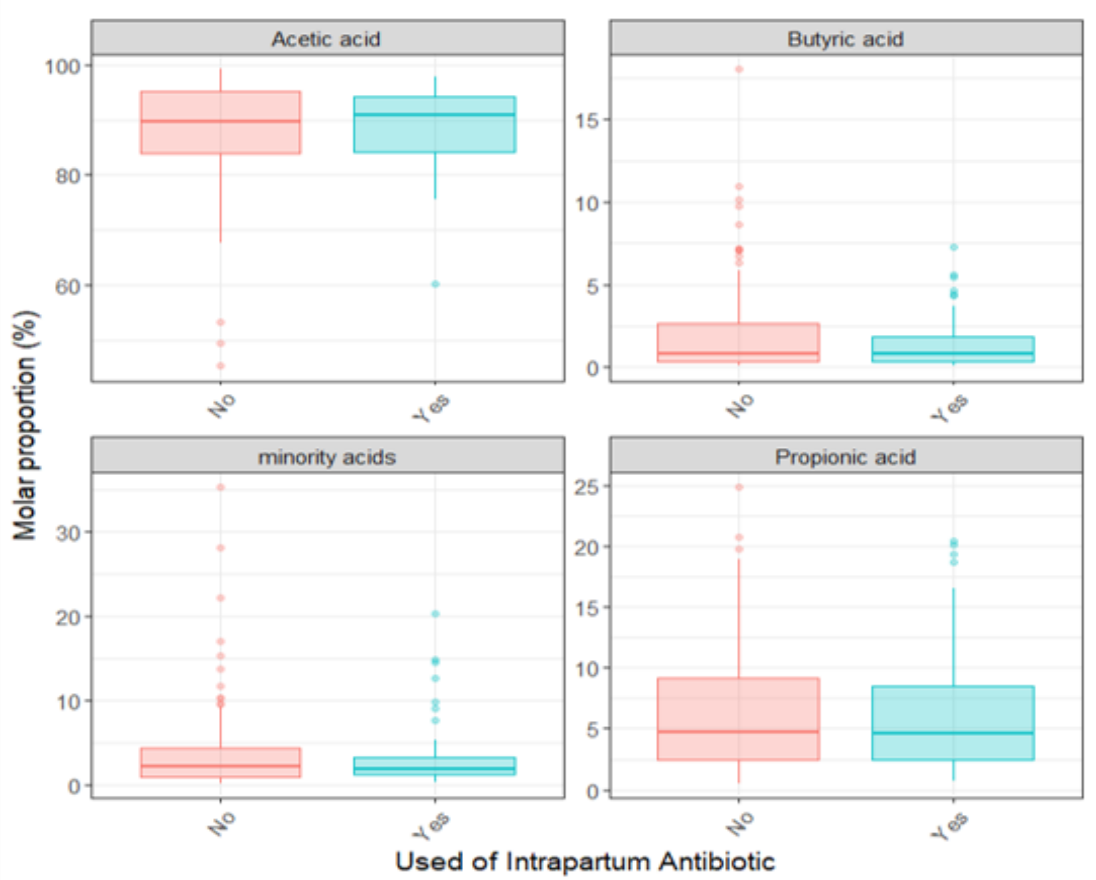


Figure 43. SCFAs profile of infants at 3 months of age, comparing between those whose mothers have been exposed to IPA during delivery (n=51) and those who have not (n=124).

An analysis of the SCFAs was also performed, comparing their molar proportion between the INEIPA and IEIPA within the control group and the case group independently (**figure 44**) and no significant differences were observed in any of the fatty acids studied.

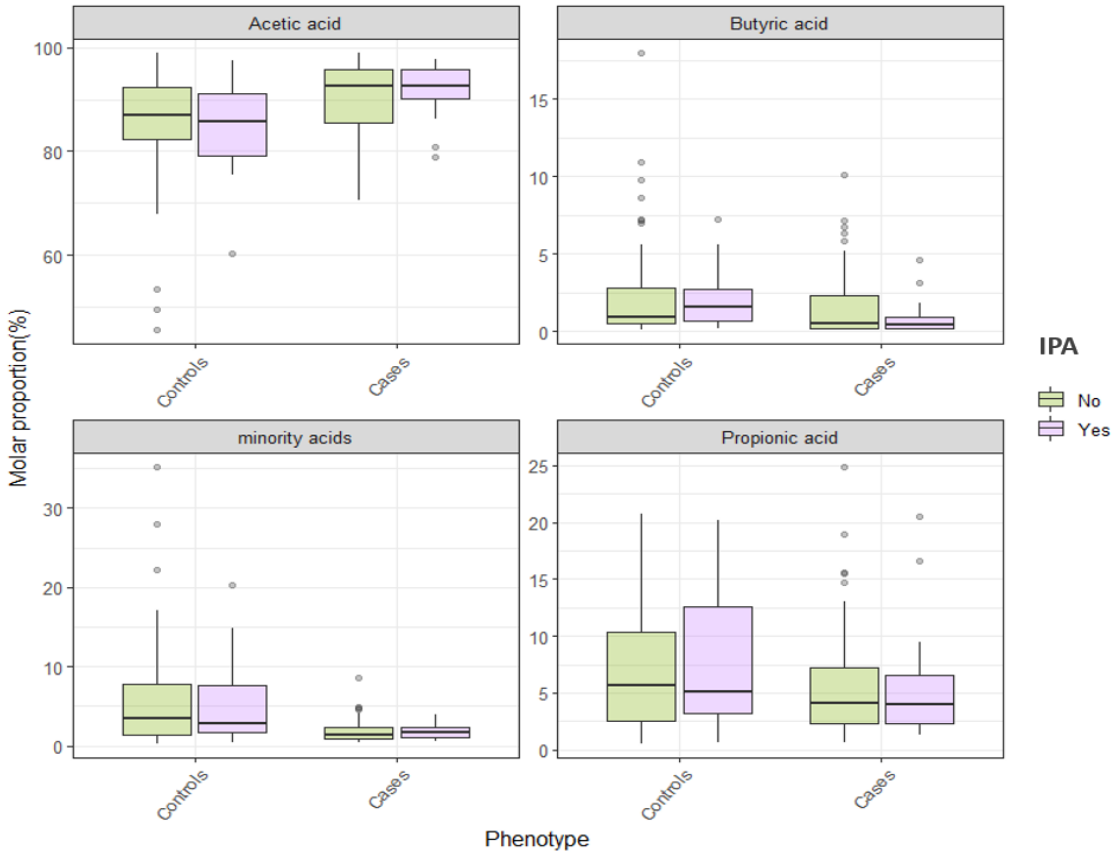


Figure 44. SCFAs profile of infants at 3 months of age, comparing within the control group or the case group between infant mothers have been exposed to IPA during delivery and those who have not.

Only the study carried out by Arboleya *et al.,* 2016 [359] investigates the SCFAs profile of infants as a function of IPA exposure. The authors concluded that at 30 days of age, infants not exposed to antibiotics at all showed a trend towards higher levels of acetic (p = 0.075) and total SCFAs (p = 0.060) than the newborns whose mothers received IPA. Also, the authors described that the high inter-individual variability on SCFAs levels is likely to have prevented the detection of any statistically significant difference. In our case, a high variability between individuals was also observed.

4.5.2 Mode of delivery

Mode of delivery & Infant's gut microbiota.

Figure 45 shows the median bacterial counts of infants who were born by vaginal delivery (IVD) and of infants who were born by caesarean section (ICSD). When bacterial counts were compared between both groups (IVD *vs.* ICSD),

significantly lower counts (Log genomic Eq. /g faeces) (median, (IQR)) were observed in the ICSD group for *Bacteroidetes* (7.70 (5.30-10.02) *vs.* 4.25 (3.87-4.79); p-value <0.01) and *B. longum* (6.65 (4.86-8.14) *vs.* 4.45 (2.98-6.47); p-value <0.01). Lower counts *of Atopobium* (4.53 (3.21-5.82) *vs.* 4.48 (3.06-4.58); p-value= 0.070) and *Clostridium cluster XIVa* (5.36 (4.20-7.43) *vs.* 4.63 (3.34-5.49); p-value = 0.089) were also observed in ICSD group but these results were not statistically significant. In the case of the prevalence percentage, no significant differences were observed between both groups (**data not show**).

When the effect of the mode of delivery on the gut microbiota of infants was analysed, taking into account the phenotype of infants (controls or cases) (**figure 46**), only higher counts of *Bacteroidetes* (7.70 (5.30-10.02) *vs.* 4.25 (3.87-4.78); p-value <0.01) and *B. longum* (6.65 (4.86-8.14) *vs.* 4.45 (2.98-6.47); p-value <0.01) were observed in the IVD compared with ICSD when they also belonged to the control group. No statistically significant differences were observed in any of bacterial counts of IVD *vs.* ICSD when all infants belonged to the case group.

With regard to determining the impact of the mode of delivery on the composition of the infant's gut microbiota, researchers have conducted numerous investigations on the diversity of the gut microbiota of infants from birth to 7 years of age, and this factor has been demonstrated to have a strong influence on early gut colonization [92]. Specifically, several researchers agree that infants born by CS delivery do not have direct exposure to the faecal and vaginal microbiota of the mother, and as a result, a lower total microbial diversity has been observed [86,93], as well as a lower abundance and diversity of *Bacteroides* and *Bifidobacterium* [21,66,86–89,125,208,360]. Our results referring to a lower colonization of *Bacteroides* and *Bifidobacterium* are similar to that observed in these studies.

Also, ICSD have also been found to have less *E. coli* colonization and a greater presence of *Clostridium cluster I* and *C. difficile* [87–89,93,361]. In contrast, the microbiota of IVD had a higher diversity and prevalence of vaginal-related microbes such as *Lactobacillus*, *Prevotella*, *Escherichia*, *Bacteroides, Bifidobacterium* and *Sneathia* [21,87–89,208].

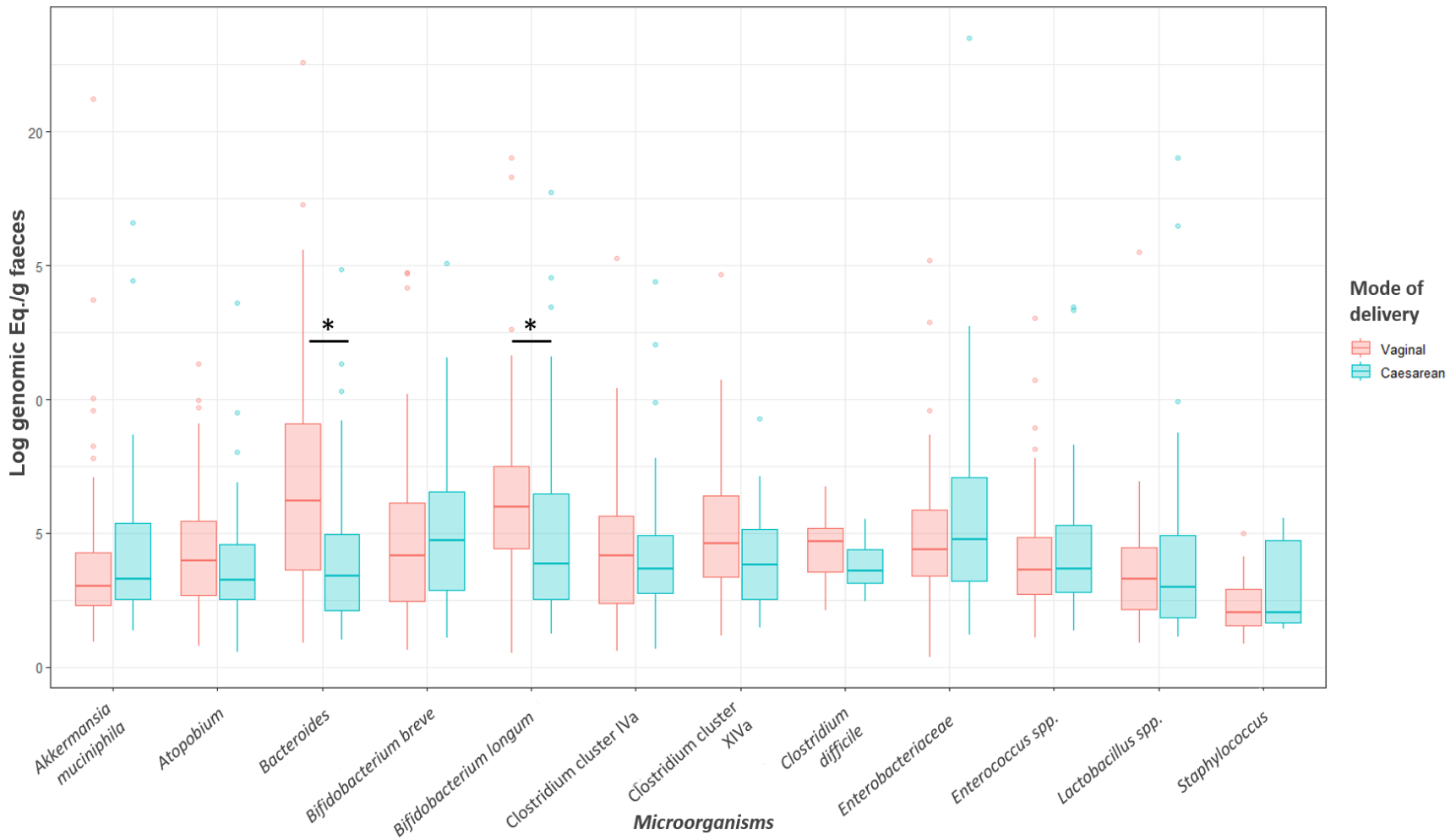


Figure 45. Gut microbiota profile of infants at 3 months of age, representing the counts of 12 microorganisms of interest and comparing between those who were born by vaginal delivery (n = 151) and those who were born by caesarean delivery (n = 45).

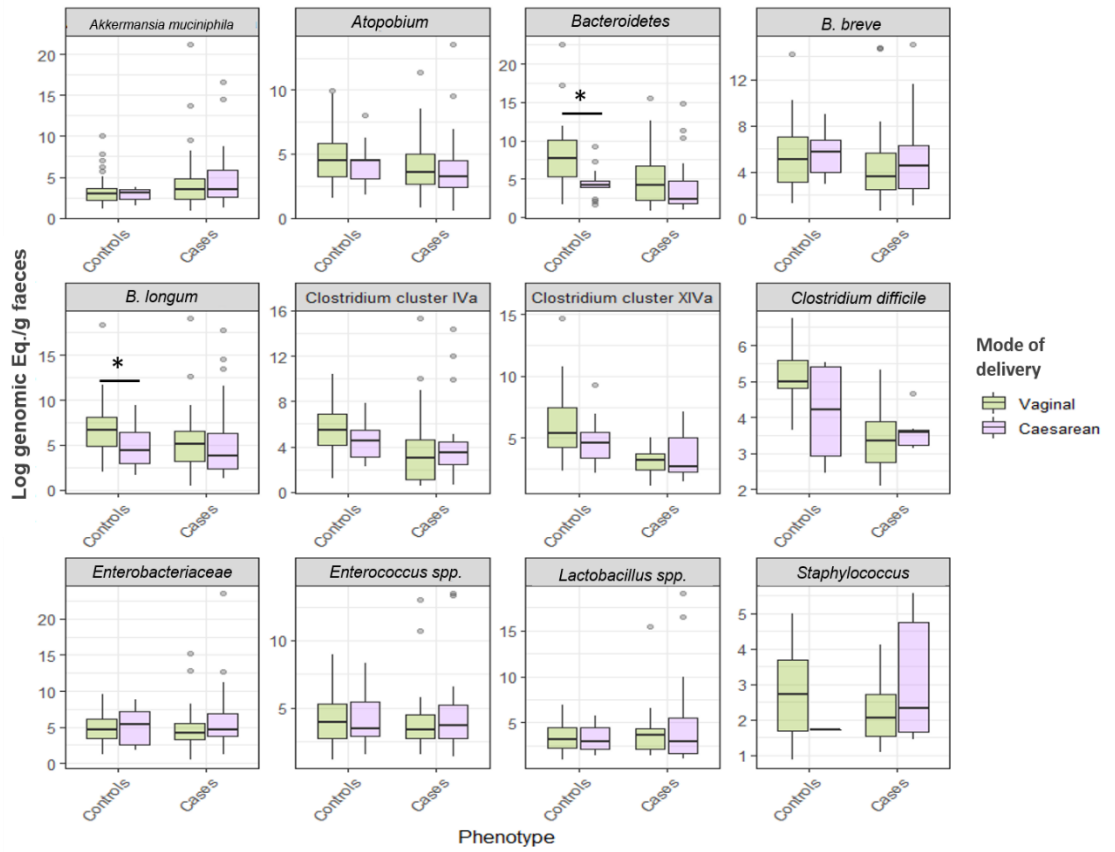


Figure 46. Gut microbiota profile of infants at 3 months of age, comparing within the control group or the case group between infants born by vaginal delivery and those who were born by caesarean delivery.

It should be noted that differences in colonization patterns are only observed in control infants and that these differences disappear when infants belong to the case group. This allows us to think that the microbiota of the cases, regardless of the type of delivery, has already been affected by other factors, probably prenatal factors, or that for some reason. Finally, as Kumbahare *et al., (2019)* [55] indicated in their review, the mode of delivery and type of lactation have combined effects on the infant's gut microbiome specifically over *Bifidobacterium* species since breastmilk contain several stimulating factors that help to develop their growth.

Mode of delivery & Infant's SCFAs

Regarding the analysis of SCFAs according to the type of delivery, no significant differences were observed between the molar proportions of any of the fatty acids when IVD and ICSD were compared (**figure 47**). Neither significant differences were observed between the molar proportions of the IVD and ICSD when the infants of both groups belonged to the control category (**figure 48**).

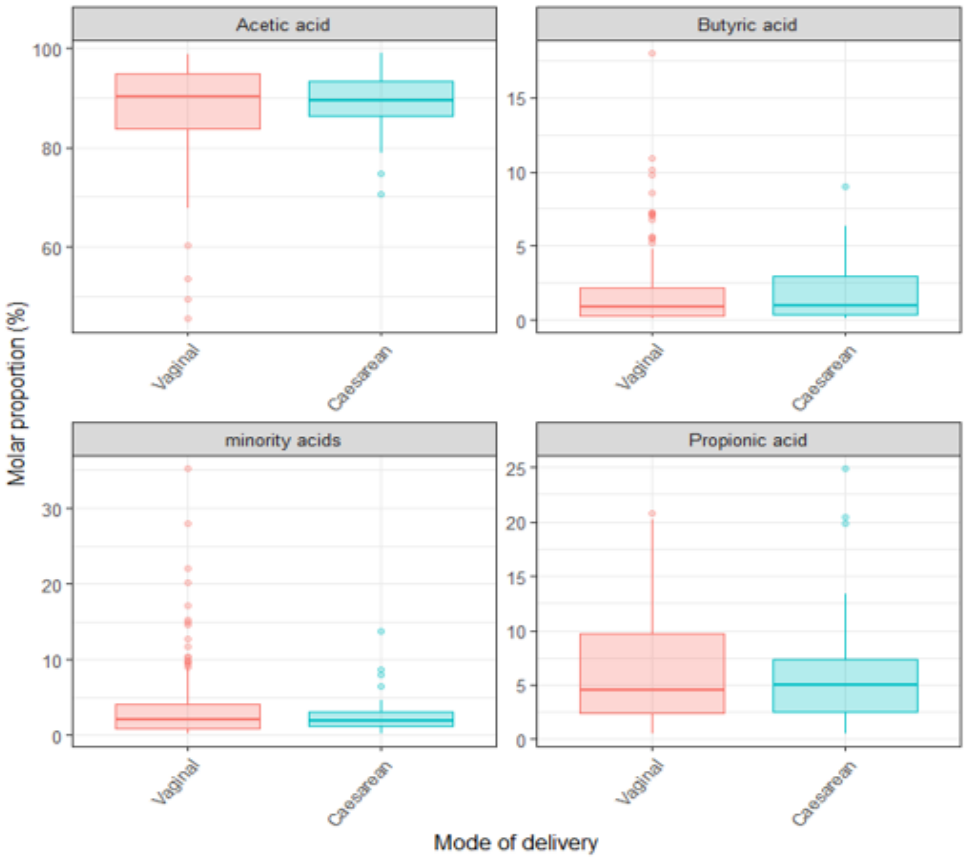


Figure 47. SCFAs profile of infants at 3 months of age, comparing between those who were born by vaginal delivery (n = 152) and those who were born by caesarean delivery (n = 45).

Significant differences were only observed in the molar proportions of minority fatty acids between IVD and ICSD (1.20 % (0.86-2.18) *vs.* 1.94 % (1.28-2.96); p-value <0.05) when all of them belonged to the group of cases.

The results observed in our study do not agree with that observed by Mueller *et al.,* in 2021 [360]. The authors noted that ICSD had higher faecal butyrate concentration at 3 months. These differences, as has been commented on other occasions, may be due to the influence of the type of lactation and also to the great interindividual difference.

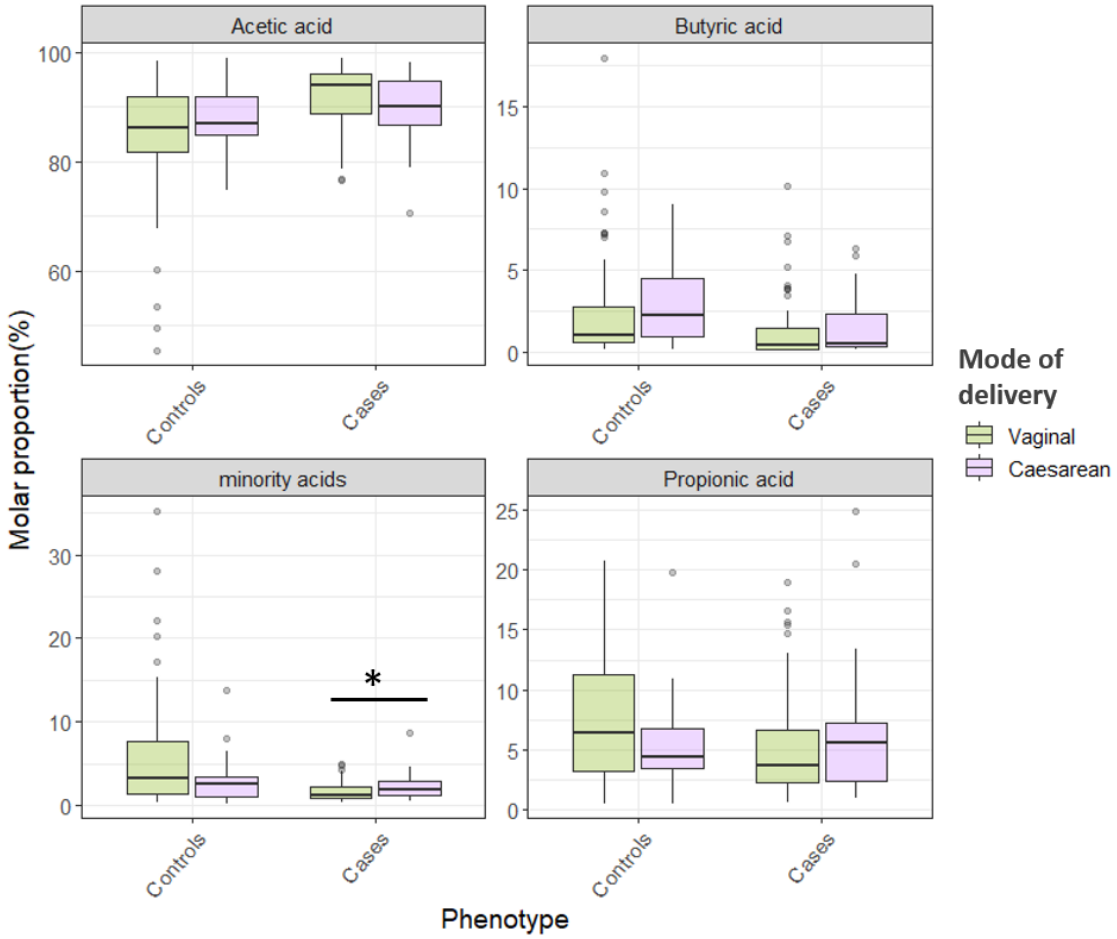


Figure 48. SCFAs profile of infants at 3 months of age, comparing within the control group or the case group between those who were born by vaginal delivery and those who were born by caesarean delivery.

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4.5.3 Weeks of gestation: Full term infants (FTI) vs. preterm infants (PTI).

Weeks of gestation & Infant's gut microbiota.

When the bacterial counts of full-term infants (≥ 37 weeks of gestation) (FTI) were compared with counts of preterm infants (<37 weeks of gestation (PTI), higher counts were generally observed in most of the bacteria quantified in the first group, but only the colonisation of *Atopobium (*Log genomic Eq./g faeces) (median (IQR)) (3.96 (2.66-5.37) *vs.* 2.89 (2.65-3.14); p-value <0.01) resulted significantly higher (**figure 49**). Nor were significant differences found in the prevalence of each of the bacteria comparing FTI *vs.* PTI.

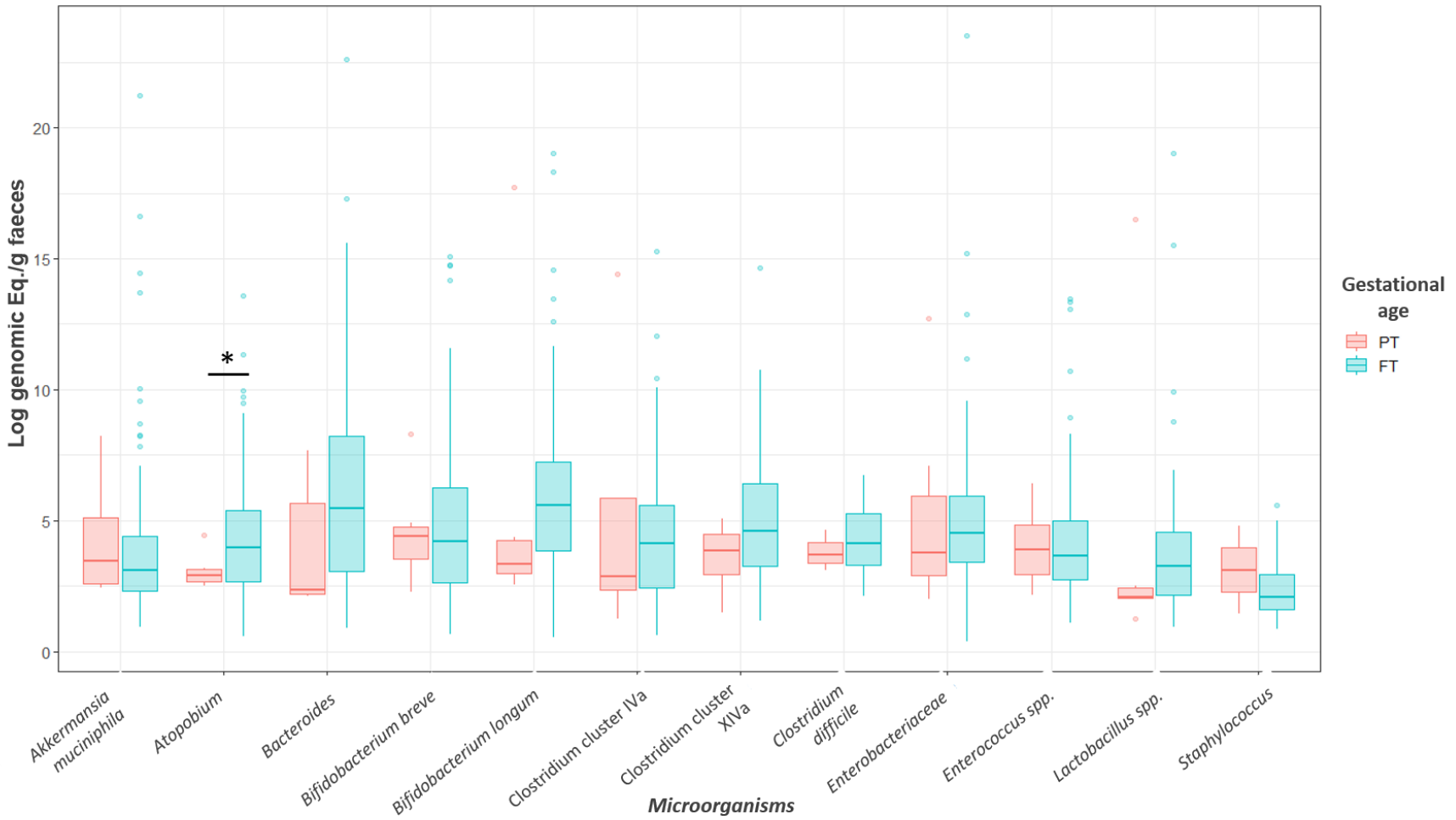

Figure 49. Gut microbiota profile of infants at 3 months of age, representing the counts of 12 microorganisms of interest and comparing between premature (PTI) (n=8) and full-term infants (FTI) (n=188).

Preterm delivery is often associated with both CS and antibiotic usage. Independent of these confounders, the pattern of gut microbial colonisation in PTI varies when compared with the pattern of a healthy, breastfeed FTI [34,85,87]. Moles *et al.,* [16] showed that the microbiota present in meconium of PTI was distinct to that of FTI. The main patterns of gut microbial colonisation were higher counts of *Enterococcus* together with Proteobacteria, and lower levels of Bifidobacteria, compared to that expected for children delivered at term. Also, several authors observed that in PTI (< 33 weeks), the microbiota is characterized by reduced bacterial diversity [16,84,362] and higher levels of potentially pathogenic bacteria and lower numbers of *Bifidobacterium*, *Bacteroides* and *Atopobium* compared with breastfeed full-term infants [362,363]. These results coincided with that observed by Barret *et al.,* in 2012 [364]. Using 16s sequencing techniques, these authors studied the gut microbiota composition of 20 faecal samples collected from 10 CS delivered PTI (<33 weeks) and observed that PTI were characterised by a lack of detectable *Bifidobacterium* and *Lactobacillus* genera commonly associated with the healthy infant gut. At the same time the authors observed a large interindividual variation in the faecal microbiota diversity of PTI, suggesting that the preterm microbiota is individual-specific and does not display a uniformity among infants [364]. Both the factor type of lactation, the mode of delivery and the interindividual variety could explain the differences in the observed results. Low *Atopobium* counts in preterm infants may be due to an association with cesarean delivery, as this bacterium is typical of the mother's vaginal microbiota.

With the intention of dilucidate how gestational age can influence the gut microbiota of infants taking into account their phenotype (cases or controls), we intended to make a comparison of the counts between PTI and FTI for the same category controls or cases but this analysis could not be carried out due to the small sample size.

Weeks of gestation & Infant's SCFAs

Regarding SCFAs, no statistically significant differences were observed between FTI and PTI with respect to their molar proportions (**figure 50**).

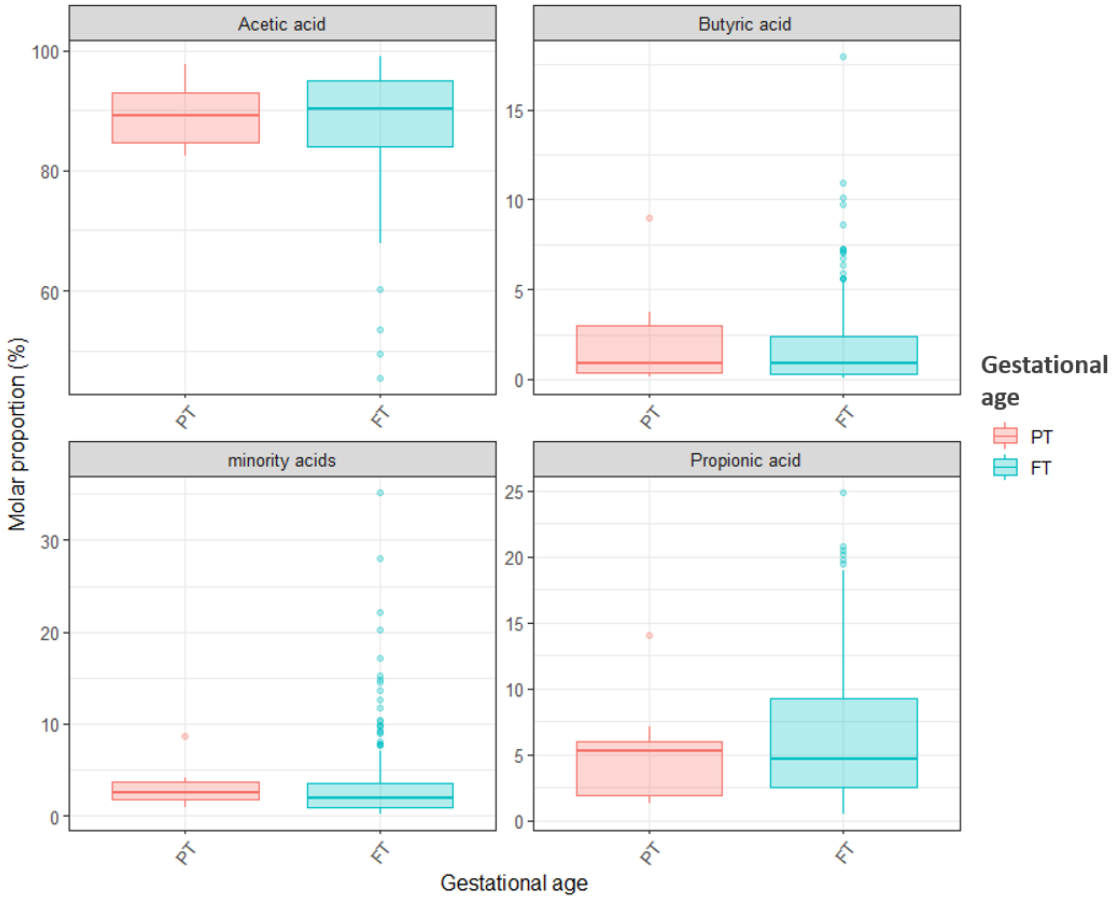

Figure 50. SCFAs profile of infants at 3 months of age and comparing between premature infant (PTI) (n=8) and full-term infants (FTI) (n=189).

Only one study has been found in which SCFAs are compared between preterm *vs.* term infants. Tauchi *et al.,* in 2019 [365] using 16s sequencing techniques, observed that PTI showed a lower acetate concentration comparing with FTI. It should be taken into account that this study examined SCFAs at 5 days of life and in our case, they were studied at 3 months of age, which may be the reason for the differences observed in the results. On the other hand, since no significant differences were observed in most of the bacterial groups and species studied between PTI *vs.* FTI, differences in their metabolites were not expected either.

4.6 Postnatal factors: their effect on the gut microbiota and SCFAs profile of the infant

4.6.1 Mode of lactation.

Mode of lactation & Infant's gut microbiota

In the study of how the type of lactation modulates the gut microbiota of infants, bacterial counts were compared among infants who had been exclusively breastfed (BFI), those who had been fed with mixed feeding (MXI) and those who had only been fed infant formula (IFI) (**figure 51**). In this comparison, higher bacterial counts (Log genomic Eq. /g faeces) (median(IQR)) were generally observed in the BFI compared to the other two groups, but only with statistical differences in *B. breve* (5.53 (3.56-7.14) *vs.* 3.04 (2.37-4.47) and 3.67 (2.22-5.40); p-value <0.01; (BFI *vs.* IFI and MXI, respectively)), *B. longum* (6.45 (4.35-8.14) *vs.* 4.90 (3.52-6.64); p-value <0.05; (BFI *vs.* MXI)), *Clostridium cluster XIVa* (5.06 (3.73-7.16) *vs.* 3.88 (2.80-4.88); p-value <0.05; (BFI *vs.* IFBI)) and *Lactobacillus spp*. (3.75 (2.45-4.88) *vs.* 2.64 (2.02-4.27) and 2.96 (2.03-3.27); p-value <0.05; (BFI *vs.* MXI and IFI respectively). On the other hand, comparing the prevalence of the quantified microorganisms for each of the 3 groups, only a significant higher prevalence of *Staphylococcus* was observed in BFI compared to MXI or IFI (BFI: 25 % *vs.* MXI: 11.90 % and IFI: 10.00 %; (p-value < 0.05)) (**data not shown**).

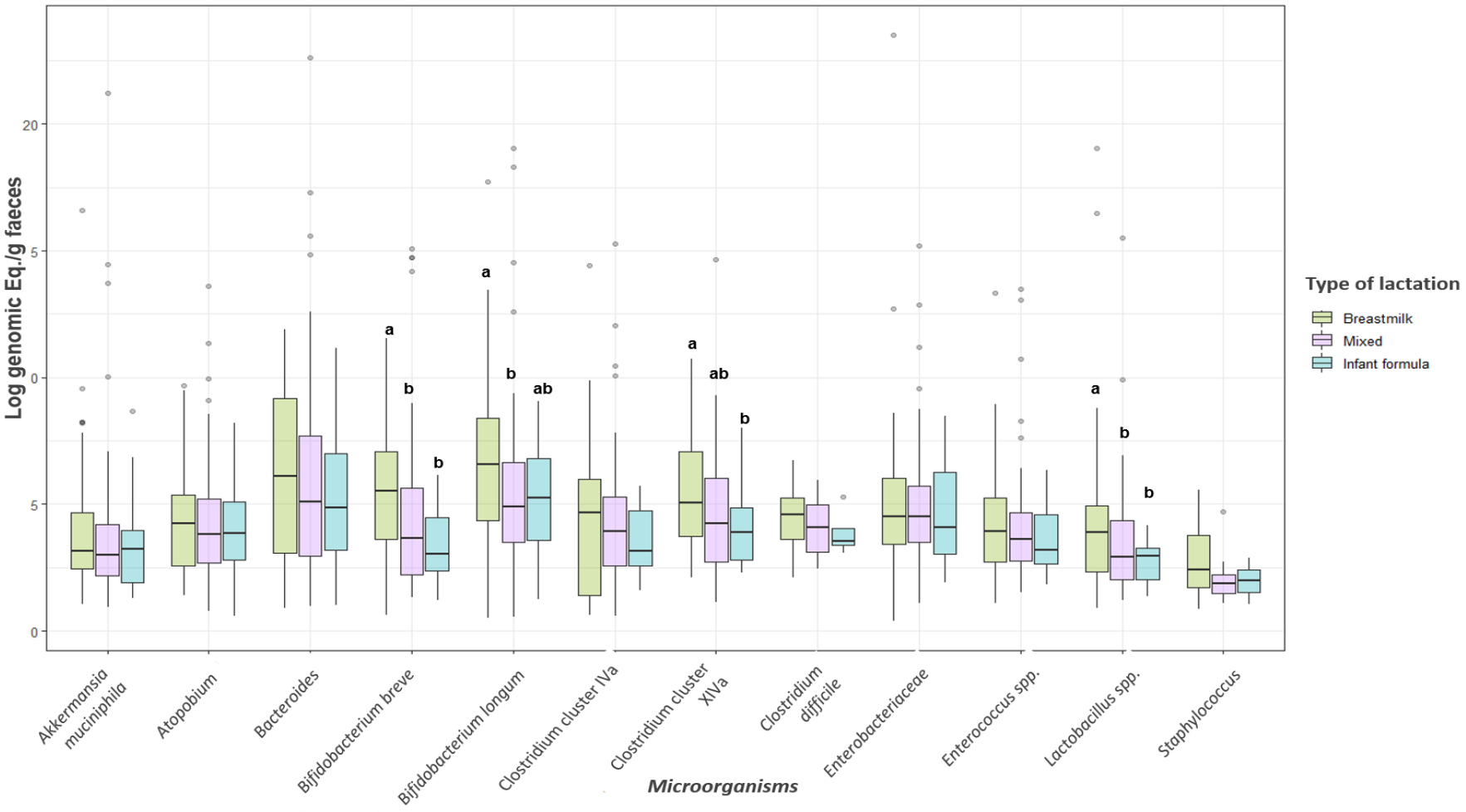

Figure 51. Gut microbiota profile of infants at 3 months of age, representing the counts of 12 microorganisms of interest and comparing among breastfed infants (n=92), mixed-lactation infants (n=84), or exclusively infant formula-fed infants (n=20).

When these groups were subdivided taking into account the phenotype assigned to the infants (cases or controls), the data represented in **figure 52** were obtained.

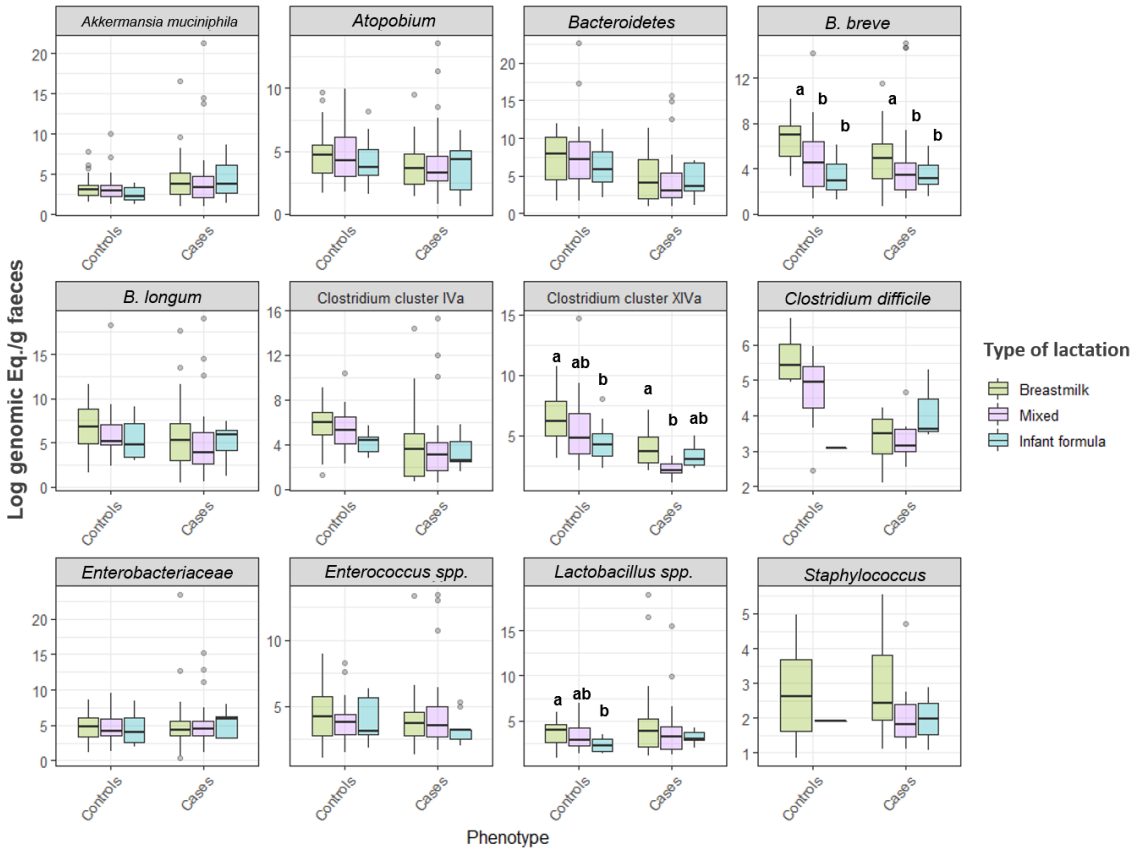

Figure 52. Gut microbiota profile of infants at 3 months of age, comparing within the control group or the case group among breastfed infants), mixed-lactation infants, or exclusively infant formula-fed infants.

Higher counts of some bacteria were observed in the BFI group compared with the other two groups (MXI and IFI) when infants were subdivided into control or cases group. Specifically, higher counts of *B. breve* (controls: BFI: 6.95 (5.12-7.59); MXI: 4.57 (2.24-6.31); IFI: 2.97 (2.12-4.47); p-value <0.01; cases: BFI: 4.94 (2.78-6.23); MXI: 3.41 (2.14-4.51); IFI: 3.12 (2.66-4.30); p-value <0.01) and *Clostridium cluster XIVa* (controls: BFI: 6.28 (4.85-8.01); MXI: 4.69 (3.41-6.49); IFI: 4.28 (3.34-5.19); p-value <0.01; cases: BFI: 3.70 (2.54-4.88); MXI: 2.40 (2.00-2.75); IFI: 3.09 (2.55-3.90); p-value <0.05). It was also observed that the counts of both *B. breve* and *Clostridium cluster XIVa* were significantly higher in the BFI control group compared to the BFI cases. Only significantly higher counts were

observed for *Lactobacillus spp.* in the BFI control group compared to the other two types of lactation (BFI: 3.75 (2.79-4.60); MXI: 2.56 (2.14-4.22); IFI: 2.22 (1.60-2.97); p-value <0.01).

It has been stated that breastfeeding confers a “beneficial” effect to infant gut microbiota which has been attributed firstly, to the prebiotic properties of HMOs, since these milk compounds stimulate the growth of specific bacterial group such as *Bifidobacteria* [53,366] and *Staphylococcus* [367], or, secondly, to the transfer of diverse microbes from mother’s gut to the infant´s (entero-mammary pathway) through breast milk [53,147]. Exclusively breast feed infants possess a microbiota characterised by a low microbial diversity being *Bifidobacteria* the predominant group, which represents up to 70-80 % of the total bacterial counts in the infant’s faeces [368]. Various authors have reported an increased colonization by *Bifidobacteria* and reduced abundance of *C. difficile* in breast-fed infants compared to formula-fed infants [55,87,89,106]. Furthermore, it has also been reported that the most prevalent *Bifidobacterium* species present in BFI are *B. breve, B. longum, B. dentium, B. infantis* and *B. pseudocatenulatum* [23,112,215]. These results coincide with that observed in our analyses where the BFI had higher counts of *B. breve, B. longum* without distinction between cases and controls.

Fallani *et al.,* [89] observed that the microbiota of formula-fed infants had significantly higher proportions of *Bacteroides* and members of the *Clostridium coccoides (Clostridium* cluster XIVa*)* and *Lactobacillus* groups. These results coincide with our results with respect to *Lactobacillus spp.* and *Clostridium* cluster XIVa but not regarding to *Bacteroides*, since we have observed a higher prevalence of these two bacteria in IFI, although without statistically differences. Finally, as we commented above, breast milk has its own microbiota, which mainly includes *Streptococcus* and *Staphylococcus* species followed by other bacteria [367], being one of the first colonizers of the infant gut [17]. Moreover, bacterial transfer from the mother’s skin during suckling is essentially unavoidable and another source of bacteria such as *Staphylococcus*. All of this may justify the high prevalence of *Staphylococcus* observed in the BFI (25 %) in comparison with the other two groups (MXI: 11.9 % and IFI: 10 %).

On the other hand, formula feeding has been associated with an increased microbial richness in infants at 4 months of age, with overrepresentation of *C. difficile*, a known gut pathogen [88,173,369]. In this sense, our results do not coincide with what is described in the bibliography, since no significant differences have been observed between the 3 types of lactation with respect to these bacterial counts. A certain trend towards a higher prevalence of *C. difficile* has been observed in the IFI group (BFI: 12 %, MXI: 16.7 % and IFI: 20 %), but this was not statistically significant.

With respect to the microbial profile of infants based on the allergy history of the mothers, Grönlund *et al.*, in 2007 [370] studied the microbiota in breast milk of allergic mothers *vs.* nonallergic their infants. The authors reported significantly lower amounts of *Bifidobacteria* in breast milk of allergic mothers compared with nonallergic mothers. These lower counts also influenced the gut microbiota of their offspring, with lower faecal *Bifidobacteria* counts simultaneously observed in infants of allergic mothers.

In our study, significantly higher counts of *B. breve* and *B. longum* were also observed in the control group compared to cases, both exclusively breastfed. None of the mothers belonging to the group of control infants had a history of asthma or atopy, whereas 13.54 % and 9.38 % of the mothers in the case group had a history of asthma and atopy respectively. This may account for the observed low counts of *B. breve* in that group. This is important, as we discussed above, because *Bifidobacteria* influences early immune development (including IgA production and cytokine responses) [335,336] and, consequently, an insufficient exposure to this bacteria by non-breastfed infants may develop an incorrect immune response to other microbial exposures in the future, leading to atopic disorders, including asthma. It is important to note that over time infant formulas have evolved, being modified and fortified over time. One of these changes is the supplementation with fibres with a prebiotic effect, such as galacto-oligosaccharides (GOS) and fructo-oligosaccharides (FOS) [215], which seem to induce the growth of the faecal microbiota, especially of *Bifidobacteria* species, and helping to make the gut microbiota of IFI very similar to the microbiota of BFI. On the other hand, this evolution in infant formulas and the

wide range that exists in the market can lead to obtain different results when comparing different studies.

Mode of lactation & Infant's SCFAs

Regarding the analysis of SCFAs according to the type of lactation, significant differences were observed among the molar proportions of the main short chain fatty acids when BFI, MXI and IFI were compared (**figure 53**).

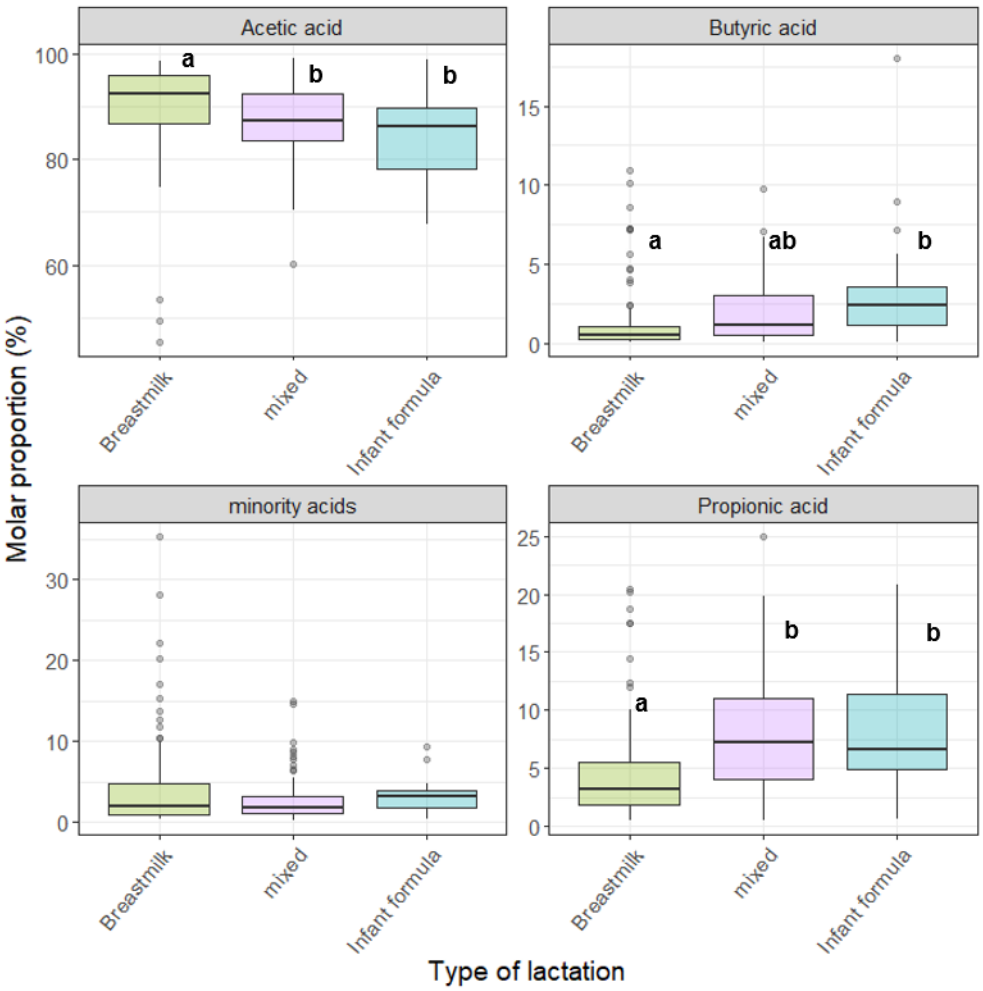

Figure 53. SCFAs profile of infants at 3 months of age, comparing among breastfed infants (n=93), mixed-lactation infants (n=84), or exclusively infant formula-fed infants (n=20).

Significantly higher molar proportions of acetic acid (and lower proportions of propionic and butyric were observed in the BFI group compared to the MXI and IFI groups.

Significant differences were observed among the molar proportions of the BFI, MXI and IFI when the infants of 3 groups belonged to the control or case category (**figure 54**).

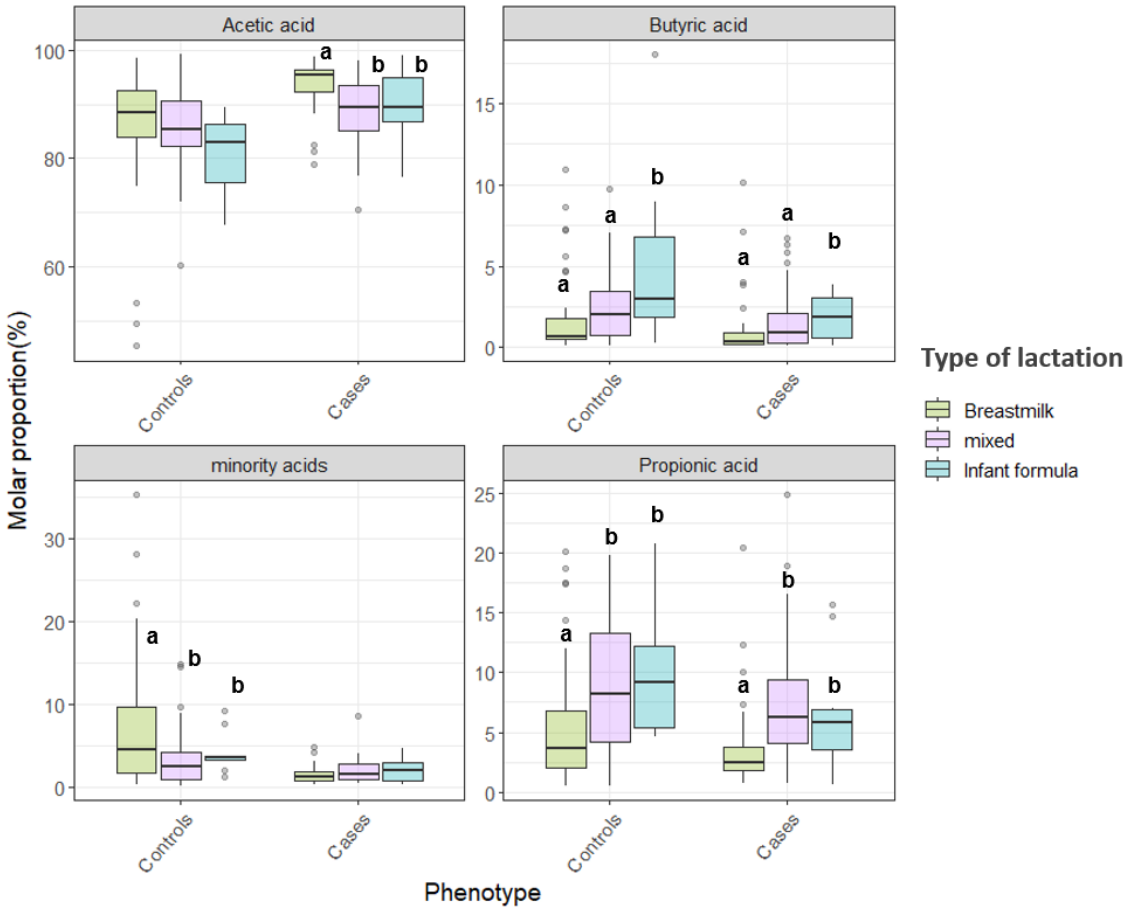

Figure 54. SCFAs profile of infants at 3 months of age, comparing within the control group or the case group among breastfed infants), mixed-lactation infants, or exclusively infant formula-fed infants.

Within the control group, breastfed infants had a lower molar proportion (%) of butyric (BFI: 0.69 % (0.50-1.77) *vs.* IFI: 2.99 % (1.89-6.78); p-value <0.01) and propionic acid (BFI: 3.60 % (2.04-6.83) *vs.* IFI: 9.14 % (5.41-12.25); p-value <0.01) compared to infants fed infant formula. On the other hand, within the case group, infants breastfed had higher molar proportions of acetic acid (BFI: 95.42 (92.33-96.42) *vs.* IFI: 89.53 (86.79-94.98); p-value <0.01) and minority acid (BFI: 4.51 (1.78-9.71) *vs.* IFI: 3.59 (3.36-3.80); p-value <0.05), and lower molar ratios of butyric (BFI: 0.31 (0.17-0.88) *vs.* IFI: 1.87 (0.59-3.03); p-value <0.01) and

propionic acid (BFI: 2.41 (1.78-3.78) *vs.* IFI: 5.81 (3.56-6.88); p-value <0.01) compared to infants fed with infant formula.

In our study total concentration of SCFAs (µM /g wet faeces) (median (IQR)) from BFI group was compared with IFI, and BFI had lower absolute concentrations of total faecal SCFAs (IBF: 85.36 (66.69-125.49) *vs.* IFI: 129.34 (106.64-173.57); p<0.001) (**data not shown)**. These results were similar to other studies [115,371] and potentially may be due to the less diverse microbiota or to a higher production of lactic acid as a secondary metabolite because as Edwards *et al.,* [372] reported, with respect to total faecal SCFAs concentrations, when lactic acid was included, there was no significant difference between breast-fed and formula-fed infants.

Regarding the molar proportions of the main SCFAs, our results coincide with what was observed by Bridgam *et al.*, in 2017 [115] and Durrani *et al.,* in 2020 [373]. Both of them found that BFI, at 3-5 months of age or 1-4 months of age respectively, had significantly higher relative proportions of acetic acid and lower proportions of butyric and propionic acid compared to those who had never been breasted. Bridgam *et al.*, [115] also concluded that infant exclusively breastfed were four times more likely (OR: 4.50; 95 % CI 158-12.82) to have a higher proportion of acetate relative to other SCFAs in their gut and showed that the association was independent of mode of delivery, IPA, infant sex, age, and maternal BMI.

4.7 Multivariate analysis of the effect of prenatal, perinatal and postnatal factors on the gut microbiota and SCFAs profile of infants using Factor Analysis of Mix Data (FAMD).

4.7.1 Multivariate study of changes in the infant's gut microbiota, based on prenatal, perinatal and postnatal factors.

In **figure 55**, referring to factor analysis, when we examined the individuals being adjusting the model according to the gut microbiota of infants parental clinical history, type of delivery, gestational age, lactation, use of antibiotics during the third trimester of pregnancy period, maternal BMI and infant sex, and differentiated them between cases and controls, (**figure 55A)**, two distinct groups

were observed being the individuals belonging to the control group more concentrated and closer together in the graphical representation.

In contrast, no statistically different groups were observed in the multivariate analysis when the individuals were grouped based on other factors such as type of delivery (**figure 55D)**, type of breastfeeding (**figure 55E)**, exposure to antibiotics during pregnancy (**figure 55C)** or IPA (**figure 55B)**.

Figure 56 shows the graphical representations of the analysis of changes in the gut microbiota of infants according to the history of asthma (**figure 56A** and **56C**) or atopy (**figure 56B** and **56D**) of the mother and father respectively. No statistically significant differences were observed in this study, but there was observed a trend towards a more stable or similar microbiota in infants whose mothers did not have a history of asthma or in infants whose fathers did not have a history of atopy compared to those whose parents did. This analysis needs to be approached with caution and only as a guideline as the number of infants whose mothers and fathers respectively have a history of asthma (n=13/9) or atopy (n=7/9) is very low.

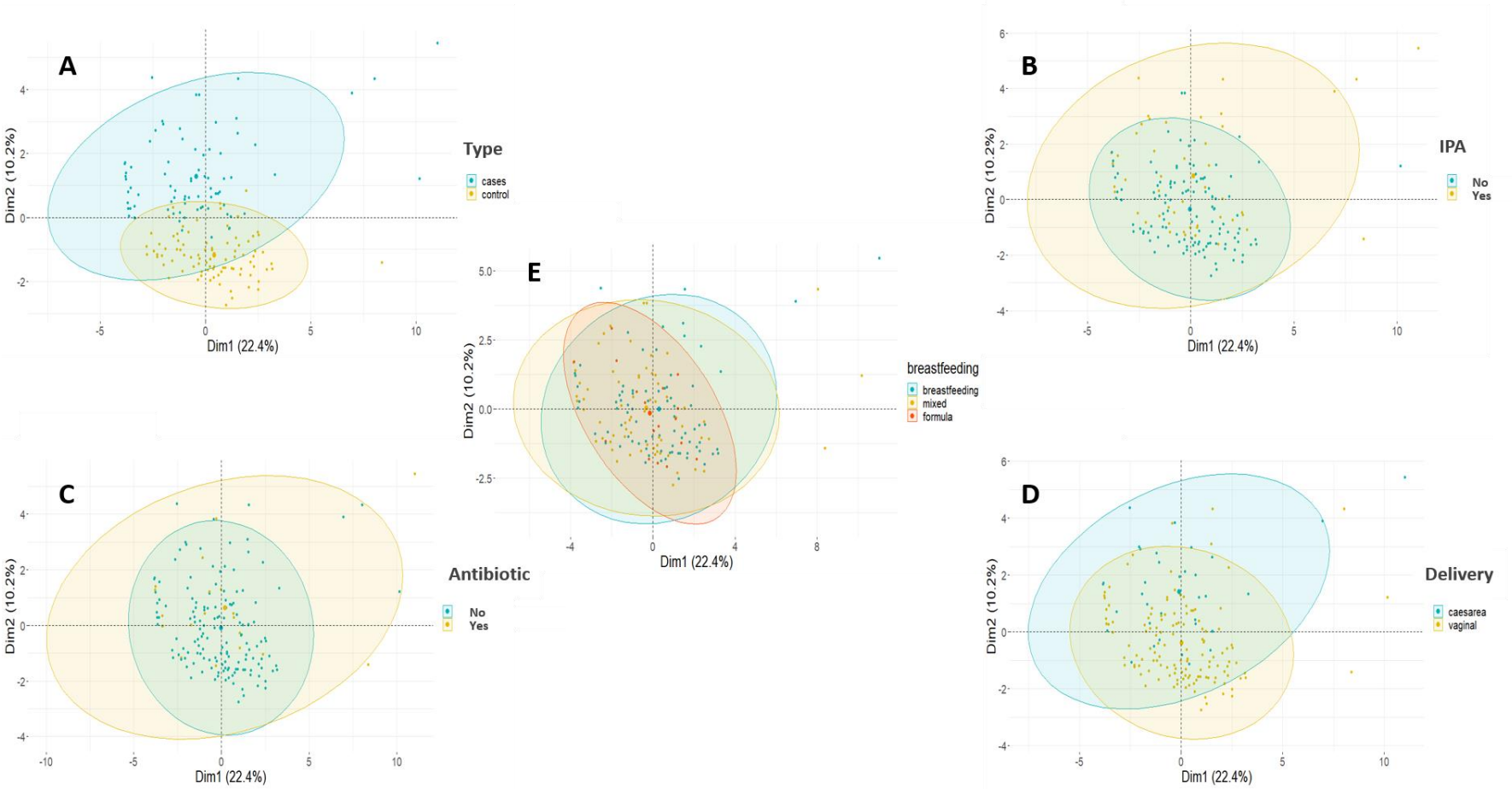

Figure 55. Factor Analysis of Mix Data (FAMD) of the gut microbiota of samples to study the similarity between the individuals taking into account all the factors study **A)** phenotype; **B)** use of IPA; **C)** antibiotic exposition during pregnancy; **D)** mode of delivery and **E)** type of lactation. Significance was calculated using PerMANOVA test.

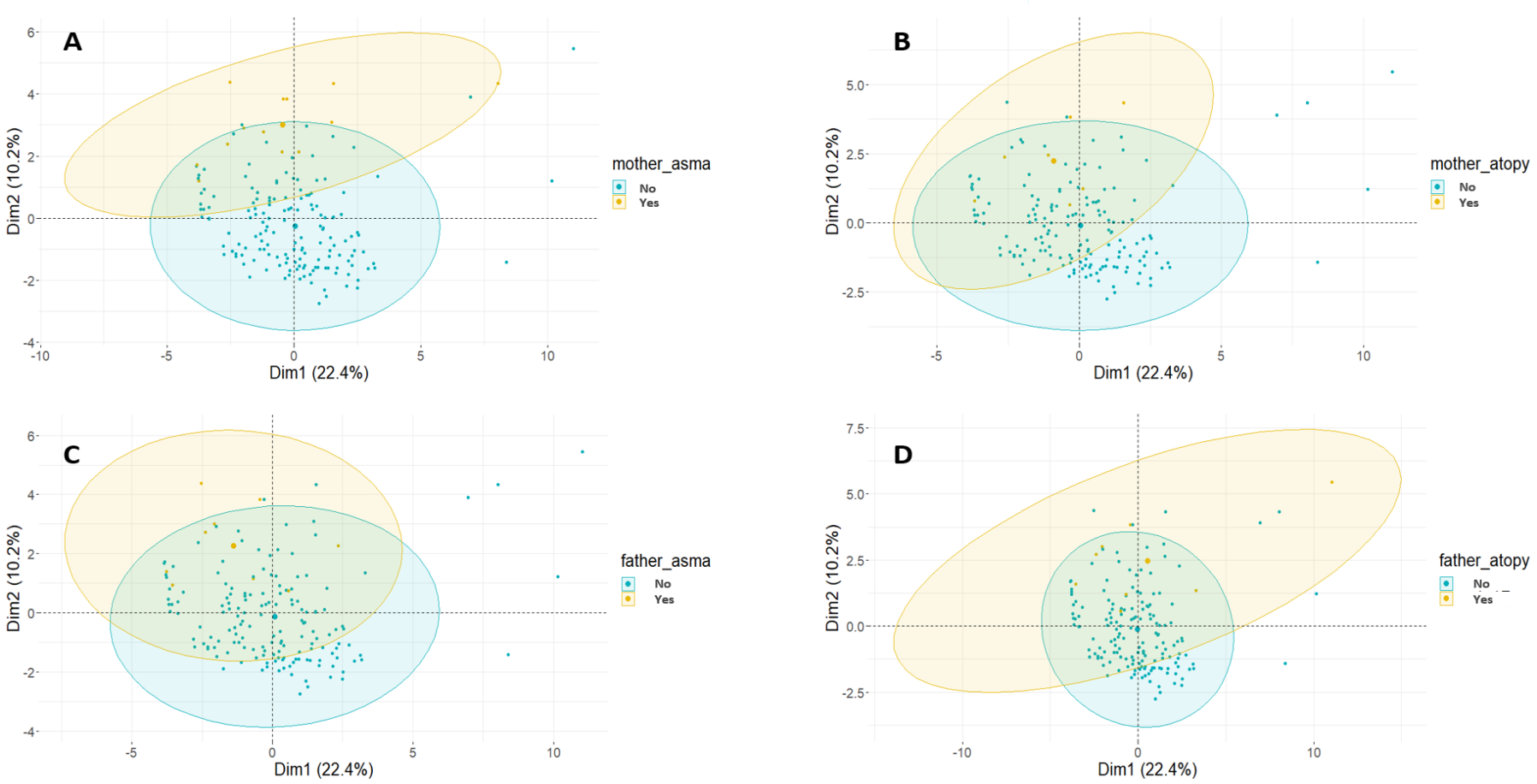

Figure 56. Factor Analysis of Mix Data (FAMD) of the gut microbiota of samples to study the similarity between the individuals taking into account all the factors study **A)** maternal history of asthma; **B)** maternal history of atopic dermatitis; **C)** paternal history of asthma; **D)** paternal history of atopic dermatitis. Significance was calculated using PerMANOVA test.

4.7.2 Multivariate study of changes in the infant's SCFAs profile, based on prenatal, perinatal and postnatal factors.

With respect to the changes in SCFAs as a function of the factors previously studied in a multivariate manner (**figure 57)**, a clear distinction was again observed in two groups when all individuals were represented and classified according to the clinical phenotype (control *vs.*. cases) (**figure 57A**), this difference being statistically significant ($p < 0.001$). Two quite distinct groups were also observed when the type of lactation is studied (**figure 57E**). Specifically, when the breastfed group and the formula-fed group of infants were compared ($p < 0.01$). No statistically differentiated groups were observed when individuals were classified according to type of delivery (**figure 67D**), antibiotic exposure during the third trimester of pregnancy (**figure 57C**) or during delivery (IPA) (**figure 57B**).

Figure 58 shows the graphics of the analysis of SCFAs changes of infants according to the history of asthma (**figure 58A** and **58C**) or atopy (**figure 58B** and **58D**) of the mother and father respectively and again no statistically significant differences were observed in this study.

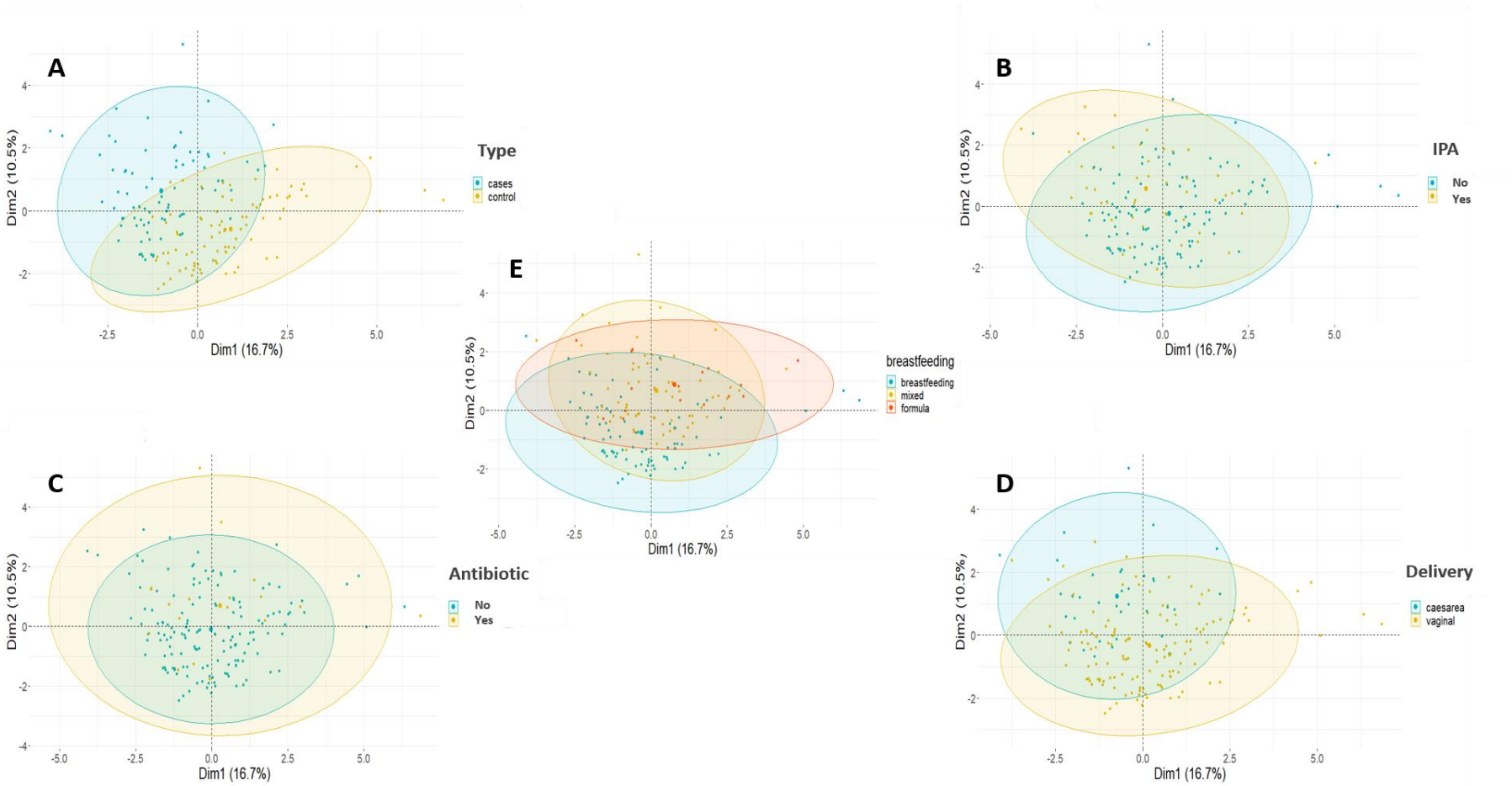

Figure 57. Factor Analysis of Mix Data (FAMD) of the SCFAS of samples to study the similarity between the individuals taking into account all the factors study **A)** phenotype; **B)** use of IPA; **C)** antibiotic exposition during pregnancy; **D)** mode of delivery and **E)** type of lactation. Significance was calculated using PerMANOVA test.

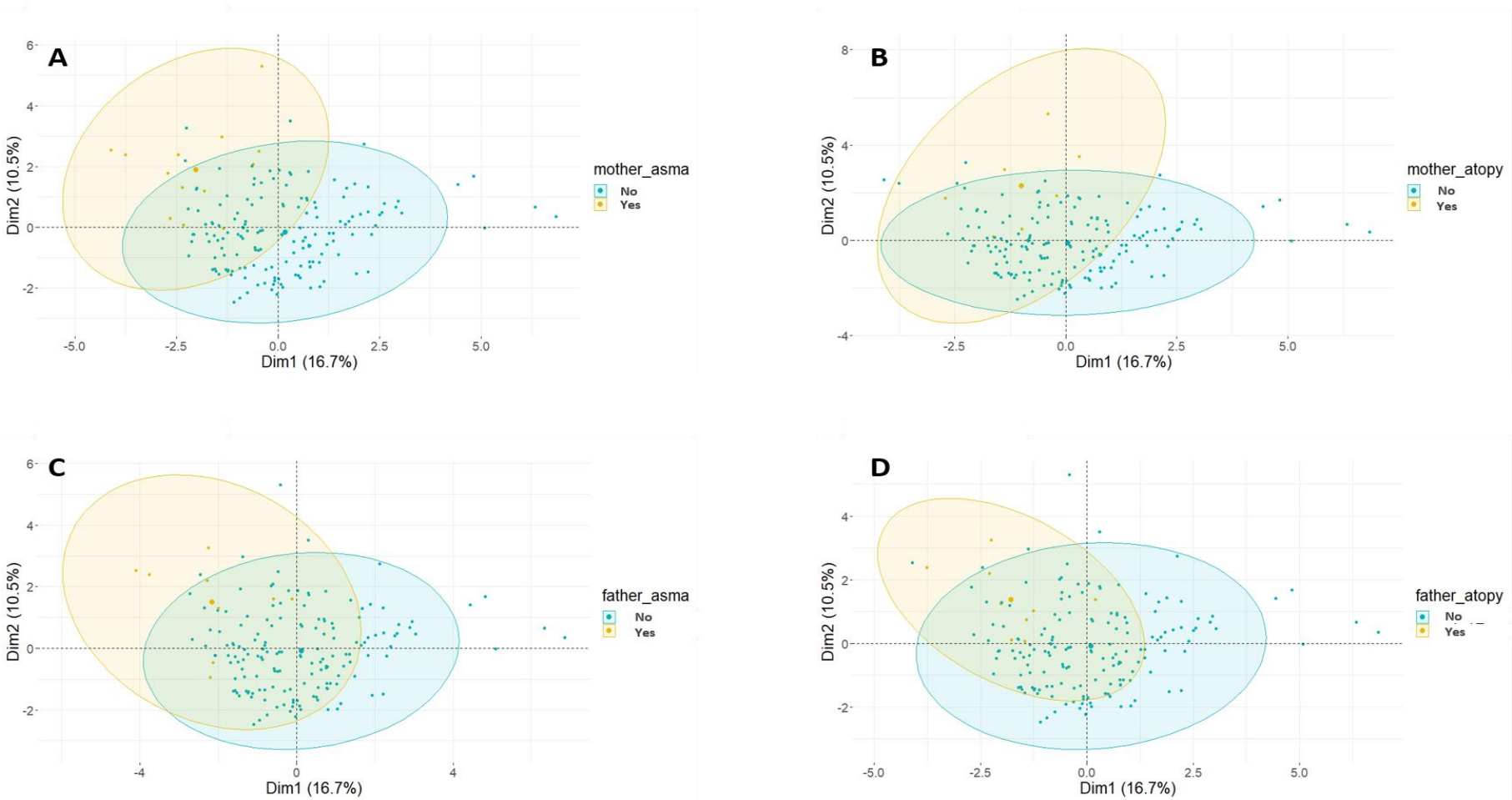

Figure 58. Factor Analysis of Mix Data (FAMD) of the gut microbiota of samples to study the similarity between the individuals taking into account all the factors study **A)** maternal history of asthma; **B)** maternal history of atopic dermatitis; **C)** paternal history of asthma; **D)** paternal history of atopic dermatitis. Significance was calculated using PerMANOVA test.

4.8 Study of prenatal, perinatal and postnatal factors and their association with an increased risk of early onset of asthma precursors symptoms.

Of all the factors discussed throughout this chapter, multivariate logistic regression analysis was performed to determine which factors were associated with an increased risk of early onset of asthmatic precursor symptoms (**table 21**).

This analysis showed that delivery by caesarean section was the only factor positively associated (OR: 3.95; 95% CI: 1.42-11.71; *P*: <0.01). That is, there is a higher propensity for early onset of asthmatic precursor symptoms when the infant was delivered by caesarean section. Regarding this factor and its association with the risk of asthma in children, there are conflicting results. On one hand, Renz-Polster *et al.,* [374] concluded that caesarean sections may be associated with an increased risk of developing asthma in childhood but this association was gender specific, with a positive association restricted to girls. Furthermore, Maitra *et al.,* [375]*,* in the Avon Longitudinal Study of Parents and Children, concluded that delivery by caesarean section was not associated with the subsequent development of asthma, wheezing or atopy in later childhood (5-7 years of age). But in 2008, Bager *et al.,*[99] carried out a meta-analysis about caesarean delivery and risk of atopy and allergic disease and concluded that delivery by caesarean section is associated with a moderately increased risk of allergic rhinitis, asthma, hospitalisation for asthma, and possibly food allergy/food atopy in the offspring, but is not associated with inhalant atopy or eczema/atopic dermatitis. Darabi *et al.,*[376]*,* in another more recent meta-analysis (2019), obtained similar results, also concluding that caesarean section increases the risk of childhood asthma.

Table 21. Association of prenatal, perinatal and postnatal factors with a higher risk of early onset of asthma precursor symptoms in infants at 3 months of age (n = 162) in the NELA cohort study.

		(95% CI)		
Prenatal and perinatal variables	**OR‡**	**Lower**	**Upper**	***P***
Body mass index (kg/m^2)	0.97	0.88	1.07	
Gestational age (weeks)	0.84	0.63	1.09	
Sex				
Boy	Ref			
Girl	0.64	0.29	1.38	
Mode of delivery				
Vaginal	Ref			
Caesarean	3.95	1.42	11.71	**
Type of lactation				
Breastfeeding	Ref			
Mixed	1.04	0.45	2.39	
Exclusive infant formula	1.86	0.48	7.35	
Mother alcohol consumption				
No	Ref			
Yes	0.57	0.08	2.74	
Mother smoke consumption				
No	Ref			
Yes	1.86	0.68	5.07	
Use of antibiotics during pregnancy				
No	Ref			
Yes	1.15	0.52	2.55	
Use of intra partum antibiotic (IPA)				
No	Ref			
Yes	0.65	0.24	1.65	

Ref: Reference; CI: confidence interval; OR: Odds ratio.
‡Multivariate logistic regression analysis. Risk of wheeze, eczema, bronchiolitis and /or bronchitis at 3 months of age modelled as categorical variable (Controls *vs.* cases); Model were adjusted for parental history of asthma and atopic dermatitis.
*p-value<0.05; **p-value<0.01; ***p-value<0.001.

A positive association was also observed between the use of antibiotics during pregnancy and a higher risk of early onset of asthmatic precursor symptoms but this association was not statistically significant. There are numerous cohort studies, case-control studies and meta-analyses that have attempted to clarify whether antibiotic use during pregnancy possess an increased risk of asthma, eczema, atopy and/or food allergies in offspring. The latest meta-analysis was recently carried out by Zhong et al. in 2021 [377]. These authors included a total of 26 studies and concluded that maternal antibiotic use during pregnancy might increase the risk of asthma/wheeze and eczema/atopic dermatitis but not food allergy in children. The researchers also indicated that

further studies with larger sample size and robust multivariable adjustment are needed to confirm their findings.

Finally, with respect to the type of feeding, an increased risk was observed with mixed breastfeeding and formula feeding, both of which were associated with an increased risk of early onset of asthmatic precursor symptoms, but neither was statistically significant. In the last meta-analysis founded and carried out by Harvey *et al*., (2021) [378], the author tried to evaluate the evidences for the association between breastfeeding and wheeze incidences and severity in high-risk infants, concluding that breastfeeding was associated with reduced odds of wheezing in high-risk infants, with the strongest protection in the first 6 months. Regarding as to whether breastfeeding can have a role in protecting against allergic disease and asthma in early childhood, there are numerous epidemiological studies in the that provide conflicting results as Oddy *et al.,* resumed in their review [110]. While some studies reported a protective effects of breastfeeding on asthma in young children [221,225,232], other studies of children show no protective effects [233–235].

4.9 Association of infant gut microbiota and SCFAs profile with early onset of asthma precursor symptoms.

Table 22 represents a multivariate logistic regression analysis (cases *vs.*. controls) to identify the possible association between the microbial profile of infants at 3 months of age (count and prevalence) and an increased probability of early onset of asthma precursor symptoms. The models were adjusted for the prenatal and postnatal factors discussed above. Significance for all tests performed was set at a p-value <0.05.

Table 22. Association of gut microbiota profile (counts and prevalence) with a higher risk of early onset of asthma precursor symptoms in infants at 3 months of age (n = 169). NELA cohort study.

	Counts†				Prevalence			
		(95% CI)				(95% CI)		
Microbiota	**OR**	**Lower**	**Upper**	***P***	**OR**	**Lower**	**Upper**	***P***
Akkermansia muciniphila	20.01	6.01	132.22	***				
No					Ref			
Yes					564.03	37.47	2.18E+04	***
Enterobacteriaceae	1.16	0.50	2.63					
No					Ref			
Yes					0.01	0.00	0.23	*
Bacteroides-Prevotella	0.58	0.36	0.85	*				
No					Ref			
Yes					0.32	0.03	3.55	
Lactobacillus	0.64	0.29	1.34					
No					Ref			
Yes					0.11	0.02	0.50	**
Enterococaceae	0.37	0.15	0.75	*				
No					Ref			
Yes					0.39	0.04	3.75	
Clostridium cluster IVa	0.95	0.58	1.48					
No					Ref			
Yes					5.03	1.42	20.56	*
Clostridium cluster XIVa	0.19	0.07	0.38	***				
No					Ref			
Yes					0.09	0.02	0.29	***
Clostridium difficile	0.51	0.20	1.06					
No					Ref			
Yes					1.28	0.21	7.62	
Staphylococcus	1.15	0.46	2.97					
No					Ref			
Yes					32.87	6.38	239.66	***
Bifidobacterium longum	0.45	0.21	0.79	*				
No					Ref			
Yes					0.44	0.02	7.48	
Bifidobacterium breve	1.55	1.07	2.46	*				
No					Ref			
Yes					16.38	4.53	76.66	***
Atopobium	1.04	0.49	2.24					
No					Ref			
Yes					0.27	0.01	7.03	

Ref: Reference; CI: confidence interval; OR: Odds ratio. Multivariate logistic regression analysis to study the risk of wheeze, eczema, bronchiolitis and /or bronchitis at 3 months of age modelled as categorical variable (Controls *vs.* cases); The analyses were carried out for both the counts of each of the microorganisms (continuous numerical variable) (†) and the prevalence (categorical variable: No detected / yes detected). Model were adjusted for gestational age, BMI, parental history of asthma and atopic dermatitis, sex, mode of delivery, type of lactation, use of antibiotics during pregnancy, use of intra partum antibiotic (IPA). *p-value<0.05; **p-value<0.01; ***p-value<0.001.

Higher counts of *A. muciniphila* (OR: 20.01; 95% CI: 6.01-132.22; *P* <0.001) and *B. breve* (OR: 1.55; 95% CI: 1.07-2.46; *P* <0.05), and lower counts of *Bacteroides-Prevotella* group (OR: 0.58; 95% CI: 0.36-0.85; *P* <0.05), *Clostridium* cluster XIVa (OR: 0.19; 95% CI: 0.07-0.38; *P* <0.001), *Enterococaceae* (OR: 0.37; 95% CI: 0.15-0.75; *P* <0.05) and *B. longum* (OR: 0.45; 95% CI: 0.21-0.79; *P* <0.05) have been found to be associated with an increased risk of asthma precursor symptoms in infants at 3 months of age. An association between a low *Lactobacillus spp*. (OR: 0.64; 95% CI: 0.29-1.34; *P* >0.05) count and an increased risk of asthma precursor symptoms was also observed, but this association were not statistically significant.

On the other hand, when we performed logistic regression models based on the prevalence of the different microorganisms studied (no detected *vs.* detected), we observed that higher counts of *A. muciniphila* (OR: 564.03; 95% CI: 37.47-2.18x10^4; *P* < 0.001), *Clostridium* cluster IVa (OR: 5.03; 95% CI: 1.42-20.56; *P* < 0.05), *Staphylococcus (*OR: 32.87; 95% CI: 6.38-239.66; *P* < 0.001) and *B. breve*, (OR: 16.38; 95% CI: 4.53-76.66; *P* <0.001) and lower counts of *Enterobacteriaceae* (OR: 0.01; 95% CI: 0.00-0.23; *P* < 0.05), *Lactobacillus spp.* (OR: 0.11; 95% CI: 0.02-2.0.50; *P* < 0.01) and *Clostridium* cluster XIVa (OR: 0.09; 95% CI: 0.02-0.29; *P* < 0.001) were associated with a higher risk of wheezing, eczema, bronchitis and/or bronchiolitis at 3 months of age. Also, a high prevalence of *C. difficile* (OR: 1.28; 95% CI: 0.21-7.62; *P* > 0.05) and a low prevalence of *B. longum* (OR: 0.44; 95% CI: 0.02-7.48; *P* > 0.05) were associated with an increased risk of asthma precursor symptoms, but these associations were not statistically significant. These results are in agreement with those observed by Penders *et al.,* [379] and Kalliomaki *et al.* [331]*,* for *C. difficile* and by Sjogren *et al.,* [332] for *Lactobacillus spp..*

Overall, *A. muciniphila*, *B. breve* and *Clostridium* cluster XIVa were the bacteria for which a statistically significant association was observed in both count-based and prevalence-adjusted models. The results about the observed associations of *A. muciniphila* and *B. breve* do not correspond with the expected results, nor with what has been observed in the literature [332,380]. Both bacterial species are scientifically considered positive and a beneficial or protective effect was expected to be observed instead of the opposite. In addition, various authors

have observed that the bacteria *B. breve* and *B. longum* [335,336], induce the production of IgA and the cytokine response helping to a correct immune development. The results observed with respect to *B. longum* and *Bacteroidetes* [337] do coincide with what is described in the literature, where various authors have also observed that these bacteria also induce the production of IgA.

In the case of the SCFAs profile (**table 23**), a statistically significant association was observed between a lower concentration of butyric, iso-valeric and caproic acid and an increased risk of asthma precursor symptoms. On the other hand, a statistically significant association was also observed for iso-butyric and caproic fatty acid. In this case the association was positive and therefore higher concentrations of these two fatty acids were associated with an increased risk.

The same model was performed taking into account the molar ratios of the main SCFAs and no association was observed between any of the SCFAs and an increased risk of EOAPS.

Table 23. Association of SCFAs profile (concentration and molar proportion) with a higher risk of early onset of asthma precursor symptoms in infants at 3 months of age (n = 169) in the NELA cohort study.

	SCFAs (μM/g wet faeces)				**SCFAs (Molar proportion)**			
Variable		**(95% CI)**				**(95% CI)**		
SCFAs	**OR**	**Lower**	**Upper**	***P***	**OR**	**Lower**	**Upper**	***P***
Acetic	1.01	0.99	1.02		0.00	0.00	1.41E+21	
Propionic	0.99	0.92	1.07		0.00	0.00	1.39E+21	
Butyric	0.72	0.57	0.88	**	0.00	0.00	1.24E+21	
Minority fatty acids					0.00	0.00	9.32E+20	
Iso-butyric	16.26	3.70	96.15	***	-	-	-	-
Valeric	1.10	0.28	2.93		-	-	-	-
Iso-valeric	0.16	0.04	0.49	**	-	-	-	-
Caproic	91.08	3.49	3480.33	**	-	-	-	-
Iso-caproic	0.41	0.10	0.72	*	-	-	-	-

Ref: Reference; CI: confidence interval; OR: Odds ratio.
Multivariate logistic regression analysis. Risk of wheeze, eczema, bronchiolitis and /or bronchitis at 3 months of age modelled as categorical variable (Controls *vs.* cases);
Model were adjusted for gestational age, BMI, parental history of asthma and atopic dermatitis, Sex, mode of delivery, type of lactation, use of antibiotics during pregnancy, use of intra partum antibiotic (IPA). *p-value<0.05; **p-value<0.01; ***p-value<0.001.

The result with respect to the butyric acid concentrations are in agreement with the literature. For example, Roduit *et al.,* in 2019 [342] observed that infants (1 year of age) with the highest levels of butyric and propionic acids in faeces had significantly less atopic sensitization and were less likely to have asthma between 3 and 6 years. Besides, infants with the highest levels of butyric were also less likely to have a reported diagnosis of food allergy or allergic rhinitis.

On the other hand, although we did not observe any association between valeric acid and a higher or lower risk of early onset of asthma precursor symptoms, other publications have observed that high levels of valeric acid at 3 years of age were associated with low rate of eczema at 8 years of age [381].

5. Conclusions

The gut microbiota of infants with early onset of asthma precursor symptoms was characterized by lower counts of *Atopobium, Bacteroides, B. breve, B. longum, Clostridium* cluster XIVa and cluster IVa and *C. difficile*, and higher counts of *Akkermansia muciniphila* compared to healthy infants. Regarding the prevalence of the microorganisms studied, we conclude that the gut microbiota of healthy infants was characterized by higher prevalence of *Lactobacillus* and *Clostridium* cluster XIVa, while infants with asthma precursor symptoms presented a higher prevalence of *B. breve, A. muciniphila* and *Clostridium* cluster IVa.

About SCFAs profile the faeces of healthy infants at 3 months of age were characterized by lower concentrations of acetic acid and higher concentrations of butyric acid compared to infants with the symptoms commented previously. Concerning molar proportions, healthy infants presented lower molar proportions of acetic acid and higher proportions for butyric acid, propionic acid and all minority fatty acids grouped together.

Regarding the prenatal, perinatal and postnatal factors studied and their relationship with the gut microbiota of infants and their metabolites, it has been concluded that, only the factor "type of delivery" and more specifically caesarean delivery was associated with the early onset of asthma precursor symptoms at 3 months of age. Also, we consider necessary to carry out new works, with a larger population size, that can help to elucidate whether other factors such as the use of antibiotics during pregnancy or the type of breastfeeding are associated or not with these symptoms.

We also conclude that higher counts of *Akkermansia muciniphila* and *B. breve*, and lower counts of *Bacteroides-Prevotella*, *Clostridium* cluster IVa and cluster XIVa, and *B. longum* were associated with a higher probability of developing asthma precursor symptoms at 3 months of age. In relation to SCFAs and its possible association with these symptoms in infants, a lower concentration of butyric acid and a higher concentration of caproic acid, related to pathogenic

microorganisms such as *C. difficile*, in the stools at 3 months of age was associated with a greater probability of the appearance of these symptoms.

It is required taking in to account that these results only reflect a minimal part of the population that makes up the entire gut microbiota of infants and therefore it is considered necessary to carry out further analyses to study the gut microbiota of infants in a more comprehensive way, for example through sequencing techniques obtaining thus a global vision of the entire gut microbiota of the infant.

IV. CONCLUSIONS DERIVED FROM THIS PhD THESIS.

1. Conclusions Chapter 1.

1.1 Study I: Characterization of the level of adherence to MD in pregnant women belonging to the NELA cohort and the lifestyle and sociodemographic factors associated with a low MDA.

- The pregnant women's level of adherence to the Mediterranean diet belonging to the NELA cohort corresponded to a Medium level of adherence for both scores, aMED and rMED and for a healthy dietary pattern (AHEI-2010).
- Pregnant women with younger age, previous deliveries, low educational level, and who practice unhealthy lifestyles, such as lack of physical activity, are associated with a higher risk of low adherence to Mediterranean diet (aMED and rMED) and a healthy dietary patter (AHEI-2010).

1.2 Study II: The possible association between adherence to MD of gestational women of the NELA cohort and the risk of wheezing, atopy and eczema in their offspring at 3 months of age.

- With respect to the level of adherence to the three dietary patterns studied, no statistically significant differences were observed between mothers of healthy infants and mothers whose offspring had early onset of asthma precursor.
- In this pilot study, it was not observed an association between the degree of adherence of mothers to the Mediterranean diet during pregnancy and the early onset of asthma precursor symptoms in the offspring at 3 months of age.
- We believe it is necessary to establish or create a single index of adherence to the specific Mediterranean diet for pregnant mothers, which would allow reducing the heterogenicity of future studies.

1.3 Study III: The possible association between the consumption of food groups or specific foods by pregnant women of the NELA cohort and the risk of wheezing, atopy and eczema in their offspring at 3 months of age.

- A higher consumption of pastries and sweets by the mother during pregnancy is associated with a greater probability of early onset of asthma precursor symptoms in the offspring at 3 months old. Besides, a higher consumption of coffee, tea and / or herbal tea by the mother during pregnancy is associated with a lower probability of these symptoms.
- No association has been established between the average daily intake of some allergenic or protective foods against allergies, according to scientific evidence, and the appearance of asthma precursor symptoms in offspring at 3 months of age.
- Finally, we believe it is necessary to establish a single criteria for the definition of food groups, and also to reach an agreement about the confounding variables used to adjust the regression models, which would allow reducing the heterogenicity of future studies and thus facilitating the comparison between them.

2. Conclusions Chapter 2.

- The gut microbiota of infants with early onset of asthma precursor symptoms was characterized by lower counts of *Atopobium, Bacteroides, B. breve, B. longum, Clostridium* cluster XIVa and cluster IVa and *C. difficile*, and higher counts of *Akkermansia muciniphila* compared to healthy infants.
- Regarding the prevalence, the gut microbiota of healthy infants was characterized by higher prevalence of *Lactobacillus* and *Clostridium* cluster XIVa, while infants with asthma precursor symptoms presented a higher prevalence of *B. breve, A. muciniphila* and *Clostridium* cluster IVa.
- The faeces of healthy infants at 3 months of age were characterized by lower molar proportions of acetic acid and higher proportions for butyric acid, propionic acid and all minority fatty acids grouped together.

- In our study, of all the prenatal, perinatal and postnatal factors studied, only the factor "type of delivery" and more specifically caesarean delivery was associated with the early onset of asthma precursor symptoms in the offspring at 3 months of age.
- Higher counts of *Akkermansia muciniphila* and *B. breve*, and lower counts of *Bacteroides-Prevotella*, *Clostridium* cluster IVa and cluster XIVa, and *B. longum* were associated with a higher probability of developing asthma precursor symptoms at 3 months of age.
- A lower concentration of butyric acid and a higher concentration of caproic acid, related to pathogenic microorganisms such as *C. difficile*, in the stool at 3 months of age was associated with a greater probability of the appearance of these symptoms.
- These results only reflect a minimal part of the population that makes up the entire gut microbiota of infants and therefore it is considered necessary to carry out further analyses to study the intestinal microbiota of infants in a more comprehensive way, for example through sequencing techniques obtaining thus a global vision of the entire gut microbiota of the infant.

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VI. SCIENTIFIC PRODUCTION DERIVED FROM THIS PhD THESIS AND FROM COLLABORATIONS

1. Scientific production derived from the PhD Thesis

1.1 Scientific Articles

Suárez-Martínez, C.; Yagüe-Guirao, G.; Santaella-Pascual, M.; Peso-Echarri, P.; Vioque, J.; Morales, E.; García-Marcos, L.; Martínez-Graciá, C.; NELA Study Group, T. Adherence to the Mediterranean Diet and Determinants Among Pregnant Women: The NELA Cohort. Nutrients 2021, 13, 1248. https://doi.org/10.3390/nu13041248

1.2 Poster communications

Suárez-Martínez, C.; Pardo-Lacárcel S.; Yagüe-Guirao, G. and Martínez-Graciá, C. (2017). Influencia de la nutrición materna y microbioma materno y del neonato, en el desarrollo de asma en los niños. Proyecto N.E.L.A. (Nutrition in early life and asthma). III Jornadas Doctorales de la Universidad de Murcia. Escuela Internacional de Doctorado de la Universidad de Murcia (EIDUM). Murcia (Spain).

Suárez-Martínez, C.; Yagüe-Guirao, G. and Martínez-Graciá, C. The Role of Gut Microbiota on the development of Asthma. III Jornadas Doctorales de la Universidad de Murcia. 4th International and 5th national student Congress of Food science and Technology. Asociación Valenciana de Estudiantes y Profesionales en Ciencia y Tecnología de los Alimentos (AVECTA). Valencia (Spain).

Suárez-Martínez, C.; Yagüe-Guirao, G. and Martínez-Graciá, C. Impact of maternal Mediterranean diet adherence in pregnancy on offspring gut microbial composition and their metabolites. The NELA cohort. 8th International Human Microbiome Consortium Congress (IHMC) 2021. Barcelona (Spain).

2. Scientific production derived from collaborations

2.1 Scientific Articles

Gázquez, A.; Giménez-Bañón, M.J.; Prieto-Sánchez, M.T.; Martínez-Graciá, C.; Suárez, C.; Santaella-Pascual, M.; Galdo-Castiñeira, L.; Ballesteros-Meseguer, C.; Vioque, J.; Martínez-Villanueva, M.; Avilés-Plaza, F.; Noguera-Velasco, J.A.; Morales, E.; García-Marcos, L.; Larqué, E.; on behalf of the Nela Study Group. Self-Reported DHA Supplementation during Pregnancy and Its Association with Obesity or Gestational Diabetes in Relation to DHA Concentration in Cord and Maternal Plasma: Results from NELA, a Prospective Mother-Offspring Cohort. Nutrients 2021, 13, 843. https://doi.org/10.3390/nu13030843

Summarizing, the overall scientific production derived from this PhD has been 1 indexed article and 3 posters. As well, total research contribution apart from this Thesis has been 1 indexed article.

3. Internship in foreign Research Centre

Centre: Tegasc Moorepark. Food Research Centre (Irish Agriculture and Food Development Authority). Fermoy; Cork (Ireland).

Period of stay: 1 Mayo 2019 - 31 Agosto 2019.

Project title: Infant gut microbiota: Influence of Dietary and Environmental Factors.

Funding Organisation: ERASMUS PLUS. University of Murcia (Spain) and Escuela Internacional de Doctorado de la Universidad de Murcia (EIDUM).

ANNEXES

ANNEX 1

INFORME DEL COMITÉ DE BIOSEGURIDAD EN EXPERIMENTACIÓN DE LA UNIVERSIDAD DE MURCIA

Lucía Periago García, Jefa de Sección de Recursos Humanos de la Investigación y del Plan Propio y en funciones de Secretaria del Comité de Bioseguridad en Experimentación de la Universidad de Murcia

CERTIFICA:

Que Dª. Clara Suárez Martínez nombre presentó la memoria de trabajo la Tesis Doctoral titulada *"Influencia de la nutrición materna y microbioma materno y del neonato, en el desarrollo de asma en los niños. Proyecto N.E.L.A. (Nutrition in Early Life and Asthma)",* dirigida por D.ª Carmen Martínez Gracía y D.ª Genoveva Yagüe Girao, al Comité de Bioseguridad en Experimentación**.**

Que dicho Comité analizó toda la documentación presentada, y de conformidad con lo acordado el día treinta y uno de octubre de dos mil dieciocho, por unanimidad, se emite INFORME FAVORABLE, desde el punto de vista ético de la bioseguridad en la investigación.

Y para que conste y tenga los efectos que correspondan, firmo esta certificación, con el visto bueno del Presidente de la Comisión.

Vº Bº
EL PRESIDENTE DEL COMITÉ
DE BIOSEGURIDAD EN EXPERIMENTACIÓN
DE LA UNIVERSIDAD DE MURCIA

Fdo.: Francisco Esquembre Martínez

ID: CBE125/2018

INFORME DE LA COMISIÓN DE ÉTICA DE INVESTIGACIÓN DE LA UNIVERSIDAD DE MURCIA

Jaime Peris Riera, Catedrático de Universidad y Secretario de la Comisión de Ética de Investigación de la Universidad de Murcia,

CERTIFICA:

Que D.ª Clara Suárez Martínez ha presentado la Tesis Doctoral titulada *"Influencia de la nutrición materna y microbioma materno y del neonato, en el desarrollo de asma en los niños"*, dirigida por D.ª Carmen Martínez García y D.ª Genoveva Yagüe Girao, a la Comisión de Ética de Investigación de la Universidad de Murcia.

Que dicha Comisión analizó toda la documentación presentada, y de conformidad con lo acordado el día cuatro de junio de dos mil dieciocho[1], por unanimidad, se emite INFORME FAVORABLE, desde el punto de vista ético de la investigación.

Y para que conste y tenga los efectos que correspondan firmo esta certificación con el visto bueno del Presidente de la Comisión.

Vº Bº
EL PRESIDENTE DE LA COMISIÓN
DE ÉTICA DE INVESTIGACIÓN DE LA
UNIVERSIDAD DE MURCIA

Fdo.: Francisco Esquembre Martínez

ID: 1942/2018

[1] A los efectos de lo establecido en el art. 19.5 de la Ley 40/2015 de 1 de octubre de Régimen Jurídico del Sector Público (B.O.E. 02-10), se advierte que el acta de la sesión citada está pendiente de aprobación

ANNEX 2

Food group	Foods that compose it
Dairy products	Whole milk
	Semi-skimmed milk
	Skimmed milk
	Cooking cream
	Whole yogurt
	Nonfat yogurt
	Brugos fresh cheese
	Fresh cheese Brugos 0%
	Cured / semi-cured / creamy cheeses
	Custard, Flan, Rice pudding, etc.
	Ice creams
Eggs	Chicken eggs
Read meats	Beef, pork, lamb
White meats	Chicken with skin
	Skinless chicken
	Game meat: rabbit, quail, ...
Meat derivatives	Organ meats (pork liver, etc.)
	Chicken's liver
	Raw meat products
	Burger
	Cured meat products
	Cooked meat products
	Frankfurt type sausages
	Frankfurter type cheese sausages
Fish, shellfish and derivatives	White fish
	Blue fish: tuna, emperor ...
	Other blue fish
	Canned tuna or bonito
	Canned sardines or mackerel
	Clams, mussels, oysters
	Squid, squid, cuttlefish, etc.
	Seafood: prawns, crab, etc.
	Croquette, fried fish sticks
Potatoes	Chips
	Cooked potatoes, roasted
	Potato chips bag
Pulses	Cooked chickpeas
	Beans
	Lentils
	Boiled frozen pea
	Broad beans

Continuation	
Food group	**Foods that compose it**
Vegetables	Swiss chard or cooked spinach
	Cabbage, cabbage, or red cabbage
	Broccoli
	Cauliflower
	Brussels sprouts
	Lettuce, endive, escarole
	Tomato
	Onion
	Carrot
	Boiled green beans
	Zucchini or pumpkin
	Eggplant
	Green pepper
	Red pepper
	Mushroom and mushrooms
	Avocado
	Raw garlic
Fruit	Orange
	Tangerine
	Banana
	Apple
	Pear
	Peach
	Nectarine
	Melon, watermelon
	Grape
	Strawberry and strawberries
	Pineapple
	Mango
	Kiwi
	Natural orange juice
Nuts	Walnuts
	Peanuts
	Almonds, hazelnuts, pistachios, etc.
Cereals and refined derivatives	Normal bread
	Normal mold bread
	Normal biscote
	Rice in soup or garnish
	Rice on plate
	Noodles and other pasta in soup
	Pasta on plate

Continuation	
Food group	**Foods that compose it**
Whole grains and derivatives	Boiled corn Wholemeal bread Whole wheat loaf Whole wheat biscote
Oils and fats	Extra virgin olive oil Other vegetable oils: olive, sunflower, etc. Margarine Butter
Sauces	Mayonnaise Ketchup Ketchup Mustard
Pastries and sweets	Breakfast cereals Chocolate breakfast cereals Maria type cookies Prince type cookies Magdalena Croissant, ensaimada, donut Bollicao type bun Biscuit White chocolate Milk chocolate Pure chocolate Chocolate bar Cocoa powder Honey Fruit jam Light jam Sugar
Coffee, tea or herbal teas	Coffee Decaffeinated coffee Tea Infusions
Alcoholic drinks	Wine Beer Alcohol-free beer Beer 0% Others: whiskey, gin, rum, vodka
Sugary drinks	Commercial orange juice Commercial apple juice Commercial peach juice

	Commercial pineapple juice
	Sugary soft drinks with cola
	Orange carbonated sugary soft drinks
	Sugary sodas with lemon gas
	Sugary sodas with tonic gas
	Orange non-carbonated sugary soft drinks
	Non-carbonated sugary soft drinks lemon
	Isotonic drinks
Sweetened beverages	Light soft drinks with cola gas
	Orange carbonated soft drinks
	Light soft drinks with lemon gas
	Light sodas with tonic gas