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INSTITUTO DE NEUROCIENCIAS FEDERICO OLÓRIZ

Estructura y funcionalidad de la **microbiota intestinal** en niños nacidos de madres **obesas** y su efecto sobre el **neurodesarrollo** durante los **primeros meses de vida**

"*Gut microbial community structure and function of infants born to obese mothers and their effect on neurodevelopment in early life*"

TESIS DOCTORAL

"Mención Internacional"

Tomás Cerdó Ráez

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“*La ciencia sin religión está coja y la religión sin ciencia está ciega*”

"*Science without religion is lame, religion without science is blind*"

ALBERT EINSTEIN

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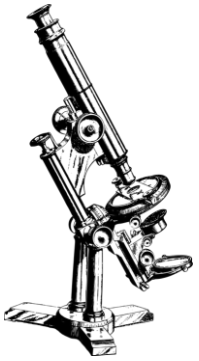
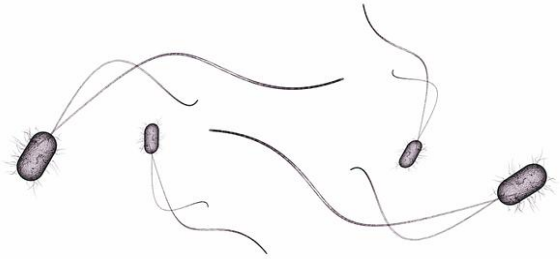


SUMMARY



Infancy is a critical period for organ maturation and growth. Observational studies have provided evidences about how influence of intrinsic and extrinsic factors in the early life can produce disturbances in the pathways that regulate energy expenditure, immune system and other systemic effects, promoting the development of certain diseases and altering the configuration and maturation of gut microbiota.

A major challenge in microbial ecology is to identify its functional members and understand how their functional and phylogenetic dynamics ultimately influence human physiology and health. Critically important are the initial stages of microbiota colonization and maturation in the gut because early dysbiosis has been shown to affect human health later in life.

Classical studies about the ethiology of obesity focused on dietary disorders and host genetic susceptibility. Recent studies have shown that mothers transmit distinct gut microbial communities to their offspring depending on maternal pre-pregnancy weight, which might favour to obesity development in infancy. Furthermore, studies in rodents provide evidence that the gut microbiota modulates brain development and synaptic related proteins showing that behaviour depends upon three psychoneuroimmune pathways, i.e., immune, hypothalamic pituitary adrenal axis and vagus nerve. Alterations of the gut microbiota impact exploratory and communicative behaviours and cognitive performance. Still, little is known about the association between gut microbiota and neurodevelopment in humans.

The present study has been developed within the PREOBE Follow-up framework with the following **objectives**:

Objective 1. To provide biological information on the relative importance of gut microbial taxa in ecosystem functioning, their collective functional pattern and the network topology in relation to host physiology during human early life.

Objective 2. To analyse the effect of maternal obesity on the composition and functionality of the gut microbiota in their offspring.

Objective 3. To test whether early gut microbioal ecosystem membership and metabolism associate with infant neurodevelopment

Results and discussion

Paper 1

Cerdó, T., Ruiz, A., Acuña, I., Jáuregui, R., Jehmlich, N., Haange, S. B., Martin von, B., Suárez, A. & Campoy, C. (2018). **Gut microbial functional maturation and succession during human early life**. *Environmental microbiology*. https://doi.org/10.1111/1462-2920.14235

The evolutional trajectory of gut microbial colonization from birth has been shown to prime for health later in life. Here, we combined cultivation-independent 16S rRNA gene sequencing and metaproteomics to investigate the functional maturation of gut microbiota in faecal samples from full-term healthy infants collected at 6 and 18 months of age.

Phylogenetic analysis of the metaproteomes showed that *Bifidobacterium* provided the highest number of distinct protein groups. Considerable divergences between taxa abundance and protein phylogeny were observed at all taxonomic ranks. Age had a profound effect on early microbiota where compositional and functional diversity of less dissimilar communities increased with time. Comparisons of the relative abundances of proteins revealed the transition of taxon-associated saccharolytic and fermentation strategies from milk and mucin-derived monosaccharide catabolism feeding acetate/propanoate synthesis to complex food-derived hexoses fuelling butanoate production. Furthermore, co-occurrence network analysis uncovered two anti-correlated modules of functional taxa. A low-connected *Bifidobacteriaceae*-centred guild of facultative anaerobes was succeeded by a rich club of obligate anaerobes densely interconnected around *Lachnospiraceae*, underpinning their pivotal roles in microbial ecosystem assemblies.

Paper 2

Cerdó, T., Ruiz, A., Jáuregui, R., Azaryah, H., Torres-Espínola, F. J., García-Valdés, L., Segura, M.T., Suárez, A. & Campoy, C. (2018). **Maternal obesity is associated with gut microbial metabolic potential in offspring during infancy**. *Journal of physiology and biochemistry*, *74*(1), 159-169. doi: 10.1007/s13105-017-0577-x

Children born to obese mothers are at increased risk for obesity, but the mechanisms behind this association are not fully understood. Our study aimed to investigate differences in the functions

encoded by the microbiome of infants at 18 months of age when the transition from early infant-feeding to solid family foods is established. To investigate the impact of maternal prepregnancy body mass index on infants' gut microbiome, faecal samples from infants born to normoweight (n = 21) and obese mothers (n = 18) were analysed by 16S rRNA gene sequencing and a functional-inference-based microbiome analysis.

Our results indicated that *Firmicutes* was significantly enriched in infants born to normoweight mothers whereas *Bacteroidetes* was significantly enriched in infants born to obese women. In both microbiomes, the greatest number of genes (>50%) that were assigned a function encoded for proteins involved in "metabolism" among tier 1 KEGG Orthology (KO) categories. At lower KO functional categories, the microbiome of infants born to normoweight mothers was characterized by a significant enrichment in the abundances of "pentose phosphate pathway" (p = 0.037), "lysine biosynthesis" (p = 0.043), "glycerolipid metabolism" (p = 0.042), and "C5-branched dibasic acid metabolism" (p = 0.045). Notably, the microbiome of infants born to obese mothers was significantly enriched in "streptomycin biosynthesis" (p = 0.047), "sulphur metabolism" (p = 0.041), "taurine and hypotaurine metabolism" (p = 0.036), and "lipopolysaccharide biosynthesis" (p = 0.043).

Paper 3

Cerdó, T., García-Valdés, L., Altmäe, S., Ruíz, A., Suárez, A., & Campoy, C. (2016). **Role of microbiota function during early life on child's neurodevelopment**. *Trends in food science & technology*, *57*, 273-288. https://doi.org/10.1016/j.tifs.2016.08.007

There are critical periods during pregnancy and early life when child's neurodevelopment can be altered, where different factors including hormones, stress, genetics, and diet have an important role. Novel studies are indicating that also gut microbiota and maternal obesity can influence child's neurodevelopment.

This review summarises the current concepts related to microbiota-gut-brain axis, including microbiota modulation of the eating behaviour, child's cognitive function and brain structure, microbiota analysis techniques and neurodevelopment assessment in children. Further, we propose and present knowledge about potential mechanisms of action and ways to intervene for disease prevention and treatments, opening up an exciting area with important medical and industrial applications.

This novel and fast developing research area is indicating that gut microbiota in association with body weight might have an important impact on foetal and child neurodevelopment. However, the exact mechanisms are not known and further research in the field is warranted. Within the MyNewGut Project we aim to analyse the impact of microbiota in association with body weight on cognitive and behaviour development in children. We will study the phylogeny and function of the gut microbial communities in overweight, obese and gestational diabetes pregnancies and in their progeny, in association with infants and children's cognitive and behavioural outcomes. As well, the impact of gut microbiome on brain structure and function during childhood will be evaluated. Results from this study will shed light on the impact of maternal and offspring gut microbiome and body weight on child's neurodevelopment, brain structure and function, and will suggest potential mechanisms for intervention.

Paper 4

Cerdó, T., Ruíz, A., Suárez, A., & Campoy, C. (2017). **Probiotic, Prebiotic, and Brain Development**. *Nutrients*, *9*(11), 1247. https://doi.org/10.3390/nu9111247

Recently, a number of studies have demonstrated the existence of a link between the emotional and cognitive centres of the brain and peripheral functions through the bi-directional interaction between the central nervous system and the enteric nervous system. Therefore, the use of bacteria as therapeutics has attracted much interest. Recent research has found that there are a variety of mechanisms by which bacteria can signal to the brain and influence several processes in relation to neurotransmission, neurogenesis, and behaviour. Data derived from both in vitro experiments and in vivo clinical trials have supported some of these new health implications. While recent molecular advancement has provided strong indications to support and justify the role of the gut microbiota on the gut–brain axis, it is still not clear whether manipulations through probiotics and prebiotics administration could be beneficial in the treatment of neurological problems. The understanding of the gut microbiota and its activities is essential for the generation of future personalized healthcare strategies. Here, we explore and summarize the potential beneficial effects of probiotics and prebiotics in the neurodevelopmental process and in the prevention and treatment of certain neurological human diseases, highlighting current and future perspectives in this topic.

Draft Paper 5

Cerdó T., Ruiz, A., Acuña, I., Torres-Espínola, F.J., Jáuregui, R., Jehmlich, N., Haange, S. B., Martin von, B., Suárez, A. & Campoy, C. **Intestinal bacteria are associated with the cognitive development of children at 6 months of age**.

Compelling evidence suggest that gut microorganisms influence neurodevelopment in mice. To test this hypothesis in humans, we conducted a longitudinal study in full-term healthy infants where cognitive function assessed with Bayley III was associated with gut microbial composition, structure and metabolism. Children were categorized according to their Bayley scores within each domain into two groups, above and below the median (50^{th} percentile). Composite cognitive scale (CCS) was the only test in which both study groups, divided according to the mean, showed significant differences in gut microbial composition.

Higher evenness ($p<0.004$), Shannon ($p<0.011$) and Simpson ($p<0.021$) diversity and reduced dominance ($p<0.021$) values in gut microbiota characterized the gut microbiota of infants with above median CCS. Principal coordinate analysis based on weighted UniFrac metrics of β-diversity showed that the gut microbiota of infants clustered by CCS ($p<0.014$), indicating significant phylogenetic dissimilarities in the microbial profile of highly abundant taxa. Taxa within *Lactococcus* and *Lachnnospiraceae_Incertae_Sedis* were significantly enriched in infants with below the median CCS. Conversely, taxa within *Bacteroides* showed a higher abundance in children with above the median CCS.

Metaproteomic analyses suggested mechanisms that might underlie microbial effects on infant neurodevelopment. In main COG category, proteins involved in "Intracellular trafficking" were more abundant in children with below the median CCS while those involved in "Carbohydrate transport" were enriched in children with above the median CCS. In children with below the median CSS, there was increased abundance of "aspartate carbamoyl transferase" and "dihydroorotase". Interestingly, in children with above the median CSS "histidine ammonia lyase" was significantly enriched, an enzyme involved in histamine metabolism.

Conclusions

Conclusion 1. Our metaproteomics data revealed that the gut microbiota harbours a distinctive subset of biologically active microorganisms, indicating considerable discordance between microbial composition and phylogenetic origin of proteins at all taxonomic levels and suggesting that using bacterial taxa or even metagenomics as input information to build predictive theoretical models of microbial activity may be highly misleading.

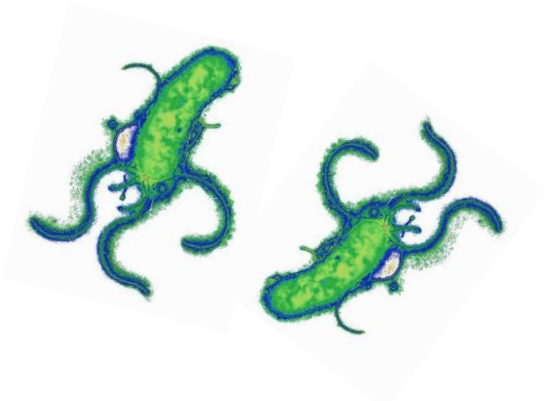
Conclusion 2. The detailed reconstruction of the gut microbial carbon metabolism by metaproteomic analysis, including the assignment of enzymes to microbial taxa, revealed alternate temporary microbial and metabolic configurations where community-wide metabolic relationships to harvest energy by fermentation of prevailing dietary and host-derived carbon substrates, mainly glycans, differentiated chronological states in infant early life.

Conclusion 3. Our results show that the maturation of the gut microbiota during the first 18 months of life is a non-random process where two mutually exclusive modules of functional families, built around *Bifidobacteriaceae (6 months)* and *Lachnospiraceae (18 months)*, which metabolically succeeded each other.

Conclusion 4. Mothers imprinted different gut microbiotas in their children depending on their pre-pregnancy weight, enriched in taxa within *Bacteroidetes* in infants born to obese mothers and in *Firmicutes* in infants born to normoweight mothers, with different predicted metabolic outcomes that may influence infants' development later in life.

Conclusion 5. Our forecoming study will show an association between gut microbiota and infant cognitive performance where regulation of histidine metabolism by gut microbiota in early life may underlie this relationship.

RESUMEN



La infancia es un período crítico para la maduración y el crecimiento de los órganos. Estudios observacionales han proporcionado evidencias sobre cómo la influencia de factores intrínsecos y extrínsecos en la vida temprana pueden producir alteraciones en las vías que regulan el gasto energético, el sistema inmune y otros efectos sistémicos, promoviendo el desarrollo de ciertas enfermedades y alterando la configuración y maduración de la microbiota intestinal. Un desafío importante en la ecología microbiana es identificar a sus miembros funcionales y comprender cómo su dinámica funcional y filogenética finalmente influye en la fisiología y la salud humana. Especialmente importantes son los periodos iniciales de la colonización y maduración de la microbiota en el intestino, ya que se ha demostrado que una disbiosis temprana puede afectar la salud en la vida adulta.

Hasta hace poco los estudios sobre la etiología de la obesidad se habían centrado en los trastornos alimenticios y la susceptibilidad genética del huésped. Sin embargo, recientes estudios han demostrado que las madres transmiten distintas comunidades microbianas intestinales a sus hijos en función del peso materno pregestacional, lo que podría favorecer el desarrollo de la obesidad en la infancia. Además, los estudios en roedores proporcionan evidencia de que la microbiota intestinal modula el desarrollo cerebral y las proteínas sinápticas relacionadas, lo cual muestra que el comportamiento depende de tres vías psiconeuroinmunes, es decir, sistema inmune, eje hipotalámico-hipofisario-adrenal y el nervio vago. Las alteraciones de la microbiota intestinal afectan los comportamientos exploratorios y comunicativos y el rendimiento cognitivo. Aún así, se sabe poco sobre la asociación entre la microbiota intestinal y el neurodesarrollo en humanos.

El presente estudio se ha realizado en el marco del Proyecto PREOBE Follow-up, y tiene los siguientes **objetivos**:

Objetivo 1. Proporcionar información biológica sobre la importancia relativa de los taxones microbianos intestinales en el funcionamiento del ecosistema, su patrón funcional colectivo y la topología de la red en relación con la fisiología del huésped durante los primeros años de vida.

Objetivo 2. Analizar el efecto de la obesidad materna en la composición y funcionalidad de la microbiota intestinal en su descendencia.

Objetivo 3. Comprobar si la comunidad microbiana intestinal inicial y su metabolismo se asocian con el neurodesarrollo infantil.

Resultados y discusión

Artículo 1

Cerdó, T., Ruiz, A., Acuña, I., Jáuregui, R., Jehmlich, N., Haange, S. B., Martin von, B., Suárez, A. & Campoy, C. (2018). **Gut microbial functional maturation and succession during human early life**. *Environmental microbiology*. https://doi.org/10.1111/1462-2920.14235

El periodo de tiempo tras el parto, en el cual se produce la colonización y establecimiento de las primeras bacterias de la microbiota intestinal, ha sido identificado como un momento crítico. Esto se debe a que dependiendo de qué tipo de comunidad microbiana se establezca podrá influir en la salud del niño en edades posteriores de su crecimiento. En este estudio, combinamos secuenciación del gen 16S rRNA independiente del cultivo y análisis metaproteómicos para estudiar la maduración funcional de la microbiota intestinal en muestras fecales de bebés sanos a término recogidas a los 6 y 18 meses de edad. El análisis filogenético de los metaproteomas mostró que *Bifidobacterium* proporcionó el mayor número de grupos de proteínas diferentes. Se observaron considerables divergencias entre la abundancia taxonómica y la filogenia de proteínas en todos los rangos taxonómicos. La edad tuvo un profundo efecto en la microbiota, donde la diversidad composicional y funcional de las comunidades más similares se incrementó con el tiempo. El análisis proteómico reveló la transición de taxones asociados a procesos sacarolíticos y de fermentación de la leche y de procesos asociados al catabolismo de monosacáridos derivados de la mucina, los cuales alimentan la síntesis de acetato/popanoato en el sistema de hexosas derivadas de la alimentación que sirven para favorecer la producción de butanoato. Además, el análisis de las redes de co-ocurrencia mostró dos módulos opuestos de taxones funcionales. Un grupo de anaerobios facultativos poco conectado entre sí, focalizado principalmente en la familia *Bifidobacteriaceae*, dio paso a un grupo de anaerobios obligados estrechamente interconectados alrededor de la familia *Lachnospiraceae*, resaltando funciones importantes de estos en el ecosistema microbiano.

Artículo 2

Cerdó, T., Ruiz, A., Jáuregui, R., Azaryah, H., Torres-Espínola, F. J., García-Valdés, L., Segura, M.T., Suárez, A. & Campoy, C. (2018). **Maternal obesity is associated with gut microbial metabolic potential in offspring during infancy**. *Journal of physiology and biochemistry*, *74*(1), 159-169. doi: 10.1007/s13105-017-0577-x

Es sabido que los niños nacidos de madres obesas tienen un mayor riesgo de sufrir obesidad, sin embargo, los mecanismos detrás de esta asociación no han sido descritos por completo. Nuestro estudio tuvo como objetivo estudiar las diferencias funcionales del microbioma intestinal de los bebés a los 18 meses de edad, periodo en el que la alimentación sólida se ha establecido casi en su totalidad en la dieta. Para investigar el impacto del índice de masa corporal materno pregestacional en el microbioma intestinal de los bebés, se analizaron muestras fecales de bebés nacidos de madres normopeso (n = 21) y madres obesas (n = 18) mediante secuenciación del gen 16S rRNA y una inferencia funcional basada en análisis del microbioma.

Nuestros resultados indicaron que el phylum *Firmicutes* fue enriquecido significativamente en los bebés nacidos de madres normopeso, mientras que *Bacteroidetes* se enriqueció significativamente en los bebés nacidos de madres obesas. En ambos microbiomas, la mayoría de los genes (> 50%) a los que se les asignó una función codificada en proteínas fueron involucrados en la categoría "metabolismo" dentro del nivel más externo de la clasificación KEGG Orthology (KO). En las clasificaciones funcionales de KO más profundas, el microbioma de los bebés nacidos de madres normopeso se caracterizó por un enriquecimiento significativo en las abundancias de la "vía de la pentosa fosfato" (p = 0.037), "biosíntesis de lisina" (p = 0.043), "metabolismo de glicerolípidos" (p = 0.042) y "metabolismo del ácido dibásico ramificado C5" (p = 0.045). Cabe destacar que el microbioma de bebés nacidos de madres obesas se enriqueció significativamente en "biosíntesis de estreptomicina" (p = 0.047), "metabolismo de azufre" (p = 0.041), "metabolismo de taurina e hipotaurina" (p = 0.036) y "biosíntesis de lipopolisacáridos" "(P = 0.043).

Artículo 3

Cerdó, T., García-Valdés, L., Altmäe, S., Ruíz, A., Suárez, A., & Campoy, C. (2016). **Role of microbiota function during early life on child's neurodevelopment**. *Trends in food science & technology*, *57*, 273-288. https://doi.org/10.1016/j.tifs.2016.08.007

Existen períodos críticos durante el embarazo y los primeros años de vida en los que se puede alterar el neurodesarrollo del niño, en los cuales diferentes factores, como las hormonas, el estrés, la genética y la dieta, tienen un papel importante. Nuevos estudios indican que también la microbiota intestinal y la obesidad materna pueden influir en el neurodesarrollo del niño.

Esta revisión resume los conceptos actuales relacionados con el eje microbiota-intestino-cerebro, incluida la modulación de la microbiota del comportamiento alimentario, la función cognitiva y la estructura cerebral del niño, las técnicas de análisis de microbiota y la evaluación del desarrollo neurológico en niños. Además, proponemos y presentamos conocimiento sobre los posibles mecanismos de acción y formas de intervención para la prevención de enfermedades y tratamientos, abriendo un área emergente con importantes aplicaciones médicas e industriales.
Esta novedosa área de investigación, la cual indica que la microbiota intestinal asociada con el peso corporal, podría tener un impacto importante en el desarrollo neurológico del feto y del niño. Sin embargo, no se conocen los mecanismos exactos y es de causa justificada la necesidad de más investigaciones en este campo.

Dentro del Proyecto MyNewGut, nuestro objetivo es analizar el impacto de la microbiota en asociación con el peso corporal en el desarrollo cognitivo y conductual en los niños. Estudiaremos la filogenia y la función de las comunidades microbianas intestinales en los embarazos con sobrepeso, obesidad y diabetes gestacional y en su progenie, en asociación con los resultados cognitivos y conductuales de los lactantes y los niños. Además, se evaluará el impacto del microbioma intestinal sobre la estructura y la función del cerebro durante la infancia. Los resultados de este estudio arrojarán luz sobre el impacto del microbioma intestinal y el peso corporal materno sobre el neurodesarrollo del niño, la estructura y la función del cerebro, y sugerirán mecanismos potenciales para su intervención.

Artículo 4

Cerdó, T., Ruíz, A., Suárez, A., & Campoy, C. (2017). **Probiotic, Prebiotic, and Brain Development**. *Nutrients*, *9*(11), 1247. https://doi.org/10.3390/nu9111247

Recientemente, varios estudios han demostrado la existencia de un vínculo entre los centros emocionales y cognitivos del cerebro y las funciones periféricas a través de la interacción bidireccional entre el sistema nervioso central y el sistema nervioso entérico. Por lo tanto, el uso de bacterias como terapia se ha convertido en un tema con mucho potencial. Investigaciones

recientes han encontrado que hay una variedad de mecanismos por los cuales las bacterias pueden enviar señales al cerebro e influir en diversos procesos en relación con la neurotransmisión, la neurogénesis y el comportamiento. Los datos derivados tanto de experimentos in vitro como de ensayos clínicos in vivo han respaldado algunas de estas nuevas implicaciones para la salud. Si bien el avance molecular reciente ha proporcionado fuertes indicaciones para apoyar y justificar el papel de la microbiota intestinal en el eje intestino-cerebro, aún no está claro si la manipulación de la comunidad microbiana intestinal a través de los probióticos y la administración de prebióticos podrían ser beneficiosas en el tratamiento de problemas neurológicos. Conocer la composición de la microbiota intestinal y sus funciones es esencial para la generación de futuras estrategias para la atención médica personalizada. Aquí, exploramos y resumimos los posibles efectos beneficiosos de los probióticos y los prebióticos en el proceso del neurodesarrollo y en la prevención y el tratamiento de ciertas enfermedades neurológicas humanas, destacando las perspectivas actuales y futuras en dicho tema.

Borrador Artículo 5

Cerdó, T., Ruiz, A., Acuña, I., Torres-Espínola, F.J., Jáuregui, R., Jehmlich, N., Haange, S. B., Martin von, B., Suárez, A. & Campoy, C. **Intestinal bacteria are associated with the cognitive development of children at 6 months of age**.

Recientes estudios sugieren que los microorganismos intestinales influyen en el neurodesarrollo en modelos de ratones. Para probar esta hipótesis en humanos, llevamos a cabo un estudio longitudinal en recién nacidos sanos a término, donde la función cognitiva fue evaluada a través del test de Bayley III y cuyos resultados se asociaron con la composición, estructura y metabolismo microbiano del intestino. Los niños se clasificaron de acuerdo con sus puntuaciones, dentro de cada dominio de dicho test, en dos grupos, por encima y por debajo de la mediana (percentil 50). La escala cognitiva compuesta (CCS) fue la única prueba en la que ambos grupos de estudio, divididos según la mediana, mostraron diferencias significativas en la composición microbiana intestinal.

La microbiota intestinal de los niños por encima de la mediana frente a los niños por debajo de la mediana en el CCS, presentó valores significativamente más altos en los índices de alfa diversidad evenness ($p < 0.004$), Shannon ($p < 0.011$) y Simpson ($p < 0.021$), mientras que el índice dominancia ($p < 0.021$) fue significativamente menor.

El análisis de coordenadas principales basado en métricas UniFrac ponderadas de beta diversidad mostró que la microbiota intestinal se agrupaba en los dos grupos de estudio del CCS de forma significativa ($p < 0.014$), indicando diferencias filogenéticas significativas en el perfil microbiano. Los géneros *Lactococcus* y *Lachnnospiraceae_Incertae_Sedis* se enriquecieron significativamente en los niños con CCS por debajo de la mediana. Por el contrario, el género *Bacteroides* mostró una mayor abundancia en niños con CCS por encima de la mediana.

Los análisis metaproteómicos sugirieron mecanismos que podrían subyacer a los efectos microbianos en el neurodesarrollo infantil. En la categoría principal de COG, las proteínas implicadas en el tráfico intracelular fueron significativamente más abundantes en los niños con CCS por debajo de la mediana, mientras que las proteínas implicadas en el transporte de carbohidratos se enriquecieron en los niños con CCS por encima de la mediana. En niños con CSS por debajo de la mediana, hubo una mayor abundancia de "aspartato carbamoil transferasa" y "dihidroorotasas". Curiosamente, en los niños con niveles superiores a la mediana de CSS, la "histidina amonio-liasa" se enriqueció significativamente, la cual es una enzima implicada en el metabolismo de la histamina.

Conclusiones

Conclusión 1. Los datos metaproteómicos obtenidos en el presente estudio han revelado que la microbiota intestinal alberga un subconjunto de microorganismos biológicamente activos, lo que indica discordancia considerable entre la composición microbiana y el origen filogenético de las proteínas en todos los niveles taxonómicos y sugiere que el uso de taxones bacterianos o incluso de metagenómica como información para crear modelos de actividad microbiana pueden ser muy engañosos.

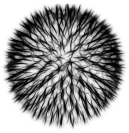
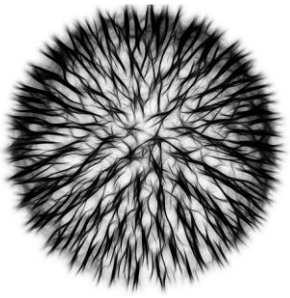
Conclusión 2. La reconstrucción detallada del metabolismo del carbono en la comunidad microbiana intestinal mediante análisis metaproteómicos, incluyendo la asignación de enzimas a taxones microbianos, demuestra un cambio temporal en la configuración microbiana y metabólica, donde la capacidad de obtener energía por parte de la comunidad se basa en la fermentación de la dieta y en los sustratos de carbono derivados del hospedador, principalmente glicanos, diferenciando estados cronológicos en los primeros años de vida.

Conclusión 3. Los resultados obtenidos muestran que la maduración de la microbiota intestinal no es un proceso aleatorio, sino que dos módulos únicos de familias funcionales,

construidas alrededor de *Bifidobacteriaceae* y *Lachnospiraceae* respectivamente, se sucedieron metabólicamente.

Conclusión 4. Las madres transmiten diferentes comunidades microbianas intestinales a sus hijos dependiendo del peso previo al embarazo; los niños nacidos de madres obesas presentan una mayor abundancia de bacterias pertenecientes al phylum *Bacteroidetes*, sin embargo, los nacidos de madres normopeso presentan mayor abundancia de bacterias pertenecientes al phylum *Firmicutes*. Estas diferencias pueden determinar potenciales resultados metabólicos diferentes y cambios en el crecimiento y desarrollo de los hijos durante la infancia y la niñez, con efectos desconocidos a largo plazo.

Conclusión 5. Nuestro próximo estudio mostrará una asociación entre la microbiota intestinal y el rendimiento cognitivo infantil, donde la regulación del metabolismo de la histidina por la microbiota intestinal en los primeros años de vida puede ser la base de dicha relación.

INTRODUCTION

Strong evidences support that the prenatal environment plays a critical role in the health and development of offspring throughout childhood and adult life (1). In this period, mother´s lifestyle, well-being and diet are closely related to a healthy pregnancy outcome (2). For a proper lactation, nutritional requirements during pregnancy, that include specific amounts of calcium, iron, folic acid, vitamins (D, C and B), and essential fatty-acids among others, must be fulfilled (3). On the opposite, harmful habits like smoking, consumption of caffeine and alcohol or use of illegal drugs have been related to adverse pregnancy outcomes (4).

Recently, mother's gut microbiota and the acquisition of a stable intestinal microbiota have emerged as modulating factors of offspring's metabolism and health (5, 6). Disruption of gut microbial ecosystem maturation and homeostasis, also referred to as dysbiosis, is being associated with childhood and later life immune diseases like necrotizing enterocolitis, eczema, asthma, inflammatory bowel diseases, irritable bowel syndrome, mental illnesses like autism and attention deficit hyperactivity disorder (ADHD), and metabolic disorders such as diabetes and obesity (7).

To date, most studies have focused on the association between gut microbial species and infant status based on 16S rRNA gene surveys and metagenomic annotations (8, 9), with the difficulty that direct association of specific microbes or functions is hampered by host genetic variation, environmental factors and genetic diversity between closely-related strains (10). These observations prompted us to test a "down to top" functional approach to define the gut microbial species and their metabolic contribution to infant's homeostasis and neurodevelopment during early life, that is, characterizing the microbiota by "what they do" to determine "who they are".

We believe that there is a need to understand the functional maturation of the gut microbiota during the critical period of early life colonization which may constitute an important research tool for microbiological indicators of future healthy or ill states and for the design of microbiota-targeted health-promoting strategies early in life.

1. HUMAN GUT COLONIZATION AND MICROBIOTA ESTABLISHMENT

Infancy is a critical period for gut microbial de novo assembly during which a constant dialogue with the immune system and a metabolic crosstalk with the host influences healthy growth and development (11, 12). The foetal programming hypothesis establishes that early exposures in utero have long-term effects on adult health (13). During pregnancy, microbial DNA has been detected in the placenta, umbilical cord, amnion fluids, and the meconium (14-17), suggesting that an invasion of the placenta by microbes may occur proposing that microbiome acquisition begins in utero (18).

Although the theory of sterility of the uterus remains controversial, other factors have been described as influential in the establishment and maturation of the intestinal microbial community such as maternal status (nutrition, BMI, microbiota composition), type of delivery (caesarean or vaginal), type of feeding later (breast milk or infant formula) and antibiotic exposure, among others (**Figure 1**).

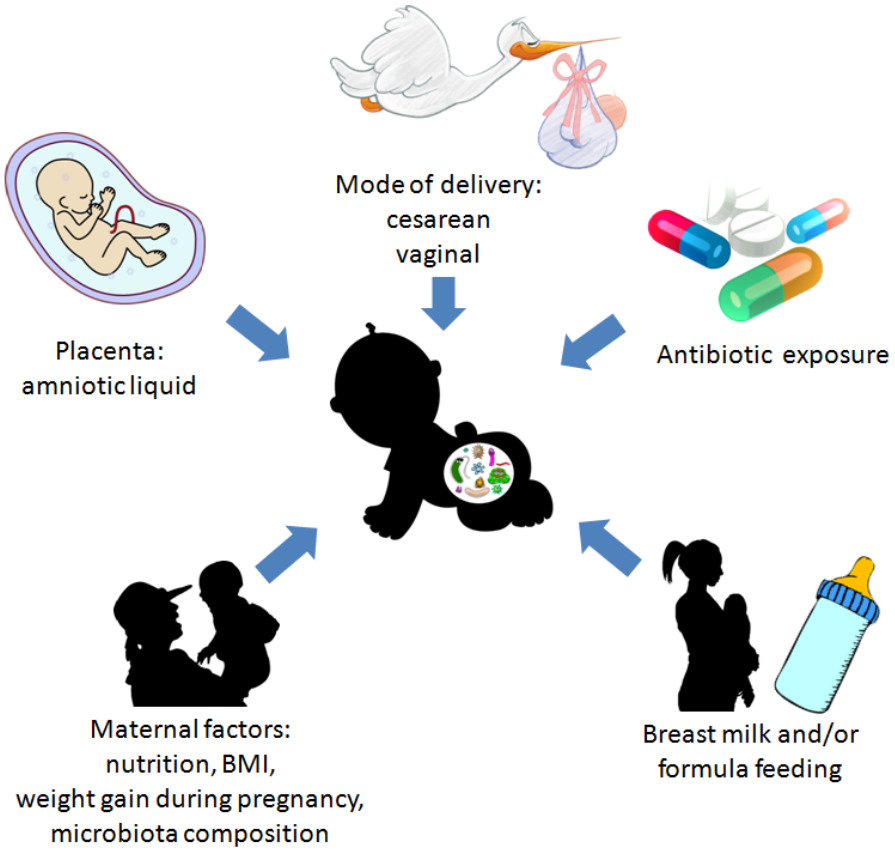


Figure 1. *Maternal and environmental elements affecting the onset and modulation of the gut microbiota in the newborn infant. A plethora of factors during pregnancy can negatively influence the neonate's gut microbiota composition and function. Furthermore, environmental factors, such as mode of delivery and feeding modality, can significantly drive the neonate's gut microbiota. Reprinted from(19).*

From an environmental point of view, at the beginning, the intestinal environment of baby is aerobic and due to dietary intake during the first three days, oxygen levels are reduced causing an environment suitable for the growth of anaerobes (20). The intestinal microbiota of neonates is characterized by low diversity and a relative dominance of mainly facultative anaerobic of the phyla *Proteobacteria* and *Actinobacteria* (21). After birth, the phyla *Firmicutes* and *Bacteroidetes* increase their diversity and dominance, reaching a total resemblance to the adult in terms of composition and diversity at 3 years of age (22).

Initially, the maternal gut microbiota is a major determinant of the infant gut microbiota because most early colonizers in the infant gut matched species found in maternal samples when babies were vaginally born. When born via C-section, infants had a gut community more similar to bacteria from maternal skin or the environment of the hospital (23). The exposition of the infant born by caesarean to the vaginal discharge, can partially restore its gut microbiota and avoid the problems that this entails (24).

Another important factor is the type of feeding in the new born and the type of solid foods that the baby will eat (**Figure 2**) which will influence on the establishment and composition of the gut microbiota along of the time (8).

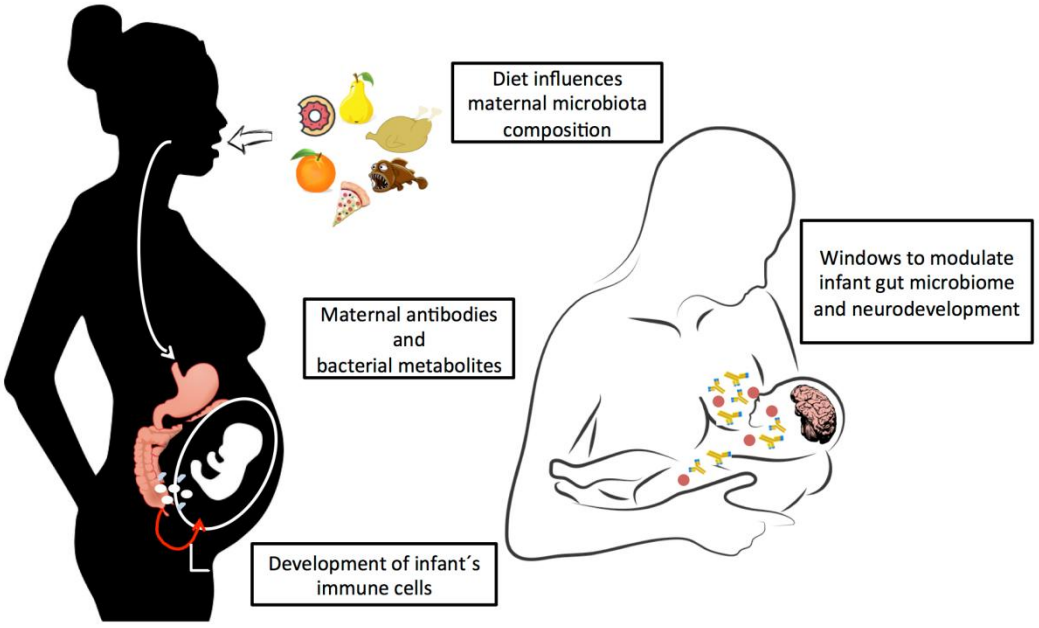


Figure 2. *Potential links between mother during and after pregnancy and influence in gut microbiota and neurodevelopment in her child. Prenatal and postnatal diet influences the fetal microbial population, via microbiota of the mother, driving early innate immune system development and neurodevelopment of the infant.*

Breastfeeding is the gold standard for infant feeding, as it provides the necessary substrates for bacterial colonization and maturation and this has an indirect effect over the morphology and physiology of the intestinal mucosa (25). Still, many babies can't be breast-fed and receive infant formulas that have been associated to a greater weight gain and increase the risk of obesity, hypertension and diabetes (26). The use of infant formula supplemented with probiotics can partially prevent this effect. Bazanella et al., in a double-blind, randomized and placebo controlled trial found that the metabolite profile in infants fed with formula supplemented with *Bifidobacterium longum* BB536 showed an improved Th1 immune response and a tendency to establish a healthy intestinal microbiota early in life compared to those who fed a non-supplemented formula (27). Similarly, preterm infants fed with formula supplemented with *Saccharomyces boulardii* improved feeding tolerance and weight gain (28).

A major problem during this period is antibiotic exposure that can reduce the phylogenetic diversity and microbial load of the gut microbiota, causing changes in the microbial community that contribute to the development of diverse diseases, such as obesity or mental illnesses (29). Indeed, infants treated with amoxicillin and gentamicin during the first week of life reduced the bacterial diversity and increased the relative abundance of *Enterobacter* in the second and third weeks of life (30). Moreover, preterm infants treated with penicillin, ampicillin, cephalexin, gentamicin, amikacin, erythromycin, vancomycin, clindamycin or teichomycin showed an increase in the presence of potentially pathogenic *Enterobacteriaceae* and a reduction in *Bifidobacteriaceae* and bacilli (*Lactobacillales spp*.), probiotic bacteria commonly linked with a healthy status and (30-32).

2. GUT MICROBIOTA AND OBESITY

Experimental evidences indicate a role for the gut microbiota in promoting obesity and it is viewed increasingly as an important target of dietary interventions and pharmacologic agents (33-35). Maternal obesity and weight gain modify the composition and metabolism of the microbiota in the gut and breast milk during pregnancy and lactation (36-38). Such microbial changes may be transferred to the offspring during delivery and lactation, altering microbial colonization of infant’s gut (39, 40). It is becoming increasingly clear that the gut microbiota in newborns and infants plays a key role in gut health and child development (37). Dysbiosis of the early infant gut has been correlated with the development of childhood obesity and type 1 diabetes (40). Several studies have shown that the gut microbial ecosystem had higher α-diversity and lower β-diversity with no distinct clustering in children born to obese mothers

compared to children born to normoweight mothers (36, 41). Mueller et al. showed that maternal obesity was associated with altered gut microbiota composition in neonates delivered vaginally, though not by C-section (42). These results disagree with those recently published by Laursen et al. (43). The authors found no association between infant's gut microbiota at 9 and 18 months of age with maternal obesity.

A key factor in obesity development is the increased capacity for energy extraction from dietary components provided by gut microbiome (44). Galley et al. observed significant effects of maternal obesity on the composition of the gut microbiome of offspring among dyads of higher socioeconomic status. Deeper analysis of the KOs revealed that carbohydrate metabolism was significantly lower in children born to obese mothers. However, these differences in KO abundances did not pass correction for multiple tests, due to low effect sizes (41). Hernandez et al. measured the activity level of GIT carbohydrate-active enzymes toward 23 distinct sugars in obese and lean subjects. They observed that both 14 d antibiotic-treated and obese subjects showed higher and less balanced sugar anabolic capacities, with 40% carbohydrates being preferentially processed as compared with non-treated and lean patients. Furthermore, metaproteome-wide metabolic reconstructions confirmed that the impaired utilization of sugars propagated throughout the pentose phosphate metabolism, which had adverse consequences for the metabolic status of the GIT microbiota (45).

3. GUT MICROBIOTA-BRAIN AXIS

Brain development in prenatal and postnatal stages is a complex and multifactorial event (46-48), crucial to determine lifelong performance in various neuropsychological domains such as cognition, language and motor functions (49, 50). The colonization by the gut microbiota simultaneously occurs with the dynamic phase of postnatal brain development, including cell differentiation, axon myelination and synaptogenesis, and the rapid emergence of infant cognitive functions (51). Subsequently, the diversity of commensal species within less-heterogeneous communities increases with age as well as the metabolic landscape of of microbiota-derived molecules (52) that have been shown to influence the fine maturation of the brain with long lasting effects (53-57). The gut microbiota influences brain function through the neuroendocrine, neuroimmune and autonomic nervous systems and via microbiotic toxin production (**Figure 3**) (58, 59).

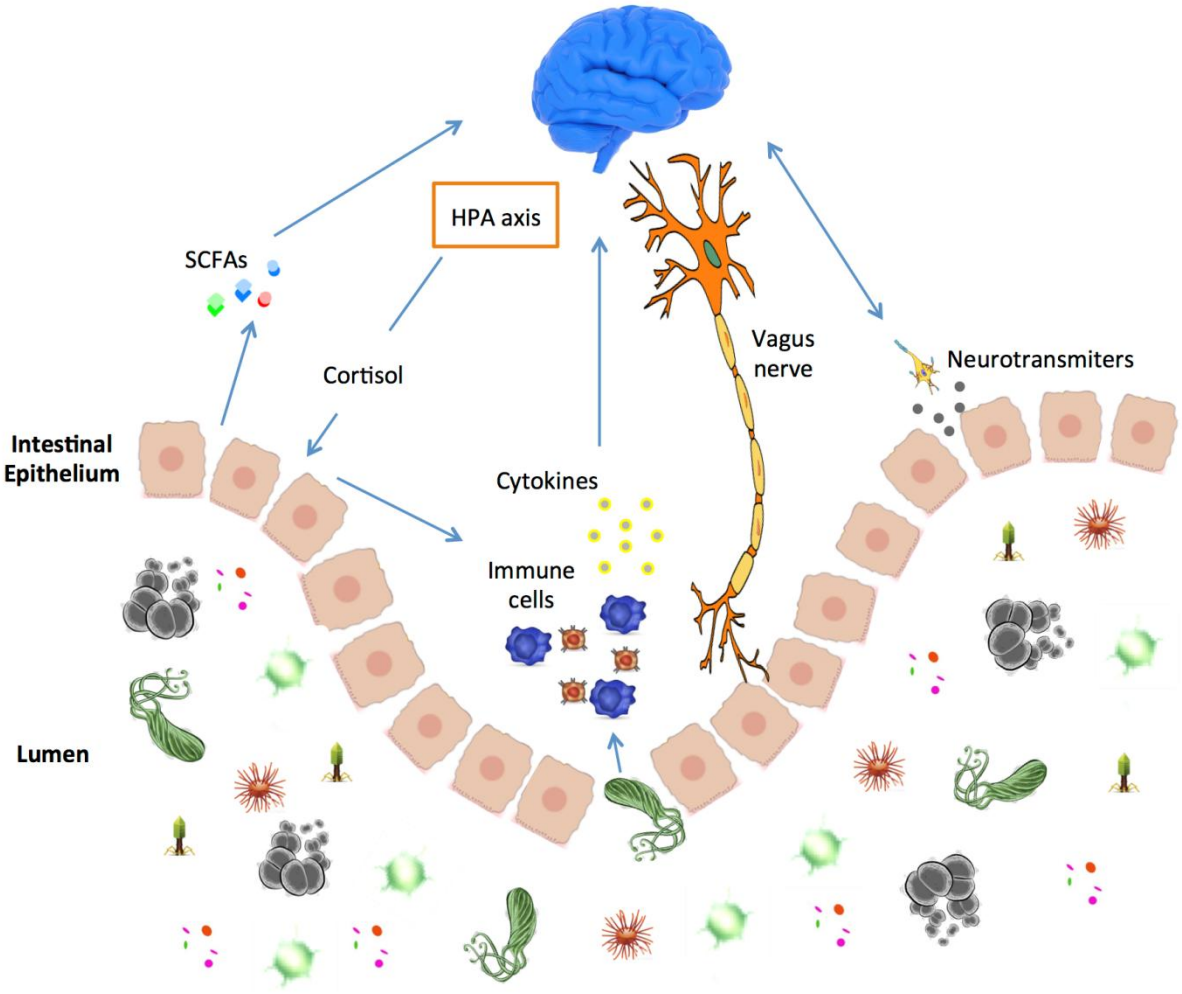
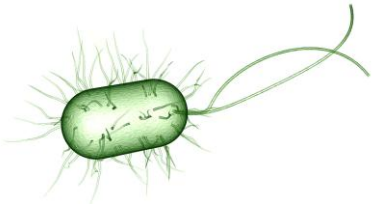


Figure 3. *The gut-brain axis pathway. Direct and indirect pathways support the bidirectional interactions between the gut microbiota and the central nervous system (CNS):Cytokine balance and microglia activation (immune pathway), cortisol (endocrine pathway) and vagus and enteric nervous system (neural pathway). In addition, short-chain fatty acids (SCFAs) are neuroactive bacterial metabolites of dietary fibers that can also modulate brain and behavior.*

Dysbiosis of the intestinal microbiota produce changes in neuromodulators/neurotransmitters and brain volumes that impact central nervous system (CNS) function and social behaviour in rodents (60-63) while associations of human gut microbial composition with autism (64), temperament (65), cognition (66) and depression (67) have been shown. For instance, germ free mice, that are mice produced and raised without any bacteria, display changes in anxiety-like behaviours (68) and appear unusually sensitive to stress (69). Reduction in depression-like behaviours was observed in different rodent models with normal gut microbiota, following

administration of a probiotic (70, 71). A recent study showed that brain regions controlling emotional and sensory processing were altered in a group of healthy females provided with a probiotic yoghurt formula compared with controls, showing a link between gut microbiota and brain function in humans (72). Numerous reports have addressed a possible role of the gut microbiota in the development of autism since onset of the disease usually follows antimicrobial therapy and oral vancomycin treatment showed a decrease in autistic symptoms (73, 74). Regarding stress, infants of mothers with high cumulative stress during pregnancy had significantly higher relative abundances of *Proteobacteria* and lower relative abundances of lactic acid bacteria (75). Carlson et al. identified three clusters of infants whose microbial composition associated with significant differences in Mullen scores of early learning at 2 years (76). Interestingly, Fernandez-Leal et al. identified a specific gut microbiota–brain map profile that discriminated obese individuals from nonobese subjects (77). These complex relationships between gut microbiota and the host are currently being referred to as the microbiota-gut-brain axis (78, 79).

OBJECTIVES



The specific composition of the gut microbiota in the early age can be related to the subsequent predisposition to the suffering of important pathologies. Links between the environmental microbiota, antibiotic treatments, composition of the intestinal microbiota, neurodevelopment and immune function in the early stages of life have been identified. Therefore, the need to advance in the knowledge about the effects of the composition and functionality of the microbiota on the early programming of obesity, neurodevelopment and behavior alterations in the infants is justified.

In view of the above, the objectives of the present thesis are:

Objective 1. To provide biological information on the relative importance of gut microbial taxa in ecosystem functioning, their collective functional pattern and the network topology in relation to host physiology during human early life.

Objective 2. To analyse the effect of maternal obesity on the composition and functionality of the gut microbiota in their offspring.

Objective 3. To test whether early gut microbioal ecosystem membership and metabolism associate with infant neurodevelopment.

THE PREOBE STUDY

The PREOBE study is based on a prospective observational mother-offspring cohort, trying to obtain genetic, nutritional, biochemical, microbiological, immunological, anthropometric, neuropsychological and neuroimaging markers to clarify the long-term influences of early programming on growth-adiposity, cognition and behaviour development.

The study recruitment was performed between 2008 and 2012, through collaboration with the Clinical University Hospital San Cecilio and the Mother-Infant University Hospital of Granada, Granada, Spain and their peripheral health centers. The cohort was established with 331 pregnant women recruited between 12 and 20 wks of pregnancy; from them 265 completed the follow-up till delivery, forming the current cohort based on 4 mother-children pairs groups: 1) healthy and Normal weight (18.5≤BMI<25), 2) Overweight (25≤BMI<30), 3) Obesity (BMI≥30), 4) Gestational Diabetes *(including normal weight, overweight and obese women)*. Mothers were included in the study when they were aged between 18 and 45 years, had single and non complicated pregnancies and did not participate in other study.

Pregnant women were supervised throughout gestation up to the delivery, and then up to 18 months postpartum. Standardized fetal anthropometry was performed, as well as in the neonates; infants and children were followed-up until 8 years old (3, 6, 12 and 18 months, and at 2, 3.5, 6.5 and 8 years old) (**Figure 4**). Important information has been obtained, such as maternal body composition and weight gaing during pregnancy, maternal and offspring dietary intake, lifestyle including physical activity, maternal and umbilical cord biochemical and immunological biomarkers, offspring's growth and neurodevelopment, between others.

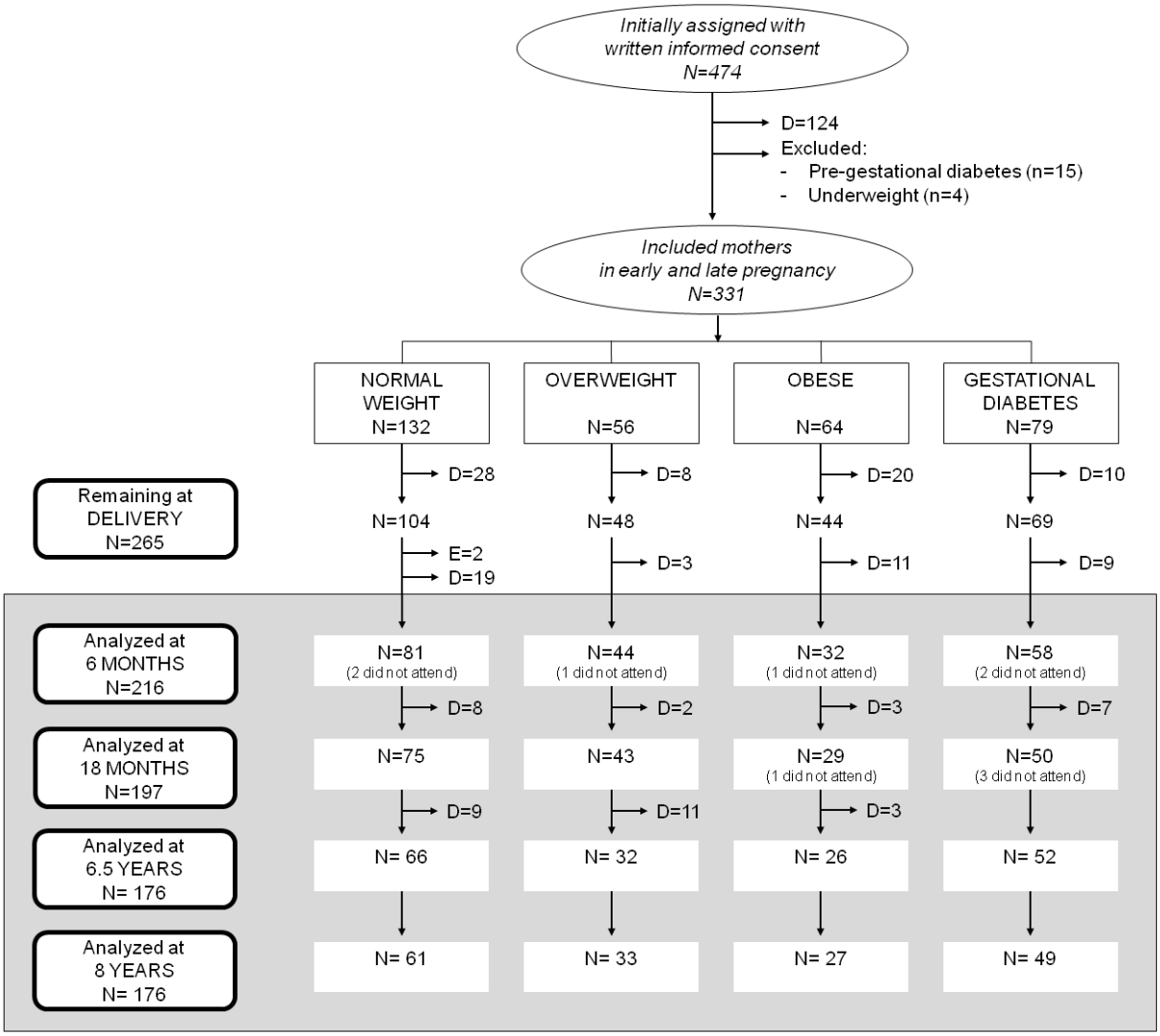


Figure 4. Trial profile. The study included 331 pregnant women at different stages of pregnancy but before 34 weeks of gestation. Included mothers were allocated to four different groups based on their pre-gestational BMI and their gestational diabetes status. Infants were followed-up in different visits until 8 years old. *D=drop outs and E=exclusions.*

In the frame of this Project, the present study will focus in the analysis of gut microbiota during the first 18 months of life. Associations between the maternal and infant data collected during the follow-up and the composition and functionality of the gut bacterial community at 6 and 18 months of age will be established (**Figure 5**). These periods of early life are relevant in the development of the intestinal microbiota, since at 6 months there is a change of feeding from milk, breastfeeding or formula, to complementary feeding and solid foods. On the other hand, 18 months of age is a period in which solid foods have been established in almost 90% of the

children's diet, determining a microbial community more similar to that found in adults.

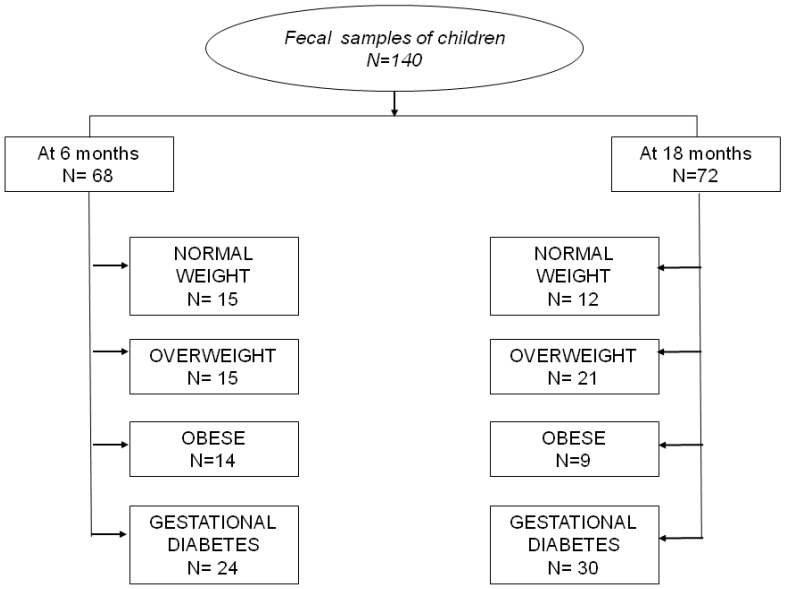


Figure 5. Stool samples were obtained from 140 infants participating in the PREOBE Follow-up study; 68 samples were collected at 6 months and 72 at 18 months. Within the Gestational Diabetic group, there were normal-weight, overweight and obese mothers.

Results

Paper 1

Cerdó, T., Ruiz, A., Acuña, I., Jáuregui, R., Jehmlich, N., Haange, S. B., von Bergen, M., Suarez, A. & Campoy, C. (2018). **Gut microbial functional maturation and succession during human early life**. *Environmental microbiology*. https://doi.org/10.1111/1462-2920.14235

Impact factor: 5,395; Q1; Category: MICROBIOLOGY; Ranking: 18/125

Paper 2

Cerdó, T., Ruiz, A., Jáuregui, R., Azaryah, H., Torres-Espínola, F. J., García-Valdés, L., Segura, M.T., Suarez, A. & Campoy, C. (2018). **Maternal obesity is associated with gut microbial metabolic potential in offspring during infancy**. *Journal of physiology and biochemistry*, *74*(1), 159-169. doi: 10.1007/s13105-017-0577-x

Impact factor: 2,444; Q2; Category: PHYSIOLOGY; Ranking: 37/84

Paper 3

Cerdó, T., García-Valdés, L., Altmäe, S., Ruíz, A., Suárez, A., & Campoy, C. (2016). **Role of microbiota function during early life on child's neurodevelopment**. *Trends in food science & technology*, *57*, 273-288. https://doi.org/10.1016/j.tifs.2016.08.007

Impact Factor: 5,191; Q1; Category: FOOD SCIENCE & TECHNOLOGY; Ranking: 4/130

Paper 4

Cerdó, T., Ruíz, A., Suárez, A., & Campoy, C. (2017). **Probiotic, Prebiotic, and Brain Development**. *Nutrients*, *9*(11), 1247. https://doi.org/10.3390/nu9111247

Impact Factor: 3,550; Q2; Category: NUTRITION & DIETETICS; Ranking: 23/81

Draft paper 5

Cerdó T., Ruiz, A., Acuña, I., Torres-Espínola, F.J., Jáuregui, R., Jehmlich, N., Haange, S. B., Martin von, B., Suárez, A. & Campoy, C. **Intestinal bacteria are associated with the cognitive development of children at 6 months of age.**

PAPER 1

Gut microbial functional maturation and succession during human early life

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Abstract

The evolutional trajectory of gut microbial colonization from birth has been shown to prime for health later in life. Here, we combined cultivation independent 16S rRNA gene sequencing and metaproteomics to investigate the functional maturation of gut microbiota in faecal samples from full-term healthy infants collected at 6 and 18 months of age. Phylogenetic analysis of the metaproteomes showed that *Bifidobacterium* provided the highest number of distinct protein groups. Considerable divergences between taxa abundance and protein phylogeny were observed at all taxonomic ranks. Age had a profound effect on early microbiota where compositional and functional diversity of less dissimilar communities increased with time. Comparisons of the relative abundances of proteins revealed the transition of taxon-associated saccharolytic and fermentation strategies from milk and mucin-derived monosaccharide catabolism feeding acetate/propanoate synthesis to complex food-derived hexoses fuelling butanoate production. Furthermore, co-occurrence network analysis uncovered two anti-correlated modules of functional taxa. A low-connected *Bifidobacteriaceae*-centred guild of facultative anaerobes was succeeded by a rich club of obligate anaerobes densely interconnected around *Lachnospiraceae*, underpinning their pivotal roles in microbial ecosystem assemblies. Our findings establish a framework to visualize whole microbial community metabolism and ecosystem succession dynamics, proposing opportunities for microbiota-targeted health-promoting strategies early in life.

Introduction

The human gut is a bioreactor with a microbiota that consists of hundreds or thousands of bacterial species-level taxa, dominated by the phyla *Firmicutes* and *Bacteroidetes* with the less abundant phyla *Proteobacteria*, *Actinobacteria* and *Verrumicrobia* (Harmsen and de Goffau, 2016). The composition of the gut microbiota is influenced by genetic and environmental factors starting early in life (Charbonneau et al., 2016; Milani et al., 2017). Considerable efforts have focused on cataloguing the composition of the infants' gut microbiota (Palmer et al., 2007; Qin et al., 2010; Gosalbes et al., 2011; Koenig et al., 2011; Franzosa et al., 2014; Valles et al., 2014; Backhed et al., 2015; Asnicar et al., 2017; Cerdó et al., 2017) because the evolutional trajectory of gut microbiota from birth has been shown to prime for health later in life (Cryan and Dinan, 2012; Cox et al., 2014; Cerdó et al., 2016; Tamburini et al., 2016). These studies have shown that the initial microbiota evolves over time, increasing diversity and adapting to the anaerobic environment and nutrient availability.

However, current knowledge on infants' gut microbiota has been almost exclusively obtained from 16S rRNA gene sequencing, metagenomics and metatranscriptomics analyses. A major limitation of these DNA-based studies is that they infer potential functions, providing limited insight into the metabolic landscape and dynamic interplay of the

gut microbiota. Because protein abundance is a reflection of specific microbial activities in a given ecosystem, metaproteomics exploits the power of high performance mass spectrometry (MS) to simultaneously address composition and function in microbial communities (Hettich et al., 2013). So far, three intestinal metaproteomics studies on preterm neonates and one on term infants have been carried out (Klaassens et al., 2007; Brooks et al., 2015; Young et al., 2015; Zwittink et al., 2017), leaving the dynamics of microbial functional maturation during early life largely unexplored. Here, we combined cultivation-independent 16S rRNA gene sequencing and metaproteomics to investigate the functional differentiation of gut microbiota in faecal samples from full-term healthy infants collected at 6 and 18 months of age. We used the metaproteomics data in a systematic comparative strategy to provide direct evi- dence of active microbial taxa, functional signatures and topological architecture of gut microbial interactions char- acteristic to each chronological state.

Results and discussion

Considering the metaproteome analysis as indicators of current metabolic activity and physiological adaptation provides microbial ecologists with a robust framework, facilitating a closer understanding of the complex dynamics that drive ecosystem functional and compositional responses to environmental pressures (Hettich et al., 2013). Our hypothesis is that the metaproteome provides insight about the relative importance of its members in ecosystem functioning, their collective functional pattern and the network topology in relation to host physiology. Our goal was to address those questions during early human life.

Taxonomic profiling of gut microbiota and their proteins

We collected faecal samples from healthy infants of 6- and 18-months of age (Supporting Information Table S1) to characterize the gut microbial composition by 16S rRNA gene sequencing and the expressed proteins by shotgun metaproteomics. The phylogenetic composition and categorical breakdown of identified OTUs and proteins in our samples are presented in the supporting material (Supporting Information Table S2). After quality filtering, 7,890,853 read sequences rendered a gut microbial profile consisting of 679 species-level bacterial operational taxonomic units (OTUs) that narrowed to 89 distinct genera belonging to 40 families after high confidence phylogenetic annotation (Fig. 1A). In total, 11,901 peptides were identified, of which 9,173 bacterial protein groups were assigned and unambiguously quantified. These protein groups were assigned to 134 genera belonging to 61 families (Fig. 1A). This is the highest number of distinct proteins groups identified in human gut metaproteomics studies published so far, but indicate the high variability thorough the investigated cohorts. Moreover, taxonomic analysis of metaproteomics data showed that protein coverage and abundances within each phylum were highly diverse (Supporting Information Fig. S1). In *Actinobacteria*, *Bifidobacterium* accounted for most protein group abundances. Notably, the greatest number of distinct protein groups annotated to a genus belonged to *Bifidobacterium*, which emphasizes its functional significance in the gut during early life (Charbonneau et al., 2016). *Akkermansia* within *Verrucomicrobia* accounted for 2.7% of total protein groups. *Parabacteroides*, *Prevotella* and *Alistipes* had the highest number of protein groups in *Bacteroidetes*. We were able to identify protein groups across sixty-six different genera in *Firmicutes* and forty-five genera in *Proteobacteria*. Bins of groups that represented more than 50% of proteins in *Firmicutes* were assigned to *Faecalibacterium*, *Ruminococcus*, *Veillonella*, *Roseburia* and *Eubacterium*. In *Proteobacteria*, *Pseudomonas*, *Parasuterella*, *Haemophilus*, *Bilophila*, *Escherichia* and *Desulfovibrio* were the highest contributors to protein group abundances. Our results are in agreement and further extend previous reports on the phylogenetic diversity of microbial proteins within the human gut (Klaassens et al., 2007; Verberkmoes et al., 2009; Rooijers et al., 2011; Kolmeder et al., 2012; Ferrer et al., 2013; Perez-Cobas et al., 2013; Juste et al., 2014; Brooks et al., 2015; Kolmeder et al., 2016; Tanca et al., 2017; Zwittink et al., 2017).

Comparison of metaproteomics and compositional data derived from 16S rRNA analysis

In human gut microbial ecology, a major challenge is to identify its active members whose response to environmental factors or disease-induced dysbiosis ultimately influence host homeostasis (Mao and Franke, 2015). Conventional DNA-based approaches inform about gene content and metabolic potential but do not inform about biological activity since all microbial DNA will be sequenced (Cangelosi and Meschke, 2014). Metaproteomics offers large-scale functional and phylogenetic profiling of expressed proteins and, thus, a feasible approx- imation to characterize biological activity within microbial ecosystems (Hettich et al., 2013). To estimate the biological activity of the members of the gut microbial community, we compared the log ratio of abundances between organism-origin of protein groups and 16S rRNA gene abundances. Though previous studies reported good correlations between 16S rRNA gene abundances and microbial source of protein groups (Rooijers et al., 2011; Kolmeder et al., 2016), our analysis yielded a high number of deviations even at upper taxonomic ranks (Fig. 1B), suggesting discordance between microbial membership

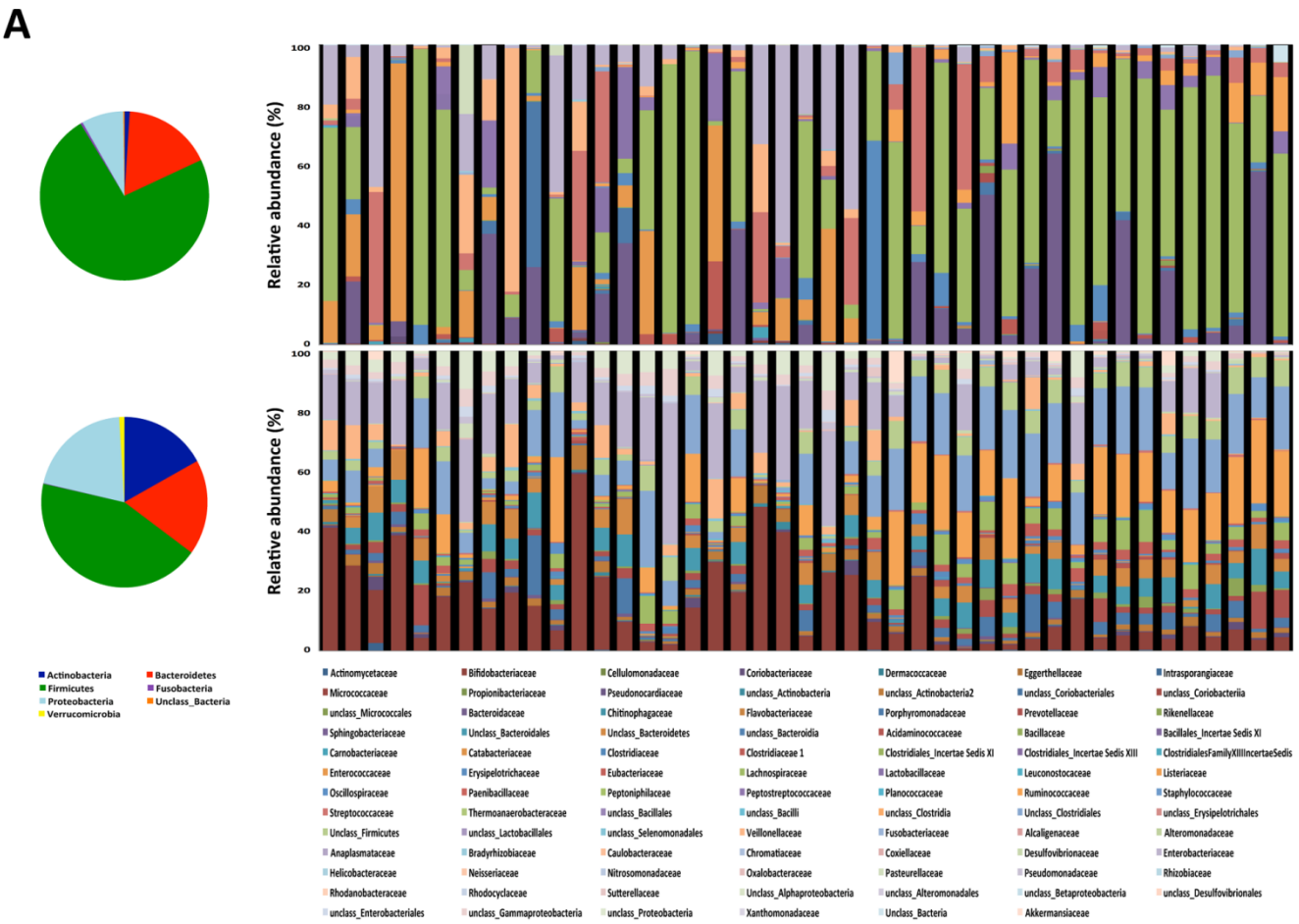

Fig. 1A. Taxonomic distribution and comparison of 16S rRNA sequences and protein groups characterized in faecal samples from the studied cohort of infants.(A) Phylogeny at phylum (circular graph) and family (bar graph) levels of total (16S rRNA gene sequences, top plots) and functional (organism- origin of protein groups, bottom plots) gut microbiota. Results for the same sample are lined up in bar plotsThe heights of the rectangles indicate mean relative abundances in the datasets. Full description of the differences is shown in Supporting Information Table S3.

and biological activity. The deviations were in a many cases of an order-of-magnitude (highly significant absolute fold change >10) either lower or higher than expected from 16S rRNA gene abundances of their corresponding taxa (Supporting Information Table S3). The most signifi- cant deviation was the high relative proportion of protein groups identified for *Verrucomicrobia* versus its null detection by 16S rRNA gene sequencing. *Actinobacteria* showed a very high log ratio with *Bifidobacterium* account- ing for high protein abundance, suggesting a high activity of this taxon in early life microbiota. Despite *Bacteroides* was the most abundant genus, most protein groups were assigned to *Parabacteroides*, *Prevotella* and *Alistipes*. Significant differences in the ratios for Lachnospiraceae and *Ruminococcaceae* in *Firmicutes* were also observed. The strongest negative ratios were observed in *Streptococcus*, *Veillonella*, *Enterococcus* and *Blautia* whereas the positive ratios observed for Faecalibacterium, Flavonifractor and *Oscillibater* suggested high biological activity. Thus, our metaproteomics data revealed that the gut microbiota harbours a distinctive subset of biologically active microorganisms as consistently shown in other reports (McNulty et al., 2011; Ferrer et al., 2013; Maurice et al., 2013; Maurice and Turnbaugh, 2013; Perez-Cobas et al., 2013). While we cannot exclude analytical biases in DNA and protein extraction methods, these discordances suggest that using bacterial taxa as input information to build predictive theoretical models of microbial activity and contribution to community functioning in human gut microbial ecosystems may be highly misleading.

Temporal patterns of qualitative diversity in the gut microbiota

We initially calculated the proportion of variance (coefficient of determination, R^2) in microbiota composition and

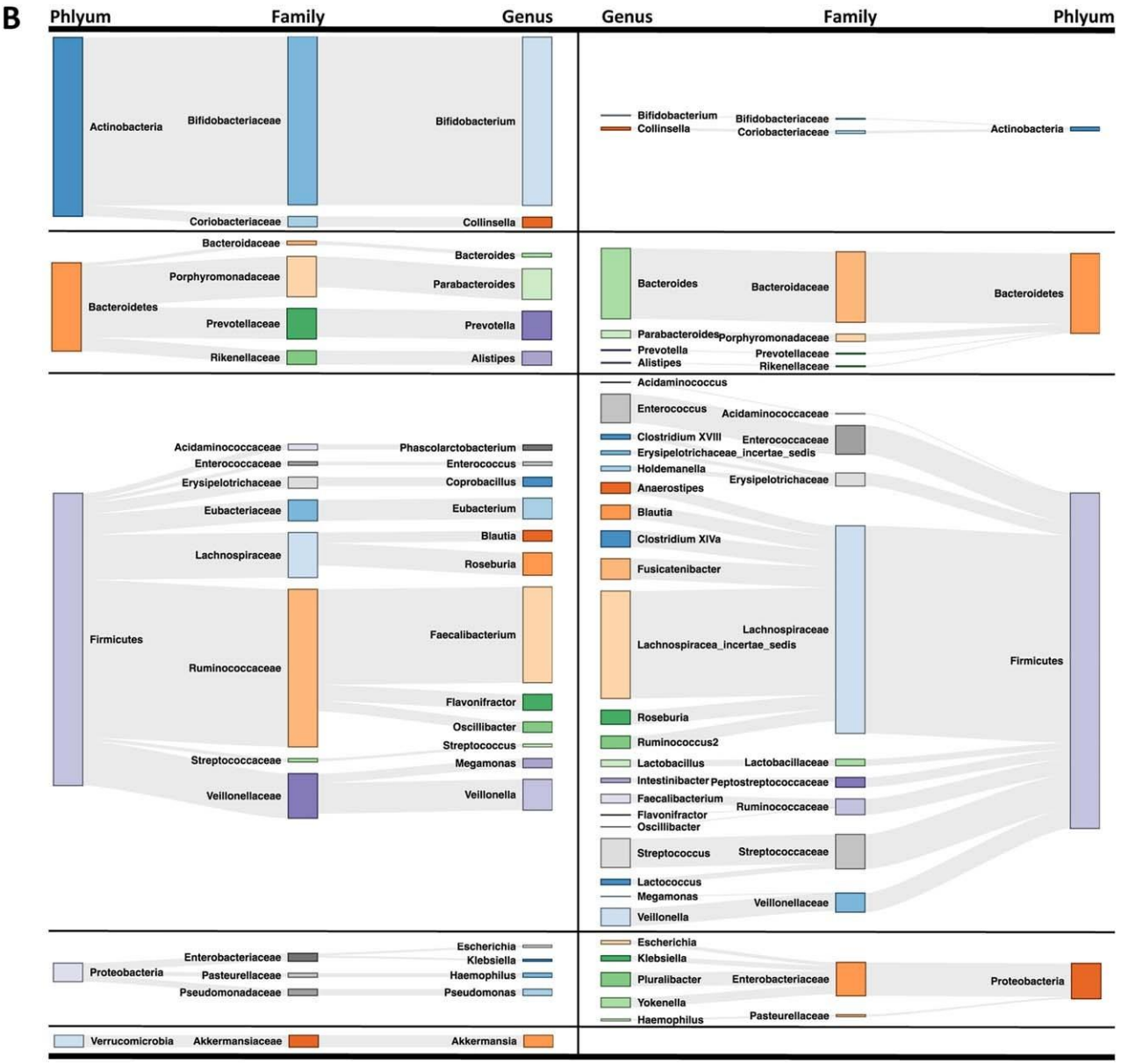

Fig. 1 B. Comparisons between the phylogeny of total (right) and functional (left) gut microbiota are tracked and confronted using a Sankey plot. To reduce the size of the Sankey plot, only highly abundant genera (mean relative abundance >1%) are shown. The heights of the rectangles indicate mean relative abundances in the datasets. Full description of the differences is shown in Supporting Information Table S3.

function that was explained by study variables and individuals (Supporting Information Table S4A and B). In microbiota composition, age and individuality explained 7 and 11% of the total variation in agreement with previous reports (Zoetendal et al., 2001; Goodrich et al., 2014; Salo- nen et al., 2014; Backhed et al., 2015; Falony et al., 2016). Mode of delivery or pre-pregnancy mother's body mass index did not influence microbial composition in our data- set. Age was the single significant variable explaining a relevant proportion (13.5%) of total variation in microbiota function.The unprecedented impact of age upon micro- biota function is remarkable, given the high variation assigned to individuality and/or mode of delivery consistently reported in compositional studies of gut microbiota. b-diversity metrics of total (phylogeny of OTUs) and functional (phylogeny of proteins) gut microbial communities confirmed that samples clustered by age (Fig. 2A–C). We observed increased a-diversity but reduced b-diversity as a function of time, suggesting that both total and functional communities accumulated diversity into less heterogeneous structures (Fig. 2D). Despite taxonomic divergence, Bray-Curtis dissimilarity metrics showed that the functional community was increasingly more con- served among infants than the total community (Fig. 2E). This result is in line with the hypothesis suggesting that gut microbiota is assembled around a between-subject more

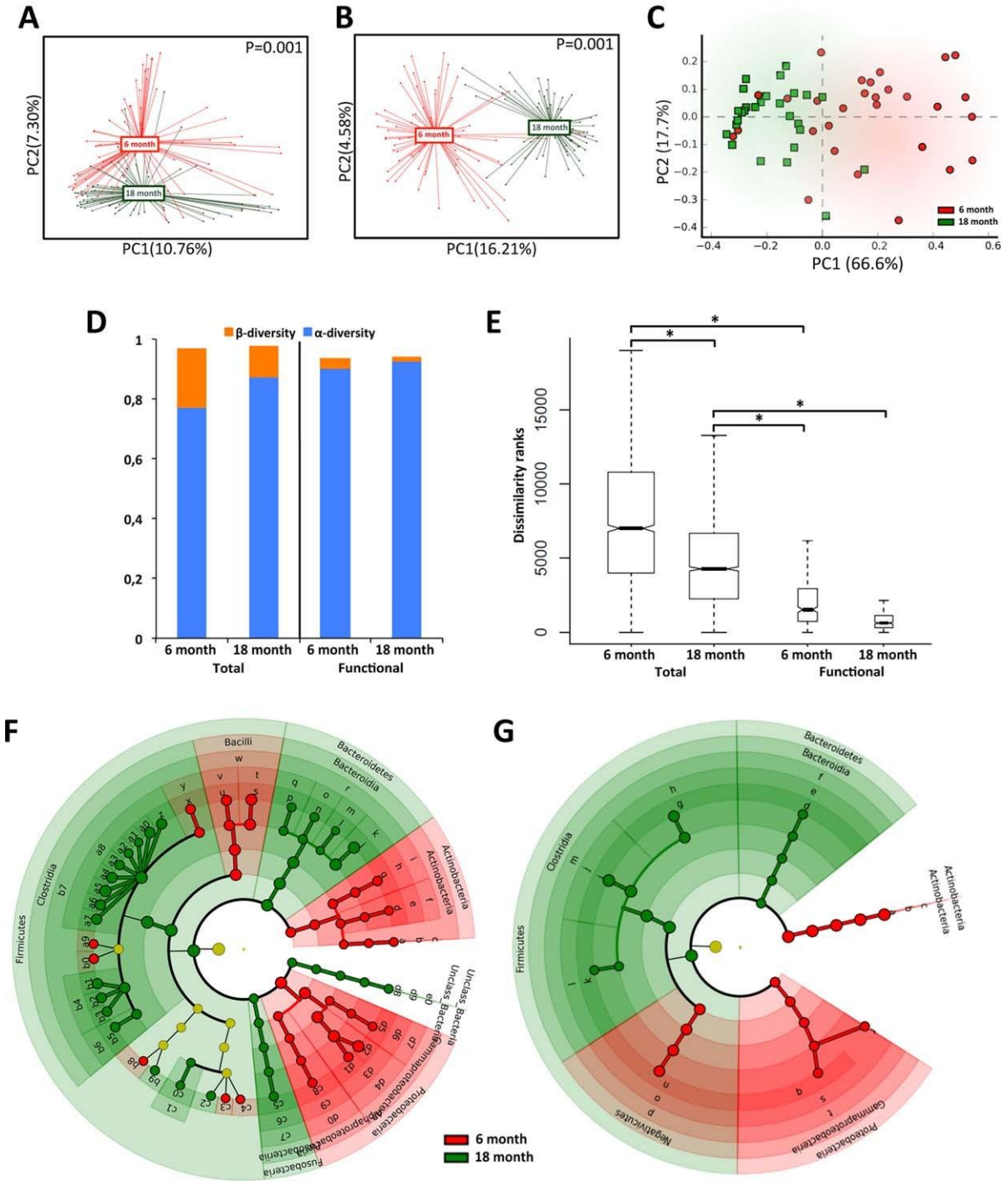

Fig. 2. Total (16S rRNA gene sequences) and functional (organism-origin of protein groups) gut microbial communities clustered according to age. (A and B) Scatterplot from principal coordinate analysis using weighted and unweighted Unifrac metrics in 6-months (red) and 18-months (green) old infants. p value for PERMANOVA test with 999 permutations. (C) Principal component analysis based on the phylogeny at phylum level of protein groups according to Bray-Curtis dissimilarity metrics in 6-months (red) and 18-months (green) old infants. (D) a-diversity and b-diversity of total and functional gut microbiota determined by Rao's diversity at phylum level. (E) b-diversity (Bray-Curtis dissimilarity metrics) of total and functional gut microbiota at phylum level. (F and G) Differentially abundant microbial taxa in total and functional gut microbiota of 6-months (red) and 18-months (green) old infants. Significantly discriminant taxon nodes are coloured and branch areas are shaded according to the highest ranked community for that taxon. If the taxon is not significantly different between sample communities, the corresponding node is coloured in yellow. For simplicity, only taxa meeting a linear discriminant analysis significant threshold >2 are shown. Full description of discriminant genera is shown in Supporting Information Table S5.

conserved consortium of biologically active microorganisms (Turnbaugh et al., 2009; Burke et al., 2011; Huttenhower et al., 2012; Franzosa et al., 2014; Moya and Ferrer, 2016; Ruiz et al., 2017). We used LEfSe, a tool for metagenomic biomarker discovery (Segata et al., 2011) to further explore age-associated shifts in total and functional gut microbiota. Many taxa at multiple phylogenetic depths were found at significantly different relative abundances between time points (Fig. 2F–G and Supporting Informa- tion Table S5). *Firmicutes* dominated the total community and its functional subset that enriched in *Bacteroidetes* and *Firmicutes* and depleted in *Proteobacteria* and *Actino- bacteria* with time. In total gut microbiota, signature highly abundant genera (>1% mean relative abundance) at 6- months were *Enterococcus*, *Lactobacillus*, *Erysipelotrichaceae_Incertae_Sedis*, *unclassified_Veillonellaceae* and *unclassified_Enterobacteriaceae* while the 18-month's total gut microbiota was significantly enriched in obligate anaerobes from the genera *Bacteroides*, *Anaerostipes*, *Blautia*, *Fusicatenibacter*, *Lachnospiraceae_incertae_sedis*, *Roseburia*, *Ruminococcus2*, *Faecalibacterium* and *unclassified_Clostridiales*. The functional gut microbiota was characterized by few signature genera due to the high inter-individual variability. In 6-month's functional gut microbiota, signature highly abundant genera were *Bifidobacterium* and *Veillonella* while *Eubacterium* and *Faecalibacterium* were enriched at 18 months. In agree- ment with previous studies (Koenig et al., 2011; Valles et al., 2014; Backhed et al., 2015), our findings reflected the shift of gut microbiota towards an adult-like structure and composition as infants grew, possibly associated to physiological fitness to persist in increasingly lower oxygen levels.

Enrichment analysis identifies age-specific functional signatures in the gut microbiota

To determine how the functional capacity of the gut microbiota developed during early life, we analysed the metaproteome using Clusters of Orthologous Groups (COG) categories, hierarchically organized in three tiers where each tier is increasingly a more specific functional assignment (main, secondary and function categories). Due to the functional redundancy of orthologous proteins in our metaproteomics dataset, 9,173 protein groups nar- rowed to 1,117 COG functions (Supporting Information Table S6). The mean number of protein groups per sample was 895 ± 49. Overall distribution exhibited a rather even pattern across the samples where the most abundant secondary COGs belonged to 'Metabolism' category: 'Carbohydrate Transport and Metabolism', 'Amino Acid Transport and Metabolism', 'Energy Production and Conversion' and 'Inorganic Ion Transport and Metabolism' (Fig. 3A). This result is consistent with previous reports on the functional profile of protein groups expressed by gut microbiota (Verberkmoes et al., 2009; Rooijers et al., 2011; Ferrer et al., 2013; Perez-Cobas et al., 2013; Kolmeder et al., 2016). A group of 30 COG functions were identified in the gut microbiota of 90% of the subjects and may represent a functional core of biological processes (Supporting Information Table S6). Interestingly, seven of these core functions were enzymes also identified in adults (Verberkmoes et al., 2009; Kolmeder et al., 2012) suggesting a high stability of these proteins across human metaproteomes. These enzymes were glutamine synthetase and glutamate dehydrogenase in 'Amino acid transport and metabolism', and enolase, glyceraldehyde-3-phosphate dehydrogenase and fucose, glucuronate and xylose isomerases in 'Carbohydrate Transport and Metabolism'.

To look for significantly over- and under-represented COG functions in the gut metaproteomes of 6- and 18- months old infants, a comparative analysis was performed. The mean number of COG functions per sample was significantly higher in the 18-months metaproteome (1118 ± 94) than in the 6 months one (767 ± 59), indicat- ing enrichment in microbial functionalities with age ($p < 0.01$). Principal component analysis based on COG functions plot revealed a clear segregation between the two time points, with 40.6% of variance explained by the first component (Fig. 3B). These results indicated that functional complexity increased with time to create more similar inter-individual functional communities.

Metaproteomics analyses revealed significant differences between the sampled time points (Fig. 3C and Supporting Information Table S7). In the 6 months' gut microbiota, we observed over-representation of COGs classified into the main COG category 'Cellular processes and signalling', distributed within 'Cell wall membrane envelope biogenesis', 'Cell motility', 'Intracellular Trafficking Secretion and Vesicular Transport' and 'Signal transduction mechanisms'. The 18-month metaproteome was enriched in COGs classified into the main COG category 'Metabolism', distributed within 'Lipid transport and metabolism' and 'Nucleotide transport and metabolism'. The most significant COG functions within 'Cell wall membrane envelope biogenesis' were a protein translocase and a S-ribosylhomocysteine lyase mostly assigned to *Bifidobacterium*, involved in the control of gut colonization and protection against pathogens during early life (Christiaen et al., 2014). Significant abundances of an outer mem- brane adhesion protein involved in b-lactam resistance, an attachment invasion locus protein and a lipopolysaccharide transport and assembly protein binned to *Enterobacteriaceae*, and an autotransporter adhesin assigned to *Veillonellaceae* were observed. In addition, we identified significant abundances of a Dps/Dpr ferritin-like protein involved in iron incorporation and six ABC-type

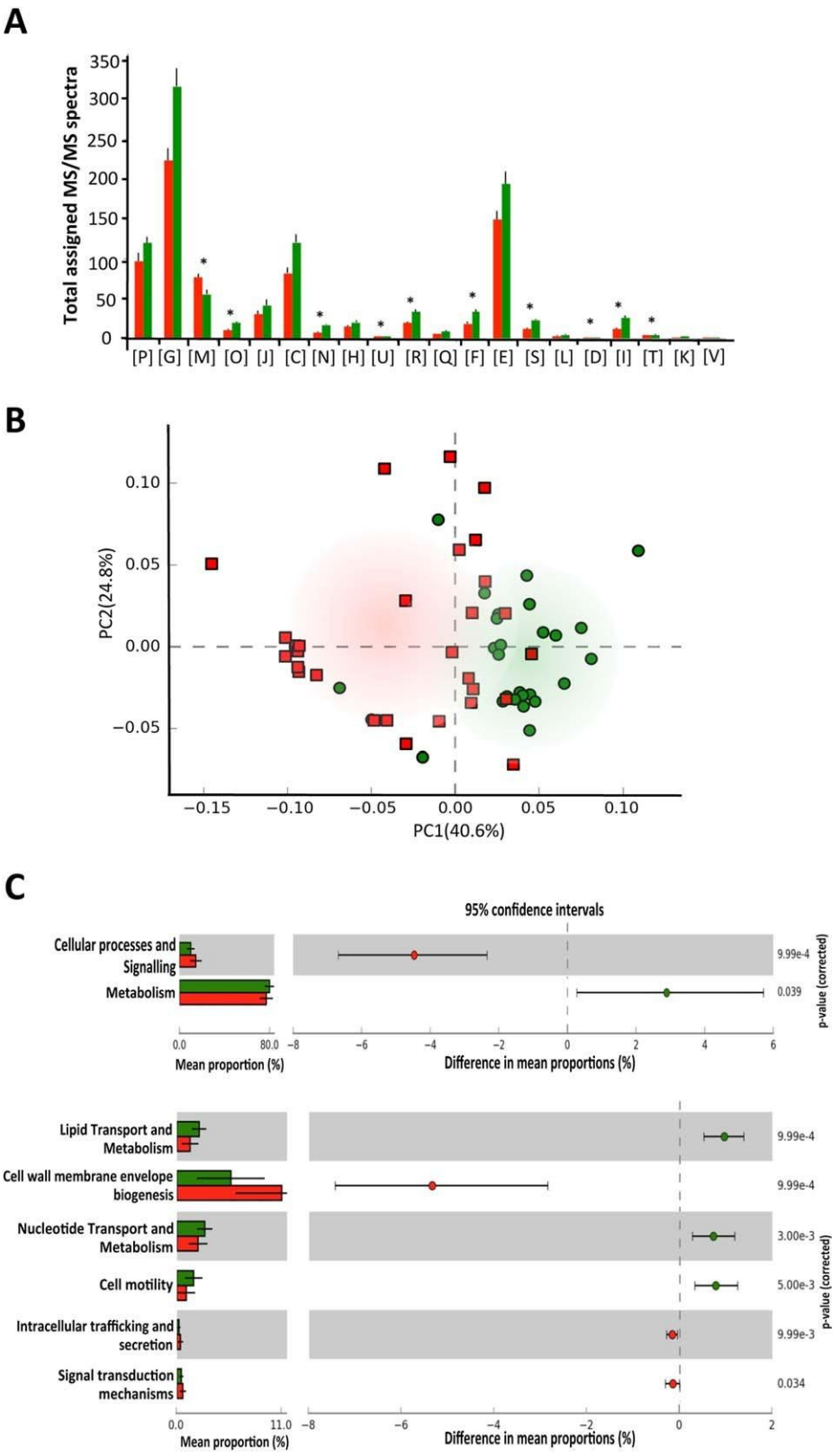

Fig. 3. Comparison of metaproteomics profiles between 6-months (red) and 18-months (green) old infants. (A) COG distribution of the protein groups detected in the metaproteomes of 6-months and 18-months old infants. (B) Principal component analysis based on COG function patterns according to Bray-Curtis dissimilarity metrics in 6-months and 18-months old infants. (C) Differences in functional comparisons of metaproteomes from 6-months and 18-months infants at main (top plot) and secondary (bottom plot) COG hierarchy levels. Left: histogram: relative mean proportions and deviations; right plot: differences between proportions and significances.

transporters for bicarbonate, ion metals (nickel), amino acids (arginine, lysine, histidine and glutamine), dipeptides (cationic peptide) and inorganic oxides (phosphate, molybdate and tungstate) (Supporting Information Fig. S2). According to its central role, these transporters were assigned to multiple taxa within *Actinobacteria* (*Bifidobacteriaceae*), *Firmicutes* (mostly *Ruminococcaceae*) and *Proteobacteria* (mostly *Enterobacteriaceae*). In contrast, only two COG functions involved in transport were enriched in the 18-months' microbiota, an oligopeptide ABC transporter binding lipoprotein binned to *Bifidobacter- iaceae* and *Ruminococcaceae*, and a C_4-dicarboxylate- transport protein assigned to *Ruminococcaceae*. The identification of proteins involved in cellular transport is consistent with the observations of previous metapro- teomes (Kolmeder et al., 2012). Different taxa within Clostridia were responsible for the abundance of lysozyme and carbon starvation protein A involved in cell defence, motility and agglutination. Taken together, this pattern of COG functions suggested that surface and signalling pro- teins of the gut microbiota were highly abundant at early stages since they regulate gut colonization and interaction with host cells.

Metabolic signatures differentiate chronological states of infants' gut microbiota

An interesting finding was that 32 COG functions within 'Metabolism' with relevant roles in polysaccharide catabolism, central carbon metabolism and fermentation were differentially abundant between 6- and 18-months gut microbiota. To facilitate the understanding of these differences, we manually parsed and illustrated these functions into a hierarchically clustered heatmap and a comprehensive model of carbon metabolism (Fig. 4). The phylogenetic assignment of the functions at phylum level is provided (Supporting Information Table S7).

Polysaccharide catabolism: In an initial step, primary fermenters provide the cocktail of glycoside hydrolases (GH) or glycosidases to breakdown host glycans and dietary polysaccharides. Several metagenomic studies have determined the diversity of GH encoding genes in the infant and adult gut microbiota that ranged from 14 to 25 GH families according to the carbohydrate-active enzymes database (CAZy) (Cantarel et al., 2009; Tasse et al., 2010; Cecchini et al., 2013; El Kaoutari et al., 2013; Backhed et al., 2015). We detected abundances of 24 GH COGs that belong to 20 GH families. To the best of our knowledge, metaproteomics results on the GH repertoire of gut microbiota have not been reported in such detail. The most abundant GH COG was β-galactosidase/β-glucuronidase, consistent with its high activity in gut microbiota (Hernandez et al., 2013), to which the largest number of peptides could be mapped in GH family. The mean number, protein abundance and catalytic activities of GH increased with age (Fig. 4A). These results reflected the high GH potential of the gut microorganisms metabolically prepared to degrade human mucin, milk oligosaccharides, plant and animal polysaccharides, even in exclusive breast-fed infants and before the introduction of solid foods (Koenig et al., 2011; Flint et al., 2012; Tailford et al., 2015). This is not surprising since mucin-adapted resident mutualists can alternatively forage on dietary plant polysaccharides to ensure gut microbial stability as the infant diet transitions to solid food (Marcobal et al., 2013). Consistent with the contribution of human and formula milk to infant diet, the microbiota of 6-months infants was enriched in β-galactosidase and arabinogalactan endo-1,4-β-galactanase, mostly expressed by *Actinobacteria*. Additionally, *Actinobacteria* and *Firmicutes* expressed an a-glucoside phosphotransferase IIC subunit, involved in the phosphorylative transport of glucose, glucosamine and n-acetylneuraminic acid while *Proteobacteria* expressed maltoporin involved in maltose and maltodextrin transport. In contrast, the higher diversity and complexity of dietary carbohydrates in 18-months' infants resulted in a significant enrichment in α-amylase, α-glucosidase and β-glucosidase that were expressed by multiple taxa within *Bacteroidetes* and *Firmicutes*. Endo-β-N-acetylglucosaminidase D involved in the hydrolysis of branched oligosaccharides was expressed only by *Bacteroidetes* while cellobiose phosphorylase involved in the phosphate-dependent hydrolysis of cellu- lose was assigned to *Firmicutes*. The determination of gut microbial β-galactosidase, α-glucosidase and β-glucosidase activities in 6- and 18-monts infants confirmed their enrichment in the metaproteomes (Supporting Information Fig. S3). Taken together, these results indicated that the gut microbiota used the proper upper glycolytic pathways depending on the availability of the carbohydrate source in a diet shifting from breast milk or formula to solid foods.

ii Central carbon metabolism: Once a monosaccharide enters a cell, it flows through the Embden-Meyerhoff-Parnas (EMP), the pentose phosphate (PP) and the Entner-Doudoroff (ED) pathways that convert monosac- charides into phosphoenolpyruvate (PEP) (Fig. 4B). As expected, all COG functions in the EMP pathway were detected in the metaproteomes due to its central metabolic role. We observed very little abundance of pyruvate kinase, indicating that synthesis of PEP was the main outcome of EMP pathway in gut microbiota. Instead, PEP carboxylase was expressed in both metaproteomes because it allows bacteria to extract the second equivalent of ATP and generate oxaloacetate in an anaerobic environment (Macy and Probst, 1979). In EMP, two COG functions were significantly enriched in the 6 months' gut microbiota:

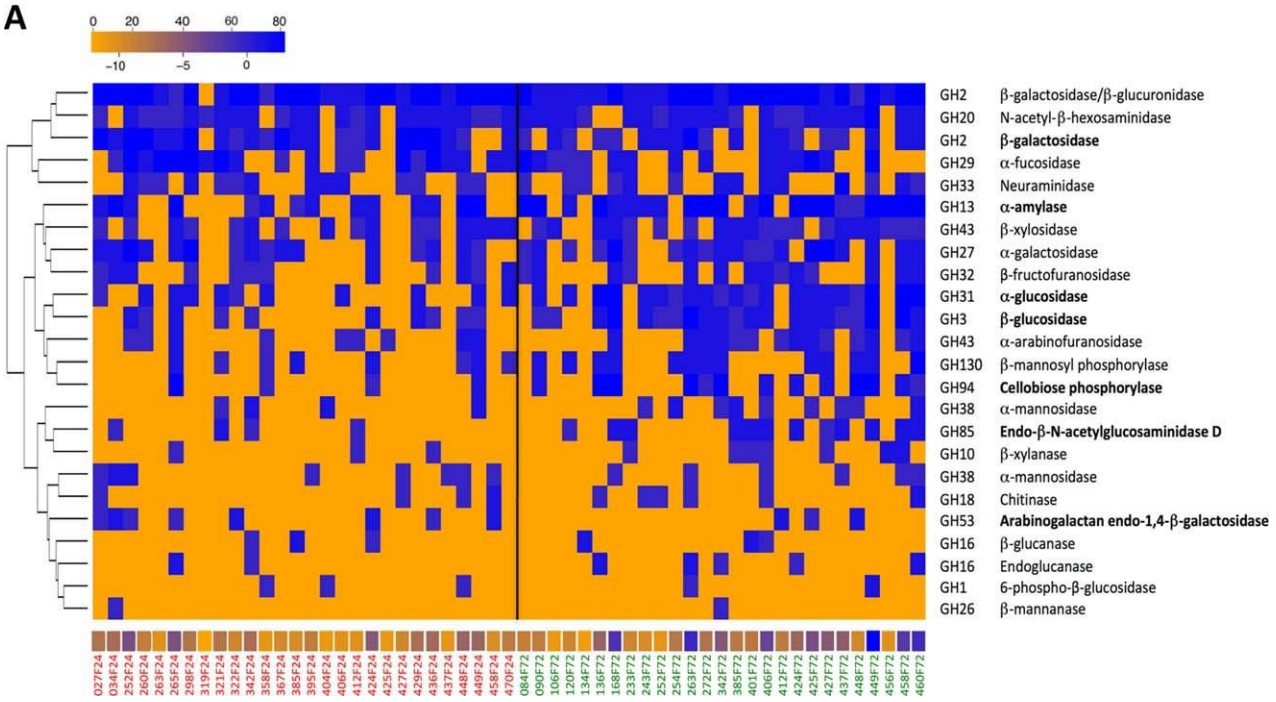

Fig. 4. Metabolic signatures that differentiate between the metaproteomes of 6-months and 18-months old infants.
(A) Hierarchical clustering and heatmap of the abundances (log_{10} values) of glycoside hydrolases (GH) characterized in the metaproteomes of 6-months and 18-months old infants. CAZy families (right) and summation of GH abundances (bottom) in samples are shown. Values are indicated by colours ranging from orange to blue in log_{10} (bottom, ≥14 to 4) and absolute scales (top, 0 to 80). GH names with significant differences between infant groups are highlighted in bold. Each vertical line corresponds to one sample, identified at the bottom of the bar plot by a code that specifies the corresponding time-point (red, F24 for 6 months; green, F72 for 18-months). (B) Enzymes with significantly different abundances between the metaproteomes of 6-months (red arrows) and 18-months (green arrows) old infants are highlighted over a schematic carbon metabolism summary based on KEGG pathway maps. Semi-transparent boxes delimitate major central metabolic pathways (Embden-Meyerhoff-Parnas, EMP; pentose phosphate, PP; Entner-Doudoroff, ED) and short-chain fatty acids. Black arrows indicate enzymes detected in both metaproteomes with no differential abundance. Orange and purple boxes mark starting carbon substrates. Enzymes are indicated in the map as follows: (1) glyceraldehyde-3-phosphate dehydrogenase. (2) Phosphoglyceromutase. (3) enolase. (4) transketolase. (5) citrate lyase. (6) isocitrate dehydrogenase. (7) succinate dehydrogenase. (8) fumarate reductase. (9) acetate:succinate CoA-transferase. (10) galactokinase. (11) galactose mutarotase. (12) gluconate/galactonate dehydratase. (13) N-acetyl- glucosamine 6-phosphate 2-epimerase. (14) 2-dehydro-3-deoxy-rhamnonate aldolase. (15) fucose dehydrogenase. (16) acetate kinase. (17) glucuronate isomerase. (18) 2-dehydro-3-deoxy-phosphogluconate aldolase. (19) succinyl-CoA reductase. (20) acetyl-CoA acyltransferase. (21) enoyl-CoA hydratase. (22) 3-hydroxyacyl-CoA dehydrogenase. (23) ethanolamine ammonia-lyase.

glyceraldehyde-3-phosphate dehydrogenase assigned to taxa within *Bacteroidetes*, *Firmicutes* and *Proteobacteria* and enolase binned to taxa in *Actinobacteria*, *Firmicutes* and *Proteobacteria*. Glyceraldehyde-3-phosphate dehydrogenase and glutamate dehydrogenase were the COG functions to which the largest number of distinct peptides could be mapped in central carbon metabolism. While glyceraldehyde-3-phosphate dehydrogenase bridges PP and ED pathways to the lower EMP pathway, glutamate dehydrogenase has been shown to link the nitrogen and the carbon-cycle and to act as an electron sink in strict anaerobes (Kengen and Stams, 1994). The 6-month's metaproteome was also enriched in transketolase, an enzyme of PP pathway necessary for the *Bifidobacterium* shunt of glycolysis that yields acetate, glyceraldehyde-3-phosphate and ATP (de Vries et al., 1967). Finally, the null-detection of citrate synthase and the enrichment in citrate lyase in TCA cycle, assigned to *Firmicutes* and *Proteobacteria*, and isocitrate dehydrogenase, expressed by *Actinobacteria*, suggested that, in an environment that does not support aerobic respiration, bacteria may use the glut of PEP and the high availability of amino acids and CO_2 in a reverse TCA cycle to synthesise oxaloacetate (Macy et al., 1978).

With age, the 18-months metaproteome was also enriched in phosphoglyceromutase in EMP, mainly assigned to *Firmicutes*. In TCA cycle, we observed the increased abundances of two structurally and functionally- related membrane-bound enzymes, succinate dehydroge- nase (SDH) and fumarate reductase (FRD). SDH, the enzyme that catalyses succinate oxidation, was mainly assigned to *Firmicutes* while *Bacteroidetes* used FRD.

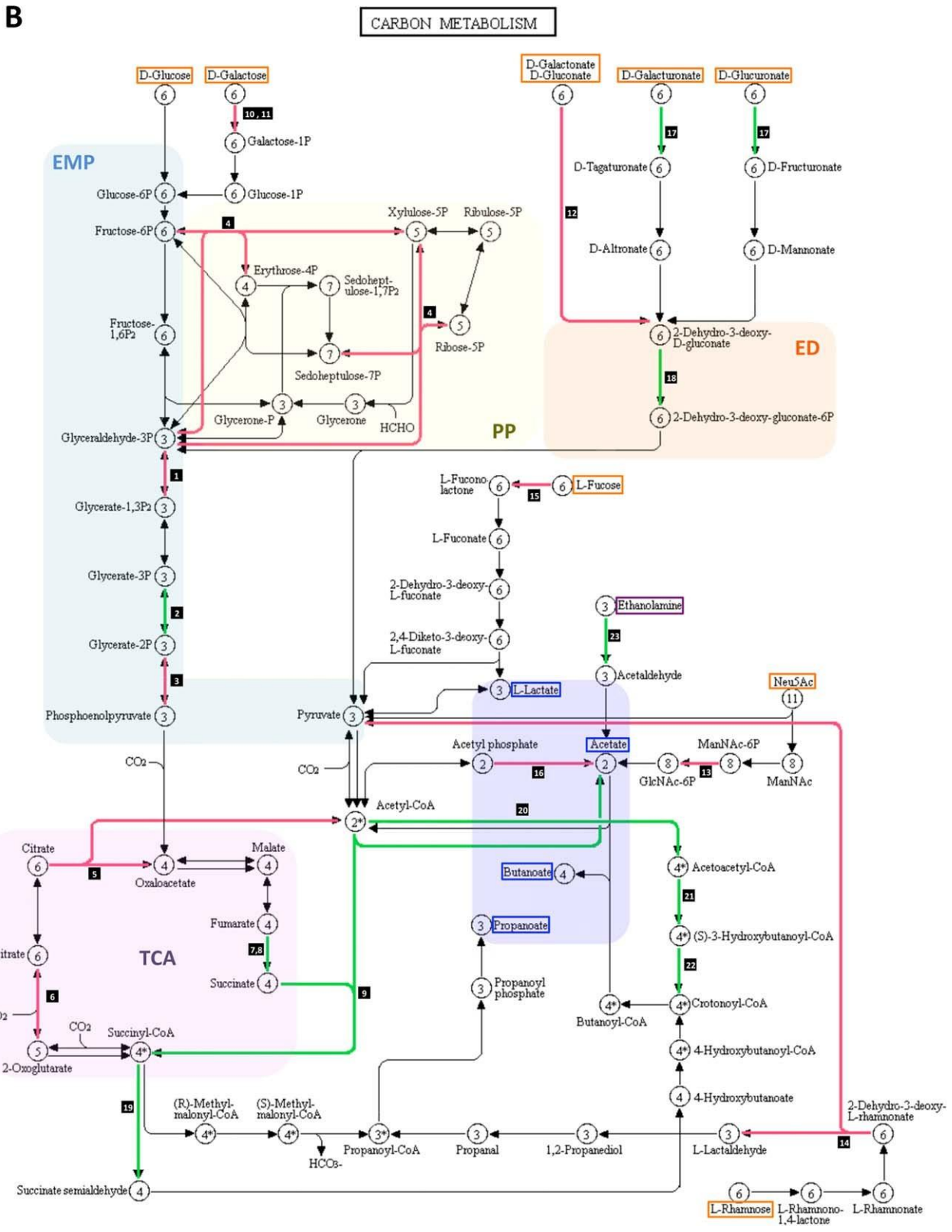

Fig. 4. (Continued.)

Fumarate reduction by FRD is the most used electron transport chain that generates ATP and succinate as a metabolic end product (Lu and Imlay, 2017). The fact that the subsequent step, succinyl-CoA synthesis, was very low abundant in *Bacteroidetes* is in line with the hypothesis of metabolic cross-feeding between *Bacteroidetes* and *Firmicutes* (Fischbach and Sonnenburg, 2011). An unexpected finding was the lack of detection of succinyl-CoA synthetase (SCS). Rather, our metaproteomics data suggested that taxa within *Firmicutes* and to a minor extent in *Bacteroidetes*, *Proteobacteria* and *Verrumicrobia* employed a variation of the classical TCA cycle based on an unorthodox enzyme, acetate:succinate CoA- transferase (ASCT) to synthesize succinyl-CoA. Kwong et al. recently showed that ASCT genes were widespread in prokaryotic genomes and functionally replaced SCS in TCA cycle of human microbial commensals (Kwong et al., 2017). In a carbon-rich anaerobic gut ecosystem, this strategy may be a result of niche specialization where gut microbiota may use acetyl-CoA, the keystone molecule of central metabolism produced from monosaccharides, amino acids, fatty acids and other secondary metabolites, as driver of a reverse TCA cycle to maintain redox balance and obtain energy for growth. These results indicated that the gut microbial community displayed distinct strategies for central carbon transformations for PEP synthesis and biosynthetic reactions.

iii Fermentation: Depending on the carbohydrate source and oxygen concentration, gut microbiota use distinct pathways of monosaccharide catabolism that end in the production of the main non-gaseous products of microbial fermentation: lactate and the short-chain fatty acids (SCFA) acetate, propanoate and butyrate (Fig. 4B).

The profile of COG functions revealed distinct pathways of monosaccharide catabolism in the infant gut at the sampled time points. In particular, the enrichment in galactokinase, galactose mutarotase, gluconate/galactonate dehydratase, N-acetyl-glucosamine 6-phosphate 2-epimerase, 2-dehydro-3-deoxy-rhamnonate aldolase (KDRA) and fucose dehydrogenase suggested active catabolism of milk and mucin-derived monosaccharides by early gut microbiota. The phylogenetic assignments of these enzymes showed that *Proteobacteria* expressed KDRA suggesting that they may contribute to propanoate fermentation by the propanediol pathway in early life microbiota. *Actinobacteria* was the major contributor to the catabolism of galactose via EMP, gluconate and galactonate via ED and the unique taxa to catabolize mucin- derived n-acetylneuraminic acid to acetate and fucose to lactate. Since we did not detect lactoyl-CoA dehydratase in the acrylate pathway, the fact that lactate dehydrogenase was highly abundant and phylogenetically assigned to all phyla suggested that fucose-derived lactate may be a cen- tral substrate for metabolic cross-feeding in early life microbiota (Pham et al., 2016). Moreover, we observed enrichment in acetate kinase that produces ATP and ace- tate as end product, a strategy mainly used by *Actinobacteria*. This result highlighted the importance of *Bifidobacteria* metabolic contribution in a gut that is starting to be colonized where they may benefit host physiology by fermenting host-derive glycans to provide acetate that reduces faecal pH and protects host epithelial cells from enterotoxins (Fukuda et al., 2011).

With age, the action of GH on solid foods with a high diversity in glycan compositions generates a richer repertoire of released monosaccharides available for microbial metabolism. Accordingly, the 18-months metaproteome was enriched in glucuronate isomerase, 5-dehydro-4-deoxy-glucuronate ketol-isomerase and 2-dehydro-3-deoxygluconokinase that channel these acid hexoses to pyruvate and glyceraldehyde-3-phosphate synthesis by 2-dehydro-3-deoxy-phosphogluconate aldolase (KDPGA) in a semi-phosphorylative ED pathway. KDPGA was the enzyme with the highest protein abundance in our metaproteomes suggesting that this catabolic pathway is metabolically important for Firmicutes and *Bacteroidetes*. In SCFA metabolism, the enrichment in succinyl-CoA reductase, acetyl-CoA acyltransferase, enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase, suggested that gut microbial metabolism shifted towards butyrate fermentation. The fact that these protein groups were mainly assigned to Clostridia support their important role in the metabolic welfare of colonocytes by releasing butyrate as a fermentation end-product (Lopetuso et al., 2013). The enrichments in ASCT and succinyl-CoA reductase suggest a link between succinate fermentation to butyrate and acetate production, as has been observed in Clostridium (Sohling and Gottschalk, 1996). Butyrate kinase protein group was not detected, confirming that butyril-CoA:acetate-CoA transferase pathway is preferred by gut microbiota (Flint et al., 2012). Notably, our metaproteome revealed an alternate route for acetate synthesis in 18-months gut microbiota. We observed an enrichment in ethanolamine ammonia-lyase that catalyzes the adenosylcobalamin (AdoCbl)-dependent conversion of ethanolamine to acetaldehyde and ammonia (O'Brien et al., 1985). Ethanolamine is abundant in the human gut because the constant renewal of the intestinal epithelium daily releases 25% of enterocytes whose membranes are rich in phosphatidylethanolamine (Snoeck et al., 2005). Thus, our data suggested that ethanolamine may be used as a source of carbon and energy under aerobic and anaerobic conditions by gut microbiota. Taken together, our metaproteomics revealed the gut microbial age-associated maturation of fermentative strategies to harvest energy from diverse carbon resources in a shifting glycobiome environment.

Bifidobacteriaceae and *Lachnospiraceae* are the hubs of succeeding anti-correlated functional co-occurrence modules in infant's gut microbiota

Analysis of microbial contributions to overall community activity indicated that the ecological network was remod- elled as the functional gut microbiota of infants evolved with time. The topology of the co-occurrence networks of active taxa collapsed at family level determined by Pearson's correlation coefficient showed two mutually exclusive modules clustered by age (Fig. 5A). A low con- nected module built by six families (20% of total nodes, 6.8% of total edges with a mean of 2 edges/node) evolved to a more complex and enriched one (80% of total nodes, 93% of total edges with a mean of 6.8 edges/node). It is plausible that these modules are built around ecologically relevant taxa whose pattern of functional interactions has a greater-than-average influence on network architecture. We calculated node parameters to identify families with the highest centrality in the modules (Supporting Information Table S8). *Bifidobacteriaceae* was the node with the largest fraction of shortest edge paths and highest betweenness centrality in 6-months microbiota.

These node properties defined *Bifidobacteriaceae* as a gate- keeper, cooperating simultaneously with different nodes of the module (Freeman, 1980). Its removal resulted in the fragmentation of this co-occurrence module, indicating that *Bifidobacteriaceae* was crucial for ecological module structure and persistence (Pocock et al., 2012). Eight Bifidobacterium species have been consistently identified in the human gastrointestinal tract (*Bifidobacterium adolescentis*, *B. breve*, *B. longum*, *B. pseudolongum*, *B. bifidum*, B. pseudocatenulanum, B. dentium and B. animalis) which protein affiliation in our metaproteomes ruled out B. dentium but included other twenty *Bifidobacterium* species, suggesting that functional diversity in the *Bifidobacteriaceae* family may be richer than taxonomic one (Turroni et al., 2009). This low connected *Bifidobacteriaceae*-cen- tred module anti-correlated with the highly connected (324 edges) and richer one (48 nodes) observed in 18-months' microbiota, suggesting competition or diversifying selection among modules. *Lachnospiraceae* was the hub (highest degree) in this cooperative module where *Desulfovibrionaceae*, *Ruminococcaceae*, *Rikenellaceae*, *Eubacteriaceae* and Porphyromonadaceae had a great importance in cooperative interactions in this module. *Lachnospiraceae*, *Eubacteriaceae* and *Clostridiaceae* may play a role in the transfer of biological information because these nodes shared the highest betweenness centrality values in this module. At the genus level, *Veillonella*, Escherichia, *Bifidobacterium* and *Pseudomonas* were mutually exclusive with *Eubacterium*, *Ruminococcus*, *Faecalibacterium*, *Alistipes* and *Bilophila*. Facultative anaerobes were not only more abundant but also were more positively correlated with obligate anaerobes in 6-months microbiota while showing many negative correlations in 18-months microbiota.

Further analysis revealed the succession of metabolic functions between taxa in these mutually exclusive consortia, suggesting a high level of functional redundancy between taxa (Moya and Ferrer, 2016; Ruiz et al., 2017). Taxa within *Bifidobacteriaceae*, *Enterobacteriaceae* and *Veillonellaceae* constituted a functional consortium responsible for an important proportion of amino acid, carbohydrate, coenzyme, energy, inorganic, nucleotide and secondary metabolisms in 6-months microbiota (Fig.5B). Age-related maturation restructured the contributions of taxa to metabolic performance where key co-occurrent families assembled an evolved functional consortium to fulfil overall gut microbial ecosystem requirements. These data supported that the maturation of the human microbiota during early life may be proposed as an example of ecological succession, in which communities undergo consecutive compositional and functional transitions in dominant taxa to establish physiological syntrophy among microbiota for niche adaptation (Koenig et al., 2011; Lozupone et al., 2012). Due to the variety of available nutrients, energy substrates and oxygen levels in the gut, it is reasonable to hypothesize that gut microbial taxa with diverse functional traits cooperate syntrophically to maximize energy yield and growth, as has been shown in other ecosystems (Morris et al., 2013). Comprehensive mathematical analysis of the characteristics of network edges between all genera pairs and their expressed functions will shed light on community-wide interactions via primary degradation, resource competition and interspecies cross-feeding between gut microbes.

Concluding remarks

Although comparisons between individual children showed great differences in the dynamics of colonization, functional changes occurred more similarly across individuals, highlighting the non-stochastic nature of the bacterial functional community succession. Our results showed that considerable discordance existed between microbial composition and phylogenetic origin of proteins at all taxonomic levels. Age was the major driver of the rewiring of networks around succeeding key functional taxa and of the restructuring of community metabolic performances. Taken together, the detailed reconstruction of the gut microbial carbon metabolism presented here, including the assignment of enzymes to microbial taxa, revealed alternate temporary microbial and metabolic configurations where community-wide metabolic relationships to harvest energy by fermentation of prevailing dietary and host-derived carbon substrates, mainly glycans, differentiated chronological states. Our data provide a proteomic catalogue of the functional maturation of early gut microbiota, which may constitute an important research tool for indicators of future healthy or diseases states and for the design of microbiota-targeted health-promoting strategies early in life.

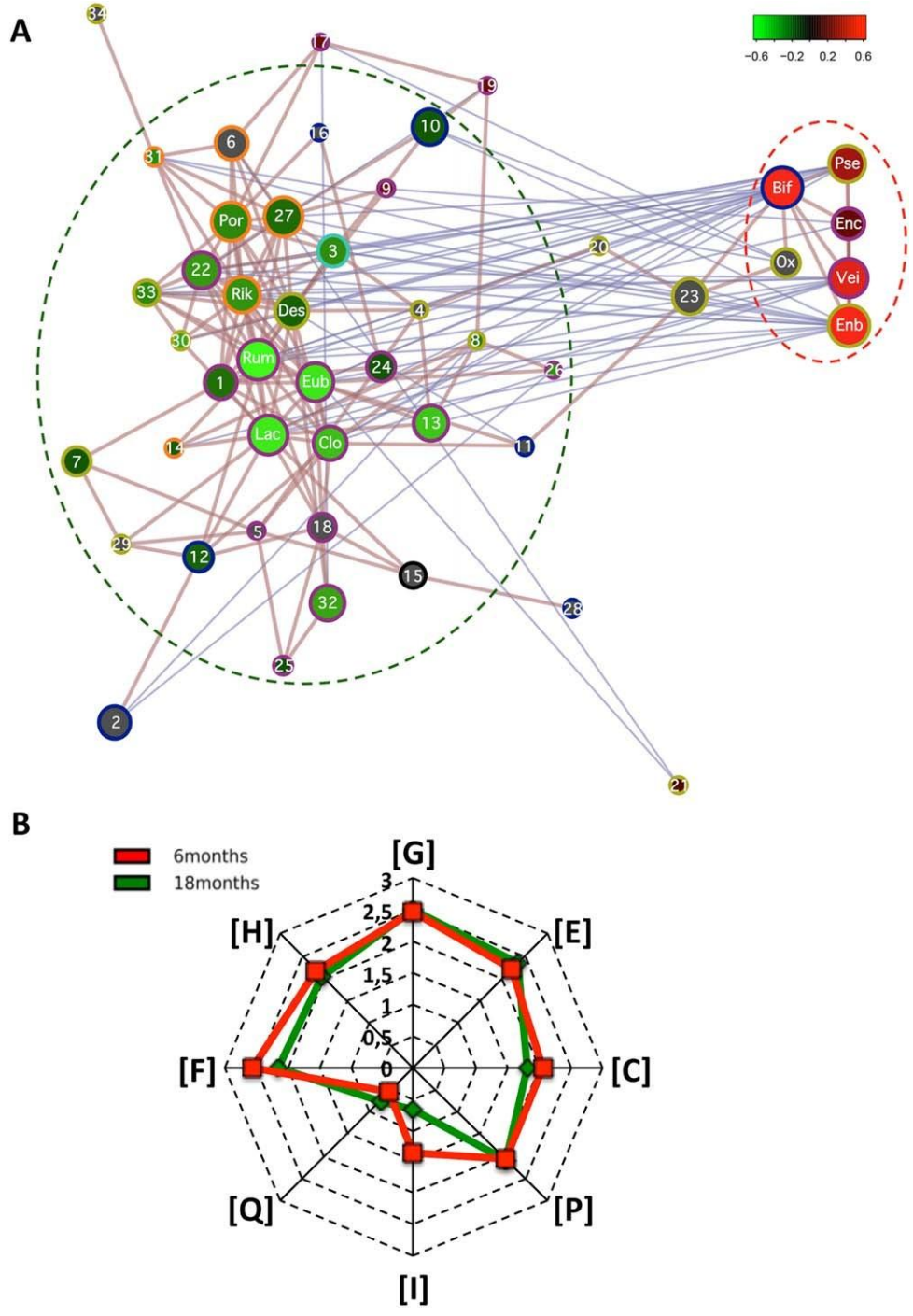

Fig. 5. Age-driven remodelling and functional succession of co-occurrent taxa in the metaproteomes.
(A) Co-occurrence network of taxa at family level in the metaproteomes of 6-months and 18-months old infants. The nodes represent families connected by significantly positive (thick brown lines) and negative (thin blue lines) and the node colour denotes association to each chronological state, both defined by the Pearson's correlation coefficient, set to a minimum of 0.6. The numbers in nodes refer to families' names shown in Supporting Information Table S5. Phylogenetic assignment at phylum level of the nodes is coloured by rings in blue for Actinobacteria, orange for Bacteroidetes, black for Fusobacteria, purple for Firmicutes, yellow for Proteobacteria and light blue for Verrumicrobia. Bif, Bifidobacteriaceae; Clo, Clostridiaceae; Des, Desulfovibrionaceae; Enb, Enterobacteriaceae; Eno, Enterococcaceae; Eub, Eubacteriaceae; Lac, Lachnospiraceae; Ox, Oxalobacteraceae; Por, Porphyromonadaceae; Pse, Pseudomonadaceae; Rik, Rikenellaceae; Rum, Ruminococcaceae; Vei, Veillonellaceae. (B) Mean relative abundances of COGs involved in metabolism expressed by key co-occurrent families in the modules. Data are expressed as $\log_{10}$ values. C, energy production and conversion; E, amino acid transport and metabolism; F, nucleotide transport and metabolism; G, carbohydrate transport and metabolism; H, coenzyme transport and metabolism; I, lipid transport and metabolism; P, inorganic ion transport and metabolism; Q, secondary metabolites biosynthesis, transport, and catabolism.

Experimental procedures

Subjects, experimental design and ethical guidelines

In the present study, full-term healthy infants aged 6- and 18-months, who did not present any intestinal disorders and had not taken antibiotics, were chosen from the panel of infants that belonged to PREOBE study cohort (Berglund et al., 2016). In this period of life, the transition from weaning to solid food consumption occurs. Characteristics of the study population are shown in Supporting Information Table S1. In this project, pregnant women were recruited between 2007 and 2012 at San Cecilio and Mother-Infant University Hospitals in Granada, Spain. The study exclusion criteria for mothers were: simultaneous participation in any other research study, any kind of drug treatment, diagnosed diseases (e.g., pre-gestational diabetes, hypertension or preeclampsia, intrauterine growth retardation, maternal infection, hypo/hyper- thyroidism, hepatic or renal disease) and vegan diet. Fresh stools were collected at 6- and 18-months after delivery and were immediately stored at -80ºC, until processing. The study included anthropometric measurements, health ques- tionnaires and medical assessments of the child. This project followed the ethical standards recognized by the Declaration of Helsinki (reviewed in Hong-Kong 1989 and in Edinburgh 2000) and the EEC Good Clinical Practice recommendations (document 111/3976/88 1990), and current Spanish legisla- tion regulating clinical research in humans (Royal Decree 561/1993). The study was explained to the participants before starting, and the parents signed an informed consent.

DNA extraction from stool samples

Genomic DNA was extracted from faecal bacteria of 6-month (n568) and 18-month (n572) old infants as previously described (Ferrer et al., 2013). Briefly, faecal samples were resuspended in 1 ml of TN150 buffer (10 mM Tris-HCl pH 8.0 and 150 mM NaCl). Zirconium glass beads (0.3 g) and 150 ml of buffered phenol were added and bacteria were disrupted with a mini bead beater set to 5000 rpm at 4ºC for 15s (Biospec Products, USA). After centrifugation, genomic DNA was purified from the supernatant using phenol-chloroform extraction. Quality was checked by agarose gel electrophoresis and quantified with Quant-iT PicoGreen dsDNA assay kit (Invitrogen, Darmstadt, Germany).

16S rRNA gene sequencing and data processing

Genomic DNA from faecal bacteria was used as templates for 16S rRNA gene amplification using 27F and 338R universal primers and two consecutive PCR reactions to integrate Illumina multiplexing sequences as previously described (Camarinha-Silva et al., 2014). The library was prepared by pooling equimolar ratios of amplicons and was sequenced using an Illumina MiSeq platform (Genetic Service, University of Granada). Reads were demultiplexed and sorted, and paired ends were matched to give 240nt reads. Data set was filtered and OTUs were defined at 99% similarity with MOTHUR programs unique.seqs and pre.cluster (Schloss et al., 2009). Taxonomic classifications of OTUs were assigned using the naïve Bayesian algorithm CLASSIFIER of Ribosomal Database Project (Wang et al., 2007). OTUs were considered unassigned when confidence value score was lower than 0.8, and were annotated using upper taxonomic ranks.

Protein extraction, separation, identification and data processing

Protein extraction was performed from faecal bacteria of 6-months (n529) and 18-months (n527) old infants as previously described (Ferrer et al., 2013). Faecal samples (0.5 g) were thawed and diluted in 1mL of 0.05% L-cysteine phosphate saline buffer solution (PBS) under anaerobic conditions. After differential centrifugation, faecal bacteria were disrupted by mechanical lysis in BugBuster Protein Extraction Reagent (Novagen) for 30 min at room temperature, followed by sonication for 2.5 min on ice. Protein extracts were centrifuged for 10 min at 12.000 rpm to separate cell debris. Protein concentrations were determined with the Bradford assay (Bradford, 1976). For 1-DE analysis, two 75-lg protein samples (technical replicates denoted by a or b) were precipitated with five-fold volumes of ice-cold acetone and separated on a 12% acrylamide separating gel with the Laemmli buffer system (Laemmli, 1970).After electrophoresis, protein bands were stained with Coomassie Brilliant Blue G-250. Entire protein lanes were indi- vidually cut into one band prior to performing in-gel tryptic digestion. Peptide lysates were desalted using C18 ZipTip prior to MS analysis. Peptides were analysed by nano-HPLC system Advion NanoMate and Orbitrap Fusion mass spec- trometer (Thermo Fisher Scientific). The peptides were eluted over 115 min with a gradient of 2 to 55% solvent (acetonitrile,0.1% formic acid). MS scans were measured at a resolution of 120,000 in the scan range of 400–1600 m/z, MS2 in the Iontrap (rapid mode). Raw data files were searched with Proteome Discoverer (v1.4, Thermo Fisher Scientific) using the SequestHT algorithm against a database containing protein-coding entries of bacterial taxa selected via 16S rRNA gene sequencing. Only rank 1 peptides were considered to be identified with a threshold of FDR <1%. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Vizcaino et al.,2016) partner repository with the dataset identifier PXD009056. Higher protein abundance is represented by a higher number of MS/MS spectra acquired from peptides of the respective protein. Thus, protein abundances were calculated based on normalized spectral abundances that allow relative comparison of protein abundances over different samples (Bantscheff et al., 2012; Ferrer et al., 2013; Guazzaroni et al., 2013). 'PROteomics results Pruning & Homology group ANotation Engine' (PROPHANE) (Schneider et al., 2011) was used to assign proteins to their taxonomic and functional groups using the functional annotation of COGs. For KEGG pathway reconstructions, a BLASTP v2.2.27 search of the original protein sequences against NCBInr to retrieve KEGG Orthology identifiers was performed (Kanehisa et al., 2014). The use of a metaproteome-specific database containing fully sequenced genomes from closely related genera to the sample's strains and other documented gut genera in the database of proteomes together with the specificity of the identification procedure resulted in a high proportion of taxo- nomic and functional annotation (Denef et al., 2007).

Measurement of glycosidase activities

Glycosidase activities were quantified in protein extracts from purified faecal bacteria by measuring the release of p-nitrophenol-α-D-glucoside, p-nitrophenol-β-D-glucoside and p-nitrophenol-β-D-galactoside (Sigma Chemical Co., St. Louis, MO, USA) at 410 nm. One unit (U) of enzyme activity was defined as the amount of protein producing 1 µmol of reducing sugars in 1 min under the assay conditions.

Statistical and data analysis

Statistical analyses were carried out using SPSS version v19.0 (IBM, IL) and R statistical package (Team, 2014). San- key flow chart was created with SankeyMATIC web tool (http:// sankeymatic.com/). KEGG Mapper was used to visualize metabolic pathways. To quantify the amount of variability explained by each variable and subject in our different data sets, we calculated the coefficient of determination (R^2).

For the response of composition and function of the microbiota, multivariate analysis of variance using distance matrices was performed, based on Bray-Curtis distance metrics. The matrices were partitioned in sources of variation with subject and characteristics of the study population as explanatory variables. Significance of the pseudo-F ratios was assessed by permutation test (999 permutations, using the adonis function from the R package vegan) (Oksanen et al., 2011) . b- diversity for compositional data was calculated as Unifrac dis- tance with GUnifrac package. Permanova analysis of the distance between different time points was calculated with adonis function from vegan package. Bray-Curtis dissimilarity measures were calculated with vegan package and anosim test was used to establish significant differences between time points. Statistical Analysis of Metagenomic Profiles v2.0 was used to compare the abundances of taxa, COG categories and subcategories between time points (Parks et al., 2014). a-diversity indices were calculated with PAST software (Hammer et al., 2001). Significant differences were identified with the White's non-parametric t test. Benjamini & Hochberg FDR method was used to correct for multiple comparisons, and results with a q-value (corrected P-value <0.05) were retained. Pearson's correlation network analysis and visualiza- tion were carried out using Calypso v8.20 (Zakrzewski et al., 2017). Network node parameters were calculated using Cytoscape v3.1.1 (Shannon et al., 2003).

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Supplementary Material

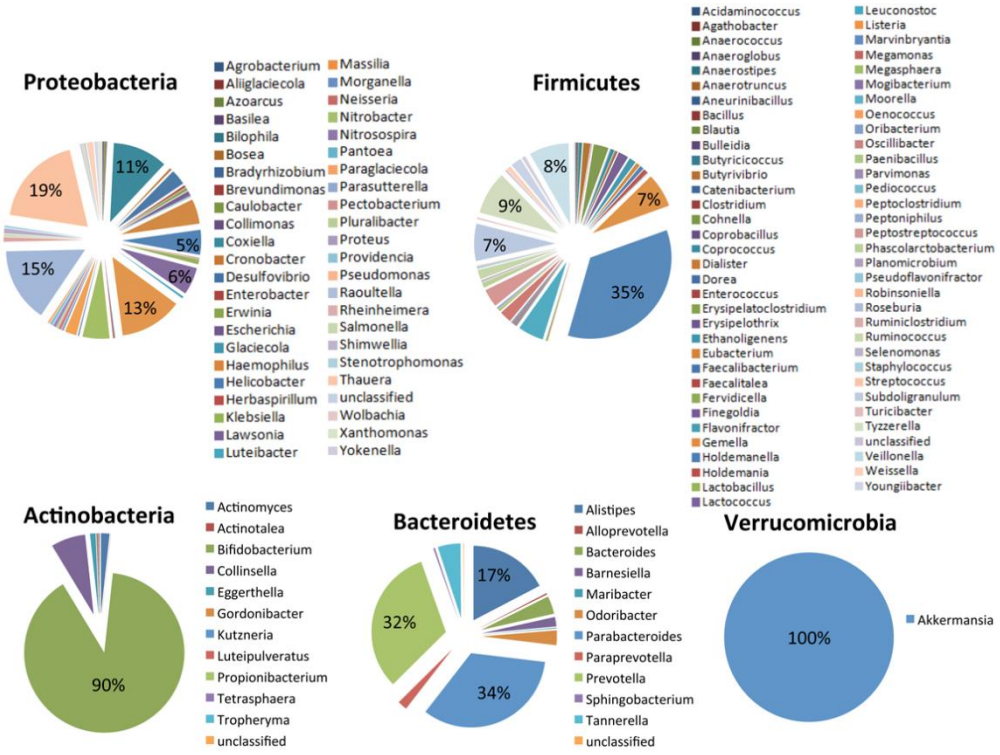


Fig. S1. Phylogeny of the functional gut microbiota (organism-origin of protein groups) at genus level.

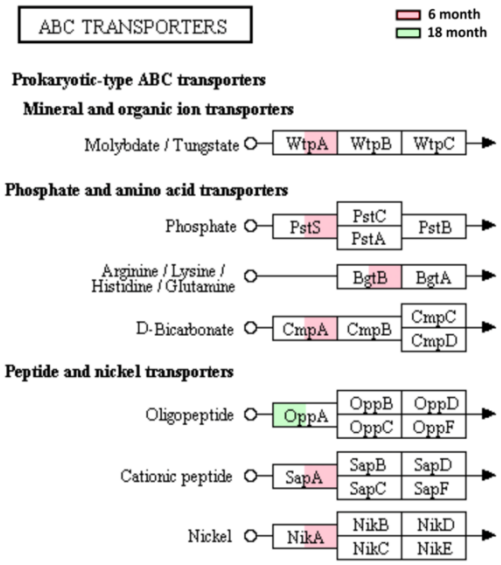


Fig. S2. Transport functions with differential abundance between the metaproteomes of 6- and 18-months old infants.

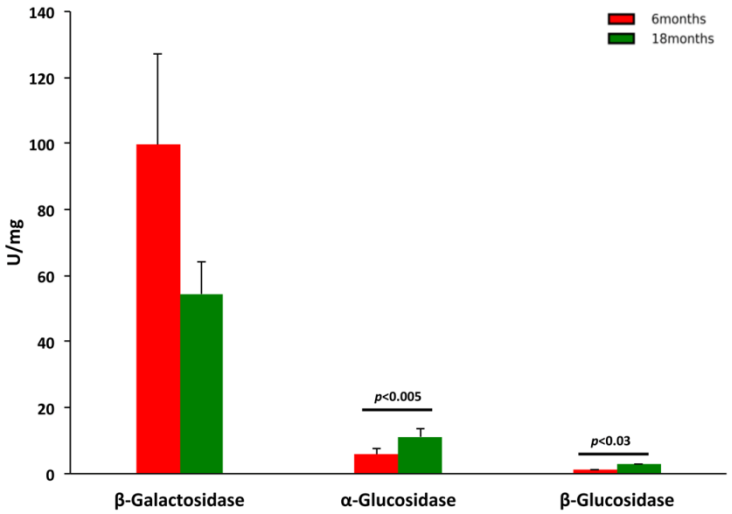


Fig. S3. Gut microbial glycosidase activities in 6- and 18-months old infants. Mean enzymatic specific activities (units per gram of total protein) ± SEM from faecal microbiota are represented.

Table S1 General Characeristics of the studied population

Mother		
Maternal Age (y)		33.17 ± 4
Pre-conceptional maternal Height		1.63 ± 0.07
Preconceptional maternal Weight		72.41 ± 16.1
Preconceptional maternal BMI		27.4 ± 5.88
Maternal education level	Primary/Secondary	54(51.43%)
	University/Doctor	51(48.57%)
Smoking during pregnancy		
	Yes	10(9.52%)
	No	95(90.48%)
Alcohol consumption during pregnancy		
	Yes	101(96.19%)
	No	4(3.81%)
Mode delivery		
	Vaginal	79(76.96%)
	Cesarea	25(24.04%)
Newborn Infant		
Gestational Age (weeks)		39.5 ± 1.6
Birth weight (g)		3293.81 ± 479.12
Lenght at birth (cm)		50.28 ± 2.02
Röhrer Ponderal Index (kg/cm3)		26.22 ± 3.54
Breast Feeding		
	Yes	73(70.19%)
	No	31(29.81%)
Infant at 6 months (n=68)		
Height (cm)		68.4 ± 2.76
Weight (kg)		7.79 ± 0.93
BMI (kg/m2)		16.63 ± 1.52
Gender		
	Male	40(58.82%)
	Female	28(41.18%)
Infant at 18 months (n=72)		
Height (cm)		81.17 ± 3.27
Weight (kg)		11.11 ± 1.35
BMI (kg/m2)		16.84 ± 1.58
Gender		
	Male	46(63.89%)
	Female	26(36.11%)

Values listed are total for the variable (percent of total value n) or Means ± SD.

Table S3. Comparisons between the phylogeny at phylum level of protein groups and 16S rDNA gene sequences in gut microbiota.

Comparisons between the phylogeny at phylum level of protein groups and 16S rDNA gene sequences in gut microbiota		
Taxonomy (Phylum)	**log10 avg Proteomic/16SrRNA**	**P-value**
Actinobacteria	2.1	<0.001
Bacteroidetes	0.5	<0.001
Firmicutes	-0.3	<0.001
Proteobacteria	0.9	<0.001
Verrucomicrobia	1.9	<0.001

Comparisons between the phylogeny at family level of protein groups and 16S rDNA gene sequences in gut microbiota		
Taxonomy (Family)	**log10 avg Proteomic/16SrRNA**	**P-value**
Acidaminococcaceae	1.2	<0.001
Actinomycetaceae	0.2	0.566
Bacteroidaceae	-2.3	<0.001
Bifidobacteriaceae	2.5	<0.001
Bradyrhizobiaceae	-0.1	0.679
Caulobacteraceae	-0.4	0.125
Enterobacteriaceae	0.6	<0.001
Enterococcaceae	-1.5	<0.001
Erysipelotrichaceae	-0.7	<0.001
Fusobacteriaceae	0.4	0.210
Lachnospiraceae	-1.0	<0.001
Lactobacillaceae	-1.8	<0.001
Leuconostocaceae	0.9	0.003
Neisseriaceae	0.0	0.950
Pasteurellaceae	0.8	0.013
Peptostreptococcaceae	-3.0	<0.001
Porphyromonadaceae	1.4	<0.001
Prevotellaceae	2.9	<0.001
Propionibacteriaceae	-0.9	<0.001
Rikenellaceae	1.6	<0.001
Ruminococcaceae	0.9	<0.001
Staphylococcaceae	-0.6	<0.001
Streptococcaceae	-1.5	<0.001
Sutterellaceae	0.1	0.542
Unclass_Bacteroidales	3.7	<0.001
Unclass_Bacteroidetes	3.5	<0.001
Unclass_Clostridiales	2.2	<0.001
Unclass_Firmicutes	2.7	<0.001
Veillonellaceae	0.2	0.131
Coriobacteriaceae	1.4	<0.001

Comparisons between the phylogeny at genus level of protein groups and 16S rDNA gene sequences in gut microbiota		
Taxonomy (Genus)	**log10 avg Proteomic/16SrRNA**	**P-value**
Unclass_Prevotellaceae	2.0	<0.001
Pluralibacter	4.4	<0.001
Yokenella	-0.3	0.182
Staphylococcus	2.8	<0.001
Neisseria	1.5	<0.001
Leuconostoc	1.6	<0.001
Finegoldia	-0.1	0.747
Faecalitalea	1.8	<0.001
Oscillibacter	2.8	<0.001
Gemella	0.7	0.476
Pediococcus	3.7	<0.001
Proteus	2.2	<0.001
Eggerthella	1.9	<0.001
Holdemania	3.4	<0.001
Coprobacillus	3.0	<0.001
Odoribacter	-0.6	<0.001
Propionibacterium	-0.9	<0.001
Megamonas	1.4	<0.001
Unclass_Bacteroidetes	0.2	0.327
Turicibacter	-0.6	0.007
Parasutterella	-0.2	0.344
Subdoligranulum	0.1	0.710
Barnesiella	1.8	<0.001
Unclass_Bacteroidales	1.1	<0.001
Megasphaera	0.4	0.290
Paraprevotella	2.5	<0.001
Flavonifractor	0.4	0.170
Acidaminococcus	1.2	<0.001
Unclass_Firmicutes	1.9	<0.001
Brevundimonas	2.2	<0.001
Phascolarctobacterium	2.7	<0.001
Butyricicoccus	2.3	<0.001
Unclass_Bradyrhizobiaceae	2.5	<0.001
Coprococcus	1.6	<0.001
Actinomyces	0.6	0.228
Prevotella	2.7	<0.001
Bifidobacterium	2.4	<0.001
Alistipes	1.2	<0.001
Dialister	-0.6	0.021
Unclass_Erysipelotrichaceae	-1.0	0.007
Haemophilus	-1.0	0.002
Fusobacterium	-0.7	0.002
Dorea	-0.6	0.095

Unclass_Ruminococcaceae	-2.0	<0.001
Unclass_Clostridiales	-0.4	0.258
Collinsella	-0.6	0.092
Ruminococcus	-0.7	0.062
Unclass_Veillonellaceae	-0.5	0.182
Holdemanella	0.9	0.017
Lactococcus	1.2	0.006
Lactobacillus	-2.2	<0.001
Parabacteroides	2.0	<0.001
Faecalibacterium	-1.6	<0.001
Anaerostipes	-2.7	<0.001
Blautia	-3.2	<0.001
Roseburia	-1.8	<0.001
Veillonella	-3.5	<0.001
Enterococcus	-3.4	<0.001
Streptococcus	-3.7	<0.001
Unclass_Enterobacteriaceae	0.6	<0.001
Unclass_Lachnospiraceae	-4.8	<0.001
Bacteroides	-2.1	<0.001
Escherichia	0.9	0.011

Supporting Information Table S4A. Associations between gut microbiota composition (16S rDNA gene sequences) and other variables of interest

	F model	R^2	*p* value
Individual	1.598	0.011	**0.019**
Age	10.274	0.07	**0.001**
Type of delivery	1.383	0.00943	0.061
Mother's pre-pregnancy body mass index#	1.227	0.01673	0.09
Type of feeding* at 3 months old	1.0383	0,01416	0.359
Type of feeding* at 6 months old	0.9422	0,01285	0.590

Groups were divided in normoweight (BMI<25 kg/m^2), over-weight (25 kg/m^2>BMI<30 kg/m^2) and obesity (BMI>30 kg/m^2).

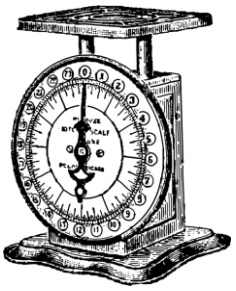
*Groups were divided in exclusively breast fed, mixed feeding, and exclusive formula feeding.

Supporting Information Table S4B. Associations between gut microbiota function (COG function) and other variables of interest

	F model	R^2	*p* value
Individual	0.5663	0.00943	0.876
Age	8.1080	0.13499	**0.001**
Type of delivery	1.1568	0.03852	0.256
Mother's pre-pregnancy body mass index#	0.9991	0.08317	0.468
Type of feeding* at 3 months old	1.0553	0.03514	0.344
Type of feeding* at 6 months old	0.9853	0.03281	0.439

Groups were divided in normoweight (BMI<25 kg/m^2), over-weight (25 kg/m^2>BMI<30 kg/m^2) and obesity (BMI>30 kg/m^2).
*Groups were divided in exclusively breast fed, mixed feeding, and exclusive formula feeding.

Tables S2, S5-S7 view online



PAPER 2

Maternal obesity is associated with gut microbial metabolic potential in offspring during infancy

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Abstract

Children born to obese mothers are at increased risk for obesity, but themechanisms behind this association are not fully understood. Our study aimed to investigate differences in the functions encoded by the microbiome of infants at 18 months of age when the transition from early infantfeeding to solid family foods is established. To investigate the impact of maternal prepregnancy body mass index on infants' gut microbiome, faecal samples from infants born to normoweight (n = 21) and obese mothers (n = 18) were analysed by 16S rRNA gene sequencing and a functionalinference- based microbiome analysis. Our results indicated that *Firmicutes* was significantly enriched in infants born to normoweight mothers whereas *Bacteroidetes* was significantly enriched in infants born to obese women. In both microbiomes, the greatest number of genes (>50%) that were assigned a function encoded for proteins involved in "metabolism" among tier 1 KEGG Orthology (KO) categories. At lower KO functional categories, the microbiome ofinfants born to normoweight mothers was characterized by a significant enrichment in the abundances of "pentose phosphate pathway" (p = 0.037), "lysine biosynthesis" (p = 0.043), "glycerolipid metabolism" (p = 0.042), and "C5-branched dibasic acid metabolism" (p = 0.045). Notably, the microbiome of infants born to obese mothers was significantly enriched in "streptomycin biosynthesis" (p = 0.047), "sulphur metabolism" (p = 0.041), "taurine and hypotaurne metabolism" (p= 0.036), and "lipopolysaccharide biosynthesis" (p = 0.043). In summary, our study showed that maternal prepregnancy obesity may imprint a selective gut microbial composition during late infancy with distinct functional performances.

Introduction

The human gut is a bioreactor with evolutionarily conserved roles in the biotransformation of dietary ingredients into products that affect our physiology, metabolism, immune function, and development [7, 24, 53]. This ensemble of organisms consists mainly of bacterial taxa of *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, and *Actinobacteria* phyla that have coevolved with the human host complementing the coding potential of our own genome [20, 27]. Although research interest has traditionally aimed at cataloguing the microbial species of the adult human gut microbiota and its relationship to complex diseases

[2, 30, 38, 49, 57], few studies have focused on the infant gut microbiota that have historically relied on culture-based enumeration and, more recently, on taxonomic profiling by16S rRNA gene sequence analyses [1, 4, 18]. Infancy is a critical period for gut microbial de novo assembly during which a constant dialogue with the immune system and a metabolic crosstalk with the host influences healthy growth and development [3, 11, 19]. These studies have shown that there is a rapid rate of colonization and expansion of gut bacteria from birth, a shift from a gut dominated by *Proteobacteria* to an adult-like one dominated by *Firmicutes* and *Bacteroidetes*, that achieves compositional diversity and stability by the third year of life [14]. Differential modes of birth (vaginal vs. C-section) and of type of feeding (breast milk vs. formula) are important aspects influencing the composition of the gut microbial ecosystem [48, 62]. Medical factors like prenatal and postnatal exposures to antibiotics and maternal factors like mother's prepregnancy weight affect the infant microbiota and increase the predisposi- tion to obesity, with long-lasting effects [12, 58]. Thus, it is important to understand how these environmental factors con- tribute to the overall composition and function of the gut mi- crobiota, what is the metabolic crosstalk of the gut microbiota and the infant body with potential long-term consequences.

Taking into account all of the above, the present study explores the gut microbial composition and function of healthy infants at 18 months of age born to mothers with prepregnancy normoweight or obesity. We have collected faecal samples to obtain the phylogenetic composition. As a novel approach, we have used a functional-inference-based (PICRUSt) analysis to identify the KEGG metabolic pathways of the microbial communities [31]. This strategy has allowed us to depict the potential metabolic pathways encoded by the microbiota of the infants and associate differential rates with mother's prepregnancy weight.

Materials and methods

Subjects, experimental design, and ethical guidelines

The infants included in this study were part of the PREOBE study cohort that recruited 331 mothers with normoweight, overweight, obesity, and gestational diabetes [5]. The pregnant women were recruited between 2008 and 2012 through collaboration with the two university tertiary hospitals in Granada, the San Cecilio and Mother-Infant Hospitals (Spain). Pregnant women with singleton pregnancies and aged between 18 and 45 years were included during their 12th to 34th weeks of pregnancy. The study exclusion criteria were simultaneous participation in any other research study, maternal drug use during pregnancy, diagnosed diseases other than obesity (e.g. pregestational diabetes, hypertension or pre-eclampsia, foetal intrauterine growth retardation, maternal infection during pregnancy, hypo/hyperthyroidism, hepatic diseases, and renal disease), or having a vegan diet. At the recruitment visit, information was collected regarding maternal prepregnancy weight and used for calculation of the prepregnancy BMI [6]. Due to drop out and availability of stool, 21 faecal samples from infants born to normoweight mothers ($18.5 \leq BMI \leq 25$ kg/m^2) and 18 faecal samples from infants born to obese mothers ($\geq$30 kg/m^2) were further used in this study.

The project was approved by the Bioethical Committees for Clinical Research of the Clinical University Hospital San Cecilio and of the Mother-Infant University Hospital of Granada, Granada, Spain. An ethical approval was also obtained by the Research Bioethical Committee of the University of Granada. Written informed consent was obtained from all participants at study entry, and after, they had received full information from a research group member.

Sample collection and DNA extraction

Parents were provided with instructions and sterile bottles for sample collection. Fresh faecal samples were collected at home by the parents when children were 18 months old and stored at −20 °C for a maximum of 24 h until delivery to the laboratory, where they were frozen to −80 °C and stored until processing. Genomic DNA was extracted from faecal bacteria as previously described [22]. Briefly, faecal samples were resuspended in 1 ml of TN150 buffer (10-mM Tris-HCl pH 8.0 and 150-mM NaCl) and incubated at 80 °C for 30 min. Lysozyme (1 mg) was added to the suspension and incubated at 37 °C for 15 min. Zirconium glass beads (0.3 g) and 150 µl of buffered phenol were added and bacteria were disrupted with a mini bead beater set to 5000 rpm at 4 °C for 15 s (Biospec Products, Bartlesville, OK, USA). After centrifugation, genomic DNA was purified from the supernatant using phenol-chloroform extraction. Phenol-chloroform extractions were performed with 150 µl of phenol buffered and 150 µl of chloroform-isoamylalcohol (24:1) (v/v) solution. After an additional chloroform extraction, DNA was precipitated with two volumes of ethanol at −20 °C for 30 min. After centrifugation and washing with 70% ethanol, the pellet was resuspended in 50 µl of milliQ water. Five units of DNase-free RNase (Promega, USA) were added, and the sample was incubated at 37 °C for 15 min. Quality was checked by agarose gel electrophoresis and quantified with the Quant-iT PicoGreen dsDNA reagent and kit (Invitrogen, Darmstadt, Germany).

16S rRNA gene sequencing and data processing

Genomic DNA from faecal bacteria was used as template for 16S rRNA gene amplification using 27F and 338R universal primers for V1-V2 region as previously described [8]. Two consecutive PCR reactions integrated the sequence of the

specific Illumina multiplexing sequencing and index primers. The library was prepared by pooling equimolar ratios of amplicons and was sequenced using an Illumina MiSeq platform (University of Granada, Spain). Reads were demultiplexed and sorted, and paired ends were matched to give 240-nt reads. The data set was filtered and operational taxonomic units (OTUs) were defined at 99% similarity with MOTHUR programs unique.seqs and pre.cluster [55]. Taxonomic classifications of OTUs were assigned using the naïve Bayesian algorithm CLASSIFIER of Ribosomal Database Project [15]. OTUs were considered unassigned when confidence value score was lower than 0.8 and were annotated using upper taxonomic ranks.

Phylogenetic investigation of communities by reconstruction of unobserved states (PICRUSt) analysis

Functional capacity of the microbiota was predicted based on the microbiota composition using PICRUSt [31]. Independent of the taxonomic analysis, 97% of the OTUs were picked using a closed-reference OTU picking protocol [10] and the Greengenes database (database/13_8) [40] preclustered at 97% identity. The obtained OTU table was normalized by 16S rRNA copy number, and functional genes were predicted from the Kyoto Encyclopedia of Genes and Genomes (KEGG) catalogue [29].

Statistical and data analysis

Statistical analyses were carried out using SPSS version 19.0 (IBM SPSS, Inc., Chicago, IL) R statistical package (R Development Core Team, 2011) and STAMP. To examine the samples' taxonomic and functional profiles more generally, Statistical Analysis of Metagenomic Profiles [46] was used to compare the abundances of taxa, KEGG categories, and subcategories between infants born to normoweight and obese mothers. Data were not normally distributed and significant differences were identified with the White's non-parametric t test. The Benjamini-Hochberg procedure was used to control the false-discovery rate due to multiple testing. Results with a q value (corrected p value) <0.05 were retained.

α-Diversity indexes were calculated with PAST software (PAleontological STatistics free software). β-Diversity was calculated as Unifracs distance with GUnifrac package and permanova analysis of the distance between different study groups was calculated with Adonis function from vegan package in R software [45]. Bray-Curtis dissimilarity measures were calculated with vegan package and anosim test was used to establish the significant difference between infants born to obese and normoweight pregnant women in R software.

Results

Characteristics of participants

Two groups of infants were defined based on prepregnancy BMI calculated from self-reported prepregnancy body weight: normoweight $18.5 \leq \text{BMI} \leq 25$ kg/m^2 and obese group ≥ 30 kg/m^2 (Table 1). All mothers and infants were in good health during pregnancy and infants were born at term. General characteristics were similar between the two study groups. The groups did not differ in maternal age, cultural level, smoking or alcohol consumption during pregnancy, sex, breast-feeding, and mode of delivery. Birth weight and Röhrer Ponderal index were 3265.71 ± 431.61 g and 26 ± 3.16 kg/cm^3 in infants born to normoweight mothers and 3428.33 ± 460.44 g and 27 ± 5.25 kg/cm^3 in infants born to obese mothers. We found lower weight gain during pregnancy in obese mothers compared to normoweight mothers, as it has been previously described [65]. At 18 months of age, weight and BMI of infants did not differ between study groups. As previously described [44], we observed a strong correlation between prepregnancy BMI and BMI at first prenatal study contact, which took place at 12 weeks of gestation (Pearson's correlation coefficient = 0.931, $p < 0.001$).

Gut microbial community structure is different in infants'gut microbiota depending on maternal prepregnancy BMI

A total of 1.848.304 high-quality 16S rRNA sequences were obtained from the 39 samples. Sequences were clustered into 662 OTUs and classified into 76 bacterial groups at genus level. We first examined the α-diversity (within sample ecological diversity) of the gut microbial communities. We used the Shannon diversity (SDI) ($p = 0.815$), Chao-1 indexes (estimates richness) ($p = 0.740$), and the taxa number (calculates unique OTUs) ($p = 0.743$). These diversity scores were not significantly different between the gut microbiota of infants born to normoweight and obese mothers (data not shown).

We calculated Unifrac distances (β-diversity) to determine the between-sample diversity in the gut microbial communities of infant groups. Permutational multivariate ANOVA using Adonis function showed that infants born to obese mothers had a significantly different microbial community structure ($p = 0.006$ for weighted and $p = 0.001$ for unweighted Unifrac distances) compared to those born to normoweight mothers. Two distinct populations representing normoweight and obese samples clustered in the principal coordinate analysis (PCoA) plot (Fig. 1a–b). Clustering analysis of individual samples using Bray-Curtis distances also showed that the gut microbial community structures were significantly different between infant groups (Fig. 1c). To exclude confounding variables

Table 1 General characteristics of the studied population		Normal weight (n = 21)	Obese (n = 18)
Mother			
Maternal age (year)		32.52 ± 4.16	33 ± 5.62
Preconceptional maternal height (m)		1.63 ± 0.07	1.61 ± 0.07
Preconceptional maternal weight (Kg)		58.18 ± 6.29[a]	91.67 ± 13.61[b]
Preconceptional maternal BMI (kg/cm^3)		21.94 ± 1.67[a]	35.05 ± 4.27[b]
Weight gain during pregnancy (kg)		10.12 ± 5.68[a]	4.83 ± 8.66[b]
Maternal education level	Primary/secondary	10(47.62%)	11(61.11%)
	University/doctor	11(52.38%)	7(38.89%)
Smoking during pregnancy	Yes	3(14.29%)	2(11.11%)
	No	18(85.71%)	16(88.89%)
Alcohol consumption during pregnancy	Yes	2(9.52%)	0(0%)
	No	19(90.48%)	18(100%)
Mode delivery	Vaginal	17(80.95%)	14(77.78%)
	Cesarean	4(19.04%)	4(22.22%)
Newborn infant			
Gestational age (weeks)		39.85 ± 1.53	39.72 ± 1.40
Birth weight (g)		3265.71 ± 431.61	3428.33 ± 460.44
Length at birth (cm)		50.38 ± 1.71	50.44 ± 2.30
Röhrer Ponderal index (kg/cm^3)		26.00 ± 3.16	27.00 ± 5.25
Breast-feeding	Yes	15(71.43%)	11(61.11%)
	No	6(28.57%)	7 (38.89%)
Infant at 18 months			
Height (cm)		81.14 ± 3.14	80.32 ± 3.68
Weight (kg)		10.96 ± 1.06	10.73 ± 1.08
BMI (kg/m^2)		16.64 ± 1.41	16.62 ± 1.13
Gender	Male	16(76.19%)	9(50%)
	Female	5(23.80%)	9(50%)

Values listed are total for the variable (percent of total value n) or means ± SD

Across rows (a, b) indicate means that are significantly different ($p < 0.05$) using analysis of variance (White's non-parametric t test)

Lack of superscript letters indicate means that are not significantly different. $p > 0.05$

that may affect the relationship between maternal prepregnancy BMI and the composition of infants' gut microbiota, we examined the effect of gender, mode of delivery, and type of feeding using Unifrac β-diversity distances and redundancy analysis (RDA). The significance of separation in RDA analysis was estimated with a permutation test using 5000 permutations. Type of feeding was categorized into three groups, exclusively breast-fed, mixed fed (formula plus breast-feeding), and exclusively formula-fed at 3 and 6 months of age. To assess the influence of duration of breast-feeding in infants' gut microbiota, infants were categorized into two groups: breast-feeding with solid foods and exclusive solid food feeding at 12 and 18 months of age. No significant effect on the total microbiota profile was found except for prepregnancy mothers' BMI (Supplementary Table S1). Although the mode of delivery was not significant, we replicated a sensitivity analysis only on data from vaginally delivered infants. We found no significant association between any of these variables except for mothers' BMI (Supplementary Table S2). Thus, our findings concluded that mother's BMI is a strong factor influencing gut microbial community structure and composition of infants.

Maternal prepregnancy BMI and phylogenetic shifts in infants' gut microbiota

To address significant differences in the mean abundances of taxa, we used the White's non-parametric t test due to skewed distributions and Benjamini-Hochberg tests for multiple comparisons with a q value set at 0.05. On a compositional level, the gut microbiota of the infant was significantly different between our study groups (Fig. 2, Supplementary Tables S3, S4, and S5). In infants born to normoweight, *Firmicutes* was the dominant phylum (81.50%), followed by *Bacteroidetes* (17.20%), *Proteobacteria* (0.72%), *Unclass_Bacteria* (0.29%), and *Actinobacteria* (0.28%) (Fig. 2a–b). In infants born to obese mothers, *Firmicutes* was also the main phyla (56.95%), followed by *Bacteroidetes* (41.83%), *Proteobacteria* (0.81%), *Unclass_Bacteria* (0.24%), and *Actinobacteria* (0.17%) (Fig. 2c–d). We observed a significant overabundance of *Bacteroidetes* ($p = 0.004$) in infants born to obese mothers whereas infants born to normoweight mothers had a significant overabundance of *Firmicutes* ($p = 0.004$) in the gut microbiota (Supplementary Table S3). At family level, *Clostridiaceae* 1 ($p = 0.007$) and *Lachnospiraceae* ($p = 0.036$) were significantly enriched in infants born to normoweight

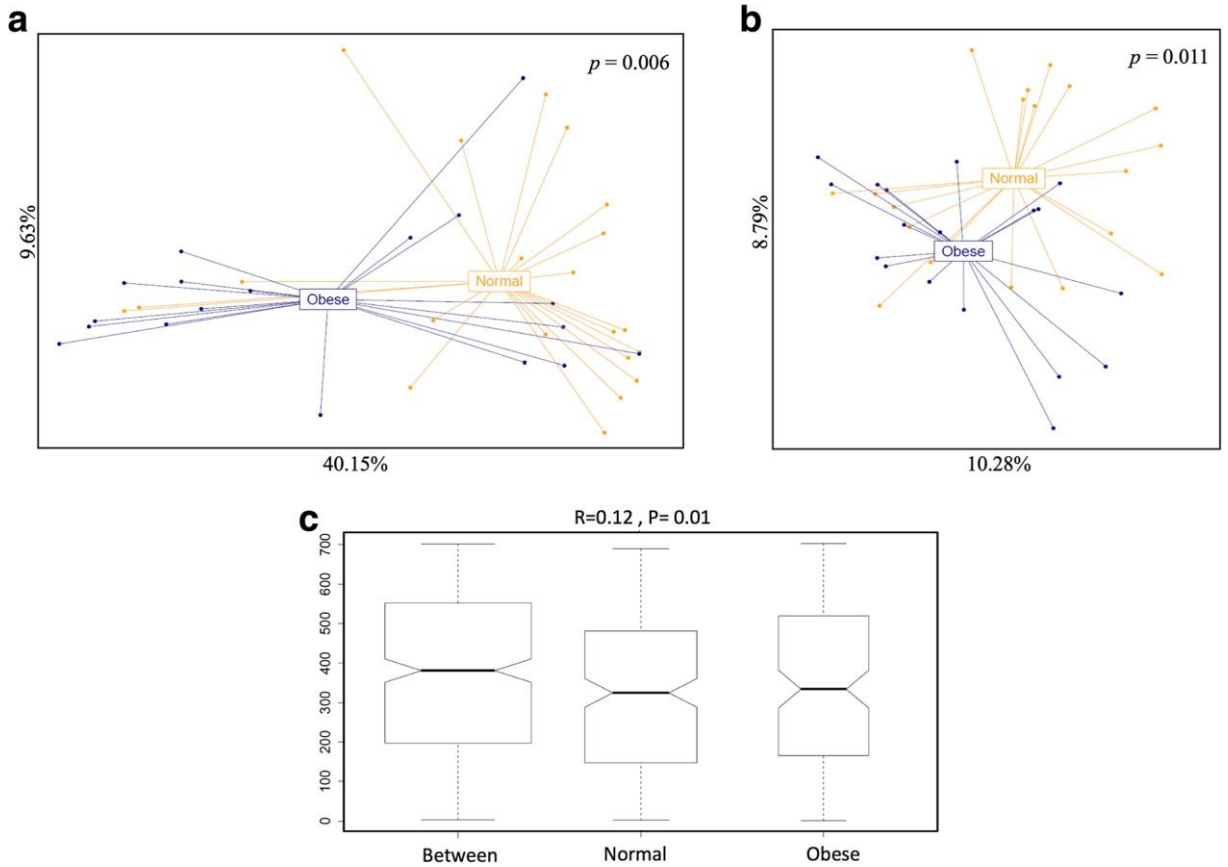

Fig. 1 Bacterial microbial community structure in infants born to normoweight and obese mothers at 18 months of age. PCoA plots of bacterial α-diversity based on weighted (a) and unweighted (b) UniFrac distances, and the Bray-Curtis dissimilarity values (c). Infants born to normoweight (normal) and obese mothers (obese) are coloured in orange and blue, respectively

mothers. Conversely, we observed a significant overabundance of *Porphyromonadaceae* (p = 0.013), *Bacteroidaceae* (p = 0.014), and *Prevotellaceae* (p = 0.037) in the gut microbiota of infants born to obese mothers (Supplementary Table S4). At genus level, the relative abundances of *Clostridium XVIII* (p = 0.031) and *Unclass_Clostridiaceae* 1 (p = 0.031) were significantly higher in infants born to normoweight mothers. In infants born to obese mothers, we observed a significant overabundance of *Parabacteroides* (p = 0.020), *Bacteroides* (p = 0.014), and *Oscillibacter* (p = 0.019) (Supplementary Table S5).

Profiling the metabolic capacity of bacterial communities

To identify microbial functions enriched or decreased in the infants gut microbiome depending on maternal prepregnancy BMI, an assessment of the microbial community functional potential was performed using PICRUSt metagenome predictions. Our analysis revealed similarities in the predicted functions of tier 1 KO functions between infant groups (Fig. 3). The greatest number of genes (>50%) that were assigned a function encoded for proteins involved in "metabolism" among tier 1 KO categories in the gut microbiome of infants born to normoweight and obese mothers. At tier 3 KO categories, we identified 226 predicted functions encoded in the microbiomes that were collapsed into higher categories using KEGG Pathways database (Supplementary Tables S6 and S7). To simplify analysis, a comparison of predicted functions involved in metabolism among tier 1 KO categories between both study groups was performed.

Between infant groups, a high number of significant differences in the abundances of second- and third-tier KO functional annotations were observed (Fig. 4a–b). At tier 2 KO functional annotations, the microbiome of infants born to normoweight mothers was characterized by a significant enrichment in the functional abundance of "nucleotide metabolism" (p = 0.039); whereas in infants born to obese mothers, the abundances of "glycan biosynthesis and metabolism" (p = 0.042), "metabolism of terpenoids and polyketides" (p = 0.036), "metabolism of other amino acids" (p = 0.042), and "biosynthesis of other secondary metabolites" (p = 0.048) were significantly increased (Fig. 4a). At tier 3 KO categories, the highest abundances of significant functional annotations in the microbiome of infants born to normoweight mothers were observed in "purine metabolism" (p = 0.04), "pentose phosphate pathway" (p = 0.037), "porphyrin and chlorophyll

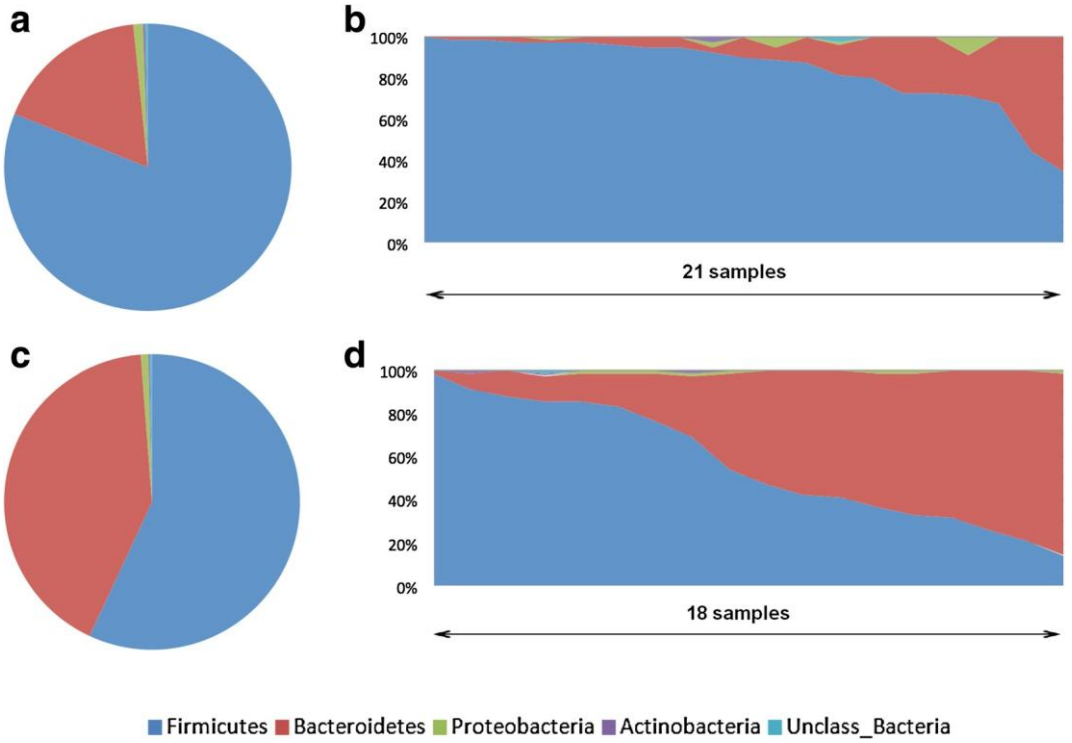

Fig. 2 Phylogenetic compositions of infants born to normoweight and obese mothers at 18 months of age. a and c Pie charts showing the distribution of phyla in infants born to normoweight mothers and obese mothers, respectively. **b** and **d** Inter-individual variation in the proportion of major phyla in infants born to normoweight mothers and obese mothers, respectively. Phyla are identified by colour shades: *Firmicutes* (blue), *Bacteroidetes* (red), *Proteobacteria* (green), *Actinobacteria* (purple), and *Unclass_Bacteria* (light-blue)

metabolism" (p = 0.038), "lysine biosynthesis" (p = 0.043), "glycerolipid metabolism" (p = 0.042), and "c5-branched dibasic acid metabolism" (p = 0.045). Notably, the microbiome of infants born to obese mothers was significantly enriched in low-abundant annotations of tier 3 KO categories. These categories were "streptomycin biosynthesis" (p = 0.047), "sulphur metabolism" (p = 0.041), "prenyltransferases" (p = 0.044), and "polyketide sugar unit biosynthesis" (p = 0.044). All categories within glycan biosynthesis and metabolism were significantly enriched in the microbiome of infants born to obese mothers: "glycosphingolipid biosynthesis globo and ganglio series" (p = 0.044, p = 0.041), "lipopolysaccharide biosynthesis" (p = 0.043), and "glycosaminoglycan degradation" (p = 0.042) (Fig. 4b).

Discussion

Maternal obesity and weight gain modify the composition and metabolism of the microbiota in the gut and breast milk during pregnancy and lactation [16, 17, 47]. Such microbial changes may be transferred to the offspring during delivery and lactation, altering microbial colonization of infant's gut [26, 58]. It is becoming increasingly clear that the gut microbiota in newborns and infants plays a key role in gut health and child development [17]. Dysbiosis of the early infant gut microbiota has been correlated with the development of childhood obesity and type 1 diabetes [11, 58]. Despite this link, few studies have addressed how maternal obesity influences the gut microbial metabolic potential during early life. Our study aimed to identify differences in the functions encoded by the microbiome of infants at 18 months of age born to prepregnancy normoweight and obese mothers. We analysed the gut microbial community composition by 16S rRNA amplicon high-throughput sequencing. Additionally, we used PICRUSt functional prediction to construct a community-level metabolic network of the microbiome and compare the abundance of pathways across infant groups [31].

Several studies have shown that the gut microbial ecosystem had higher α-diversity and lower β-diversity with no distinct clustering in children born to obese mothers compared to children born to normoweight mothers [16, 25]. These results disagree with those recently published by Laursen et al. [34]. The authors found no association between infant's gut microbiota at 9 and 18 months of age with maternal obesity. In our study, we provide evidence that maternal obesity is related to significant differences in gut microbial community structure because β-diversity metrics showed two distinct clustered groups according to maternal prepregnancy BMI. Mueller et al. showed that maternal obesity was associated with altered gut microbiota composition in neonates delivered vaginally, though not by C-section [42]. We found no effect of mode of

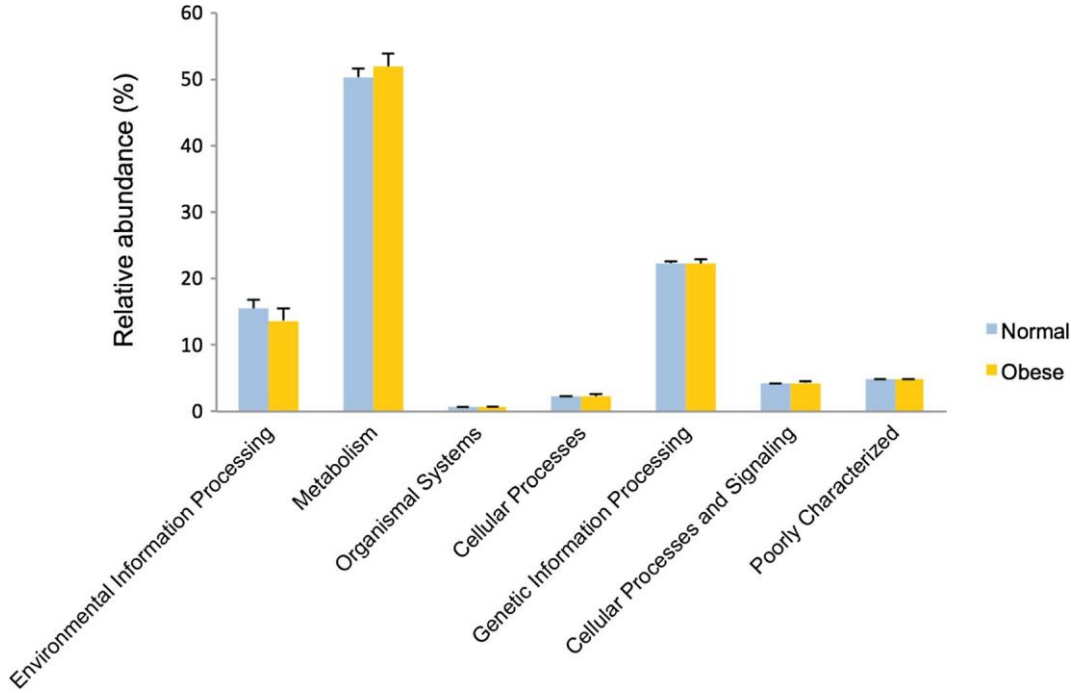

Fig. 3 Distribution of KEGG functional functional in infants born to normoweight and obese mothers. Tier 1 KEGG Orthology (KO) categories from infants born to normoweight mothers (normal) and infants born to obese mothers (obese) are identified by colour shades blue and orange, respectively

delivery or breast-feeding on gut microbiota, probably explained by the time of our sampling (18 months of age), when such variable is diluted by others like transition to complementary feeding. On a compositional level, we observed that Firmicutes was significantly enriched in children born to normoweight mothers whereas Bacteroidetes was significantly enriched in children born to obese women, due mainly to a significant increase in Lachnospiraceae and Clostridicaeae 1 and Bacteroidaceae families. At genus level, Collado et al. showed that levels of Staphylococcus and Bacteroides were significantly higher in infants born to overweight mothers during the first 6 months of life than in infants born to normoweight mothers, whereas levels of Bifidobacterium spp. were lower [16]. In our study, a significant overabundance of Bacteroides, Parabacteroides, and Oscillibacter was observed in infants born to obese mothers. Our results agree with those of Galley et al. [25]. They showed that these genera were significantly enriched in infants (aged 18 to 27 months) born to obese mothers compared to infants born to normoweight mothers. Although the genus Bacteroides is often associated with leanness and other desirable health traits [35, 51, 61, 63], some studies have linked these genera to obesity [28, 32, 56, 59]. Of note, differences in Oscillibacter have been found in prior studies of diet and obesity [13, 33, 39] that may be related to a higher consumption of fat [43]. It is plausible that differences in the sampling point (1, 6, 9, and 18 months of age) affecting feeding pattern and microbial ecosystem maturation, lifestyle, and maternal socioeconomic status explain the lack of reproducible findings. Further studies controlling for those variables in bigger cohorts are required to confirm the link between maternal obesity and offspring gut microbiota.

Our metagenomic predictions with PICRUSt showed an overall enrichment in genes involved in metabolic pathways in the gut microbiota of infants born to obese mothers. Of note, abundances of all KO modules involved in lipopolysaccharide (LPS) biosynthesis were increased. LPS derived from the outer membranes of Gram-negative bacteria has been intensively studied and is known to induce metabolic endotoxemia by promoting secretion of pro-inflammatory cytokines. The LPS from gut microbiota can induce a chronic subclinical inflammatory process and obesity, leading to insulin resistance through activation of TLR4 [9, 54]. Studies in both animal models and humans have shown that a high-fat diet can modulate the gut microbiota and increase circulating levels of LPS, probably by uptake of LPS in chylomicrons secreted from intestinal epithelial cells or through increased intestinal permeability. Therefore, the enrichment in LPS biosynthesis may predispose infants born to obese mothers to insulin resistance [58].

Our functional annotations highlight the importance of sulphur metabolism in infants born to obese mothers. This pathway is involved in the degradation of sulphur-containing compounds, such as products from the fermentation of certain amino acids (cysteine and methionine) and taurine bile acid derivatives. According to this, we also found a significantly

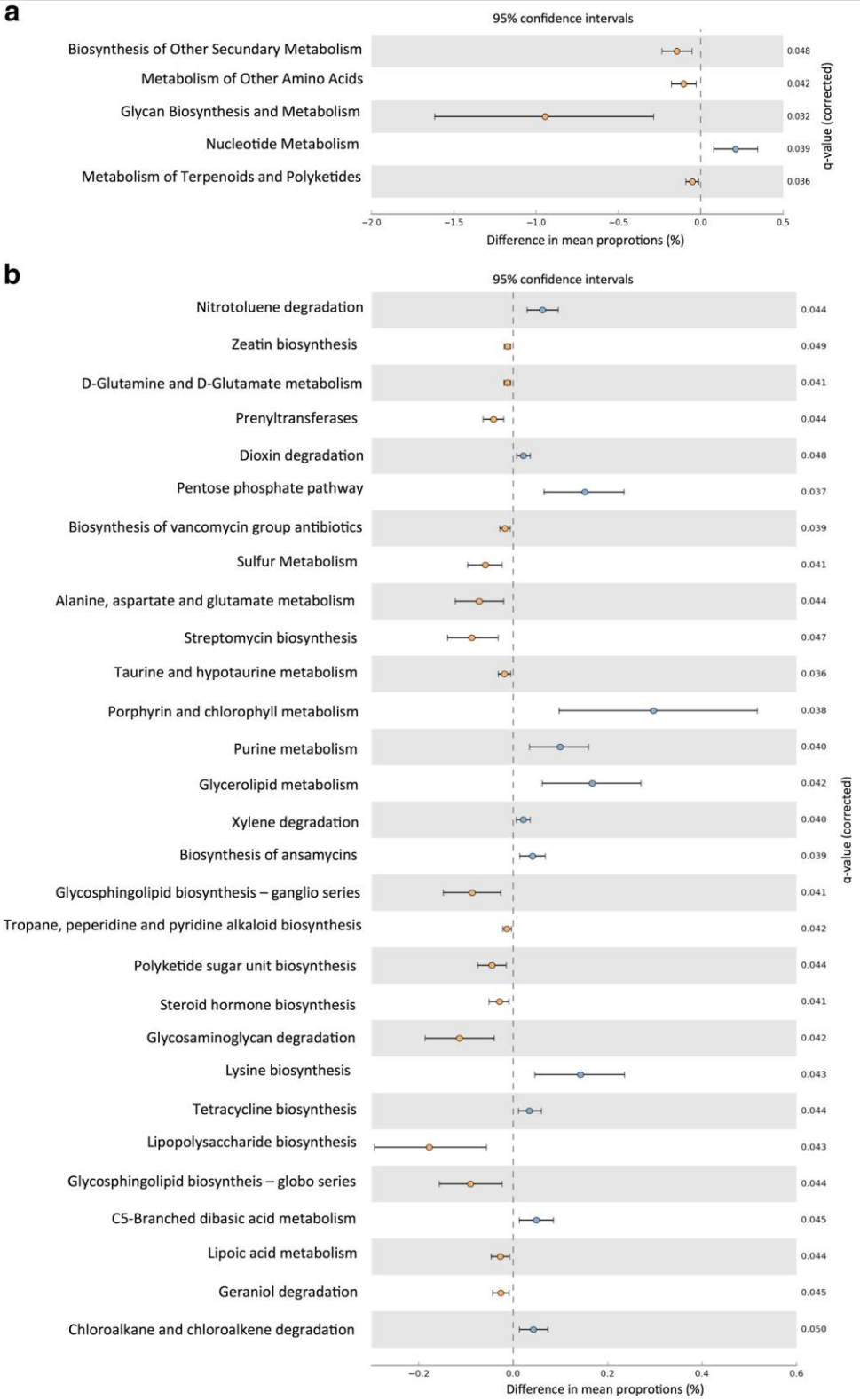

Fig. 4 Significant differences in potential metabolic capacities of the gut microbiomes between infants born to normoweight mothers (blue) and obese mothers (orange). Significance was determined by White non- parametric t test with Benjamini and Hockberg FDR correction for multiple comparisons. Only functional capacities with corrected $p < 0.05$ are shown

overrepresentation of genes involved in taurine and hypotaurine metabolism in children born to obese mothers. These pathways lead to a wide variety of compounds including hydrogen sulphide, the principal by-product of sulphur-reducing bacteria in the mammalian colon [60]. Hydrogen sulphide has well-established detrimental effects on the colonic microenvironment and epithelial health, since its excess may be detrimental for colonic epithelium energy metabolism

and DNA integrity. This result may have important long-term implications for bowel health in feeding patterns where excessive protein diets are consumed [36, 37, 64].

Despite infants born to obese mothers had higher abundances of *Bacteroides*, specialized in the degradation of dietary non-digestible carbohydrates as well as host carbohydrates including mucus, we found no enrichment in KO modules of polysaccharide degradation. The microbiota hydrolyses non-digestible carbohydrates into oligosaccharides and monosaccharides that fuel central carbon metabolism [21, 23]. Major bacterial metabolic routes are the Embden-Meyerhof-Parnas pathway (glycolysis, for six-carbon sugars) and the pentose-phosphate pathway (for five-carbon sugars) leading to phosphoenolpyruvate that is converted into fermentation products such as butyrate and propionate [41]. In our study, the microbiome of infants born to normoweight mothers has a significantly higher proportion of genes involved in lipoic acid metabolism, an essential cofactor of pyruvate dehydrogenase, and pentose-phosphate pathway. Considering that the fermentation routes were present in the gut microbiota, our results suggest an increased ability to process the larger variety of monosaccharides in the diet by the gut microbiota of infants born to normoweight mothers. Indeed, previous reports showed that normoweight children had higher levels of faecal SCFA, the end products of monosaccharide fermentation, compared to obese ones [47]. However, controversy still exists regarding the role of SCFA in obesity [50, 52]

In summary, our study showed that maternal prepregnancy obesity may imprint a selective gut microbial composition during late infancy with higher metabolic capacity. We are aware that the small sample size may preclude detecting other important differences. Still, our results showed differences in taxonomic composition and functionality of the gut microbiota across infant groups that withstood stringent correction for multiple testing. Nevertheless, future analyses enrolling a greater number of participants will help to understand the molecular factors associated to maternal prepregnancy weight that alters the microbial ecosystem during early life.

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Supplementary Material

Supplementary Table 1. *p* value for PERMANOVA analysis on weighted and unweighted Unifrac distances and for RDA analysis to assess confounding effects of the covariates.

	Weighted Unifrac	Unweighted Unifrac	RDA analysis
Pre-pregnancy BMI (Normoweight vs Obese)	0.006	0.011	0.001
Mode of delivery (Vaginal vs C-section)	0.698	0.727	0.963
Gender (Female vs Male)	0.258	0.247	0.713
Exclusively formula-fed vs exclusively breast-fed vs mixed-fed at 3 months	0.107	0.902	0.934
Exclusively formula-fed vs exclusively breast-fed vs mixed-fed at 6 months	0.201	0.480	0.254
Breast-feeding with solid foods vs exclusive solid food feeding at 12 months	0.909	0.546	0.577
Breast-feeding with solid foods vs exclusive solid food feeding at 18 months	0.774	0.735	0.773

Supplementary Table 2. *p* value for PERMANOVA analysis on weighted and unweighted Unifrac distances and for RDA analysis to assess confounding effects of the covariates using only data from those infants born by vaginal delivery.

	Weighted Unifrac	Unweighted Unifrac	RDA analysis
Pre-pregnancy BMI (Normoweight vs Obese)	0.005	0.072	0.021
Gender (Female vs Male)	0.112	0.941	0.569
Exclusively formula-fed vs exclusively breast-fed vs mixed-fed at 3 months	0.185	0.466	0.214
Exclusively formula-fed vs exclusively breast-fed vs mixed-fed at 6 months	0.137	0.425	0.418
Breast-feeding with solid foods vs exclusive solid food feeding at 12 months	0.205	0.425	0.773
Breast-feeding with solid foods vs exclusive solid food feeding at 18 months	0.144	0.251	0.792

Supplementary Table 3. Mean relative abundances of microbial taxa at phylum level in infants gut microbiota born to normoweight mothers and obese mothers (mean ± SEM)

Phylum	**Normal**	**Obese**	**Padj***
Firmicutes	81.51 ± 4.71	56.95 ± 6.47	0.004
Bacteroidetes	17.2 ± 4.76	41.82 ± 6.51	0.004
Proteobacteria	0.72 ± 0.26	0.81 ± 0.14	-
Actinobacteria	0.28 ± 0.14	0.17 ± 0.06	-
Fusobacteria	0.0059 ± 0.0015	0.01 ± 0.01	-
Unclass_Bacteria	0.29 ± 0.13	0.24 ± 0.12	-

**Comparisons between microbial phylotypes was analyzed by White's non parametrics t-test and corrected by Benjamini–Hochberg for multiple testing. Across rows (*) indicate means that are significantly different (P < 0.05)*

Supplementary Table 4. Mean relative abundances of microbial taxa at family level in infants gut microbiota born to normoweight mothers and obese mothers (mean ± SEM)

Phylum	Class	Order	Family	Normal	Obese	Padj *
Actinobacteria	Actinobacteria	Actinomycetales	**Actinomycetaceae**	0.07 ± 0.07	0 ± 0	-
			Propionibacteriaceae	0.01 ± 0	0 ± 0	-
		Bifidobacteriales	**Bifidobacteriaceae**	0.08 ± 0.07	0.03 ± 0.02	-
		Coriobacteriales	**Coriobacteriaceae**	0.12 ± 0.04	0.13 ± 0.04	-
Bacteroidetes	Bacteroidia	Bacteroidales	**Bacteroidaceae**	16.32 ± 4.63	37.01 ± 6.42	0.014
			Porphyromonadaceae	0.4 ± 0.14	2.51 ± 0.76	0.013
			Prevotellaceae	0.2 ± 0.12	1.32 ± 0.48	0.037
			Rikenellaceae	0.27 ± 0.1	0.93 ± 0.36	-
			Unclass_Bacteroidales	0 ± 0	0.05 ± 0.03	-
Firmicutes	Bacilli	Lactobacillales	**Carnobacteriaceae**	0.1 ± 0.07	0.01 ± 0	-
			Enterococcaceae	1.45 ± 0.8	0.04 ± 0.02	-
			Lactobacillaceae	0.09 ± 0.04	0.02 ± 0	-
			Leuconostocaceae	0 ± 0	0.01 ± 0.01	-
			Streptococcaceae	9.07 ± 3.23	3.57 ± 1.33	-
	Clostridia	Clostridiales	**Catabacteriaceae**	0.02 ± 0.01	0.03 ± 0.02	-
			Clostridiaceae 1	0.5 ± 0.11	0.14 ± 0.05	0.007
			Clostridiales_Incertae Sedis XI	0.01 ± 0.01	0 ± 0	-
			Clostridiales_Incertae Sedis XIII	0.04 ± 0.02	0.05 ± 0.03	-
			Lachnospiraceae	60.15 ± 5.92	42.75 ± 5.38	0.036
			Peptostreptococcaceae	1.29 ± 0.31	1.68 ± 0.49	-
			Ruminococcaceae	3.76 ± 0.69	4.5 ± 0.91	-
			Unclass_Clostridiales	1.33 ± 0.61	1.06 ± 0.24	-
	Erysipelotrichia	Erysipelotrichales	**Erysipelotrichaceae**	0.96 ± 0.15	1.93 ± 0.54	-
	Negativicutes	Selenomonadales	**Acidaminococcaceae**	0.57 ± 0.32	0.08 ± 0.04	-
			Veillonellaceae	2.09 ± 0.91	0.99 ± 0.43	-
	Unclass_ Firmicutes	Unclass_ Firmicutes	**Unclass_Firmicutes**	0.08 ± 0.02	0.08 ± 0.05	-
Fusobacteria	Fusobacteriia	Fusobacteriales	**Fusobacteriaceae**	0.01 ± 0	0.01 ± 0.01	-
Proteobacteria	Alphaproteobacteria	Rhizobiales	**Bradyrhizobiaceae**	0.01 ± 0.01	0 ± 0	-
		Unclass_Alphaproteobacteria	**Unclass_Alphaproteobacteria**	0.01 ± 0.01	0 ± 0	-
	Betaproteobacteria	Burkholderiales	**Sutterellaceae**	0.04 ± 0.02	0.04 ± 0.02	-
	Gammaproteobacteria	Enterobacteriales	**Enterobacteriaceae**	0.52 ± 0.26	0.71 ± 0.13	-
		Pasteurellales	**Pasteurellaceae**	0.13 ± 0.08	0.05 ± 0.02	-
Unclass_ Bacteria	Unclass_ Bacteria	Unclass_Bacteria	**Unclass_Bacteria**	0.29 ± 0.13	0.24 ± 0.12	-

**Comparisons between microbial phylotypes was analyzed by White's non parametrics t-test and corrected by Benjamini–Hochberg for multiple testing. Across rows (*) indicate means that are significantly different (P < 0.05)*

Supplementary Table 5. Mean relative abundances of microbial taxa at genera level in infants gut microbiota born to normoweight mothers and obese mothers (mean ± SEM)

Phylum	Class	Order	Family	Genera	Normal	Obese	Padj*
Actinobacteria	Actinobacteria	Actinomycetales	Actinomycetaceae	**Actinomyces**	0.07 ± 0.07	0 ± 0	-
			Propionibacteriaceae	**Propionibacterium**	0.01 ± 0.004	0 ± 0	-
		Bifidobacteriales	Bifidobacteriaceae	**Bifidobacterium**	0.08 ± 0.07	0.03 ± 0.02	-
		Coriobacteriales	Coriobacteriaceae	**Collinsella**	0.09 ± 0.04	0.11 ± 0.04	-
				Eggerthella	0.01 ± 0.004	0 ± 0	-
				Slackia	0.01 ± 0.01	0.01 ± 0.004	-
				Unclass_Coriobacteriaceae	0.01 ± 0.01	0 ± 0	-
Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	**Bacteroides**	16.32 ± 4.63	37.01 ± 6.42	0.014
			Porphyromonadaceae	**Barnesiella**	0.01 ± 0.01	0.33 ± 0.17	-
				Dysgonomonas	0.02 ± 0.02	0 ± 0	-
				Odoribacter	0.03 ± 0.02	0.08 ± 0.03	-
				Parabacteroides	0.34 ± 0.14	2.1 ± 0.68	0.02
			Prevotellaceae	**Paraprevotella**	0.01 ± 0.01	0.2 ± 0.18	-
				Prevotella	0.19 ± 0.12	1.11 ± 0.47	-
				Unclass_Prevotellaceae	0.00007 ± 0.0007	0 ± 0	-
			Rikenellaceae	**Alistipes**	0.27 ± 0.1	0.93 ± 0.36	-
			Unclass_Bacteroidales	**Unclass_Bacteroidales**	0 ± 0	0.05 ± 0.03	-
Firmicutes	Bacilli	Lactobacillales	Carnobacteriaceae	**Granulicatella**	0.1 ± 0.07	0.01 ± 0.005	-
			Enterococcaceae	**Enterococcus**	1.45 ± 0.8	0.04 ± 0.02	-
			Lactobacillaceae	**Lactobacillus**	0.09 ± 0.04	0.02 ± 0.004	-
			Leuconostocaceae	**Leuconostoc**	0 ± 0	0.01 ± 0.01	-
			Streptococcaceae	**Lactococcus**	1.62 ± 0.74	1.01 ± 0.52	-
				Streptococcus	7.45 ± 2.9	2.56 ± 1.31	-
	Clostridia	Clostridiales	Catabacteriaceae	**Catabacter**	0.02 ± 0.01	0.03 ± 0.02	-
			Clostridiaceae 1	**Clostridium sensu stricto**	0.14 ± 0.07	0.02 ± 0.01	-
				Unclass_Clostridiaceae 1	0.36 ± 0.09	0.12 ± 0.05	0.031
			Clostridiales_Incertae Sedis XI	**Finegoldia**	0.01 ± 0.01	0 ± 0	-
			Clostridiales_Incertae Sedis XIII	**Unclass_Clostridiales_Incertae Sedis XIII**	0.04 ± 0.02	0.05 ± 0.03	-
			Lachnospiraceae	**Anaerostipes**	2.63 ± 0.74	1.86 ± 1.21	-
				Blautia	2.8 ± 1.31	2.53 ± 0.8	-
				Clostridium XlVa	1.43 ± 0.36	2.22 ± 0.71	-
				Clostridium XlVb	0.16 ± 0.07	0.34 ± 0.26	-
				Coprococcus	0.01 ± 0.004	0.82 ± 0.63	-
				Dorea	1.01 ± 0.3	0.87 ± 0.24	-
				Eisenbergiella	0.19 ± 0.18	0.06 ± 0.04	-
				Fusicatenibacter	5.66 ± 1.36	3.3 ± 1.4	-
				Lachnospiracea_incertae_sedis	21.56 ± 4.55	16.22 ± 4.24	-
				Roseburia	6.74 ± 2.9	2.56 ± 0.76	-
				Ruminococcus2	4.15 ± 1.48	2.24 ± 0.86	-
				Unclass_Lachnospiraceae	13.82 ± 1.98	9.72 ± 1.55	-

			Peptostreptococcaceae	**Clostridium XI**	0.01 ± 0.01	0 ± 0	-
				Romboutsia	0.12 ± 0.03	0.12 ± 0.03	-
				Terrisporobacter	0.12 ± 0.06	0.13 ± 0.07	-
				Unclass_Peptostreptococcaceae	1.04 ± 0.27	1.42 ± 0.42	-
			Ruminococcaceae	**Butyricicoccus**	0.28 ± 0.13	0.05 ± 0.01	-
				Faecalibacterium	2.11 ± 0.65	2.19 ± 0.64	-
				Flavonifractor	0.07 ± 0.02	0.08 ± 0.02	-
				Oscillibacter	0.01 ± 0.004	0.05 ± 0.01	0.019
				Ruminococcus	0.59 ± 0.19	0.74 ± 0.33	-
				Subdoligranulum	0.12 ± 0.07	0.07 ± 0.04	-
				Unclass_Ruminococcaceae	0.58 ± 0.14	1.32 ± 0.47	-
			Unclass_Clostridiales	**Unclass_Clostridiales**	1.33 ± 0.61	1.06 ± 0.24	-
	Erysipelotrichia	Erysipelotrichales	Erysipelotrichaceae	**Clostridium XVIII**	0.44 ± 0.13	0.13 ± 0.05	0.031
				Coprobacillus	0.01 ± 0.004	0.03 ± 0.01	-
				Erysipelotrichaceae_incertae_sedis	0.16 ± 0.03	0.1 ± 0.04	-
				Holdemanella	0 ± 0	1.11 ± 0.53	-
				Holdemania	0.03 ± 0.01	0.07 ± 0.02	-
				Turicibacter	0.01 ± 0.01	0.17 ± 0.13	-
				Unclass_Erysipelotrichaceae	0.31 ± 0.13	0.32 ± 0.12	-
	Negativicutes	Selenomonadales	Acidaminococcaceae	**Acidaminococcus**	0.26 ± 0.18	0.01 ± 0.004	-
				Phascolarctobacterium	0.31 ± 0.26	0.07 ± 0.04	-
			Veillonellaceae	**Dialister**	0.45 ± 0.24	0.56 ± 0.42	-
				Megamonas	0.01 ± 0.01	0.07 ± 0.06	-
				Megasphaera	0.03 ± 0.01	0.04 ± 0.03	-
				Unclass_Veillonellaceae	0.39 ± 0.28	0.07 ± 0.04	-
				Veillonella	1.22 ± 0.64	0.25 ± 0.11	-
	Unclass_Firmicutes	Unclass_Firmicutes	Unclass_Firmicutes	**Unclass_Firmicutes**	0.08 ± 0.02	0.08 ± 0.05	-
Fusobacteria	Fusobacteria	Fusobacteriales	Fusobacteriaceae	**Fusobacterium**	0.01 ± 0.002	0.01 ± 0.01	-
Proteobacteria	Alphaproteobacteria	Rhizobiales	Bradyrhizobiaceae	**Unclass_Bradyrhizobiaceae**	0.01 ± 0.01	0 ± 0	-
		Unclass_Alphaproteobacteria	Unclass_Alphaproteobacteria	**Unclass_Alphaproteobacteria**	0.01 ± 0.01	0 ± 0	-
	Betaproteobacteria	Burkholderiales	Sutterellaceae	**Parasutterella**	0.04 ± 0.02	0.04 ± 0.02	-
	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	**Citrobacter**	0.01 ± 0.004	0 ± 0	-
				Escherichia/Shigella	0 ± 0	0.02 ± 0.01	-
				Unclass_Enterobacteriaceae	0.51 ± 0.26	0.69 ± 0.13	-
		Pasteurellales	Pasteurellaceae	**Haemophilus**	0.13 ± 0.08	0.05 ± 0.02	-
Unclass_Bacteria	Unclass_Bacteria	Unclass_Bacteria	Unclass_Bacteria	**Unclass_Bacteria**	0.29 ± 0.13	0.24 ± 0.12	-

**Comparisons between microbial phylotypes was analyzed by White's non parametrics t-test and corrected by Benjamini–Hochberg for multiple testing. Across rows (*) indicate means that are significantly different (P < 0.05)*

Supplementary Table 6. Mean relative abundances of predicted second-tier functional level in infant gut microbiota born to normoweight mothers and obese mothers (mean ± SEM)

1st tier	2nd tier	Normal	Obese	Padj*
Metabolism	**Amino Acid Metabolism**	19.6 ± 0.11	19.39 ± 0.15	-
	Biosynthesis of Other Secondary Metabolites	1.85 ± 0.02	1.98 ± 0.04	0.048
	Carbohydrate Metabolism	22.71 ± 0.14	22.52 ± 0.15	-
	Energy Metabolism	13.74 ± 0.07	13.53 ± 0.07	-
	Enzyme Families	4.35 ± 0.02	4.31 ± 0.03	-
	Glycan Biosynthesis and Metabolism	3.7 ± 0.18	4.62 ± 0.28	0.042
	Lipid Metabolism	6.15 ± 0.04	6.03 ± 0.05	-
	Metabolism of Cofactors and Vitamins	8.86 ± 0.06	8.81 ± 0.07	-
	Metabolism of Other Amino Acids	2.74 ± 0.02	2.84 ± 0.03	0.042
	Metabolism of Terpenoids and Polyketides	3.07 ± 0.01	3.12 ± 0.01	0.036
	Nucleotide Metabolism	8.17 ± 0.04	7.97 ± 0.06	0.039
	Xenobiotics Biodegradation and Metabolism	3.07 ± 0.04	2.97 ± 0.04	-
	Others	2 ± 0.03	1.9 ± 0.03	-

**Comparisons between functional abundances was analyzed by White's non parametrics t-test and corrected by Benjamini–Hochberg for multiple testing. Across rows (*) indicate means that are significantly different (P < 0.05)*

Supplementary Table 7. Mean relative abundances of predicted third-tier functional level in infant gut microbiota born to normoweight mothers and obese mothers (mean ± SEM) within metabolism (1st tier)

2nd tier	3rd tier	Normal	Obese	Padj *
Amino Acid Metabolism	**Alanine. aspartate and glutamate metabolism**	2.22 ± 0.01	2.3 ± 0.02	0.044
	Amino acid related enzymes	2.82 ± 0.03	2.8 ± 0.04	-
	Arginine and proline metabolism	2.45 ± 0.02	2.47 ± 0.02	-
	Cysteine and methionine metabolism	1.93 ± 0.02	1.86 ± 0.03	-
	Glycine. serine and threonine metabolism	1.58 ± 0.01	1.61 ± 0.01	-
	Histidine metabolism	1.24 ± 0.01	1.3 ± 0.01	-
	Lysine biosynthesis	1.91 ± 0.03	1.78 ± 0.04	0.043
	Lysine degradation	0.192 ± 0.003	0.192 ± 0.005	-
	Phenylalanine metabolism	0.36 ± 0.01	0.39 ± 0.01	-
	Phenylalanine. tyrosine and tryptophan biosynthesis	1.85 ± 0.03	1.76 ± 0.03	-
	Tryptophan metabolism	0.196 ± 0.005	0.18 ± 0.01	-
	Tyrosine metabolism	0.57 ± 0.01	0.59 ± 0.01	-
	Valine. leucine and isoleucine biosynthesis	1.52 ± 0.02	1.44 ± 0.03	-
	Valine. leucine and isoleucine degradation	0.28 ± 0.01	0.31 ± 0.01	-
	beta-Lactam resistance	0.082 ± 0.003	0.07 ± 0.002	-
	Butirosin and neomycin	0.113 ± 0.003	0.124 ± 0.003	-

Biosynthesis of Other Secondary Metabolites	**biosynthesis**			
	Flavone and flavonol biosynthesis	0.004 ± 0.001	0.005 ± 0.001	-
	Flavonoid biosynthesis	0.004 ± 0.001	0.005 ± 0.001	-
	Isoquinoline alkaloid biosynthesis	0.099 ± 0.003	0.109 ± 0.003	-
	Novobiocin biosynthesis	0.262 ± 0.003	0.257 ± 0.004	-
	Penicillin and cephalosporin biosynthesis	0.033 ± 0.005	0.04 ± 0.01	-
	Phenylpropanoid biosynthesis	0.36 ± 0.01	0.38 ± 0.01	-
	Stilbenoid. diarylheptanoid and gingerol biosynthesis	0.0004 ± 0.0001	0.001 ± 0.001	-
	Streptomycin biosynthesis	0.56 ± 0.02	0.64 ± 0.02	0.047
	Tropane. piperidine and pyridine alkaloid biosynthesis	0.197 ± 0.004	0.21 ± 0.003	0.042
Carbohydrate Metabolism	**Amino sugar and nucleotide sugar metabolism**	2.91 ± 0.04	3.05 ± 0.05	-
	Ascorbate and aldarate metabolism	0.34 ± 0.02	0.27 ± 0.02	-
	Butanoate metabolism	1.11 ± 0.02	1.08 ± 0.02	-
	C5-Branched dibasic acid metabolism	0.65 ± 0.01	0.6 ± 0.02	0.045
	Citrate cycle (TCA cycle)	0.91 ± 0.03	1.03 ± 0.04	-
	Fructose and mannose metabolism	2.22 ± 0.03	2.18 ± 0.04	-
	Galactose metabolism	1.86 ± 0.03	1.87 ± 0.03	-
	Glycolysis / Gluconeogenesis	2.25 ± 0.01	2.19 ± 0.02	-
	Glyoxylate and dicarboxylate metabolism	1.03 ± 0.01	1.06 ± 0.01	-
	Inositol phosphate metabolism	0.16 ± 0.01	0.18 ± 0.01	-
	Pentose and glucuronate interconversions	1.44 ± 0.04	1.39 ± 0.04	-
	Pentose phosphate pathway	2.09 ± 0.03	1.95 ± 0.03	0.037
	Propanoate metabolism	0.92 ± 0.01	0.9 ± 0.01	-
	Pyruvate metabolism	2.12 ± 0.01	2.09 ± 0.02	-
	Starch and sucrose metabolism	2.19 ± 0.02	2.21 ± 0.02	-
Energy Metabolism	**Carbon fixation in photosynthetic organisms**	1.37 ± 0.01	1.35 ± 0.01	-
	Carbon fixation pathways in prokaryotes	1.79 ± 0.02	1.87 ± 0.03	-
	Methane metabolism	2.9 ± 0.04	2.69 ± 0.05	-
	Nitrogen metabolism	1.5 ± 0.01	1.48 ± 0.01	-
	Oxidative phosphorylation	2.41 ± 0.02	2.35 ± 0.02	-
	Photosynthesis	0.8 ± 0.02	0.77 ± 0.03	-
	Photosynthesis - antenna proteins	0.00003 ± 0.00002	0.00004 ± 0.00003	-
	Photosynthesis proteins	0.8 ± 0.02	0.77 ± 0.03	-
	Sulfur metabolism	0.48 ± 0.01	0.54 ± 0.01	0.041
Enzyme Families	**Peptidases**	3.71 ± 0.01	3.74 ± 0.02	-
	Protein kinases	0.63 ± 0.01	0.58 ± 0.01	-

Glycan Biosynthesis and Metabolism	**Glycosaminoglycan degradation**	0.09 ± 0.02	0.2 ± 0.03	0.042
	Glycosphingolipid biosynthesis - ganglio series	0.07 ± 0.02	0.15 ± 0.03	0.041
	Glycosphingolipid biosynthesis - globo series	0.21 ± 0.02	0.3 ± 0.03	0.044
	Glycosphingolipid biosynthesis - lacto and neolacto series	0.0001 ± 0.0001	0.0005 ± 0.0002	-
	Glycosyltransferases	0.53 ± 0.01	0.59 ± 0.02	-
	Lipopolysaccharide biosynthesis	0.16 ± 0.03	0.33 ± 0.05	0.043
	Lipopolysaccharide biosynthesis proteins	0.36 ± 0.04	0.57 ± 0.07	-
	N-Glycan biosynthesis	0.022 ± 0.003	0.032 ± 0.004	-
	Other glycan degradation	0.7 ± 0.05	0.92 ± 0.08	-
	Peptidoglycan biosynthesis	1.55 ± 0.02	1.51 ± 0.03	-
	Various types of N-glycan biosynthesis	0.000007 ± 0.000006	0.0000004 ± 0.0000004	-
Lipid Metabolism	**alpha-Linolenic acid metabolism**	0.0012 ± 0.0004	0.0013 ± 0.0003	-
	Arachidonic acid metabolism	0.0166 ± 0.0032	0.028 ± 0.003	-
	Biosynthesis of unsaturated fatty acids	0.2004 ± 0.0036	0.19 ± 0.01	-
	Ether lipid metabolism	0.0036 ± 0.0006	0.0031 ± 0.0006	-
	Fatty acid biosynthesis	0.98 ± 0.01	0.96 ± 0.01	-
	Fatty acid metabolism	0.36 ± 0.01	0.36 ± 0.01	-
	Glycerolipid metabolism	1.02 ± 0.03	0.87 ± 0.04	0.042
	Glycerophospholipid metabolism	1.18 ± 0.03	1.09 ± 0.04	-
	Linoleic acid metabolism	0.164 ± 0.003	0.17 ± 0.004	-
	Lipid biosynthesis proteins	1.11 ± 0.01	1.13 ± 0.01	-
	Primary bile acid biosynthesis	0.085 ± 0.002	0.092 ± 0.003	-
	Secondary bile acid biosynthesis	0.083 ± 0.002	0.09 ± 0.003	-
	Sphingolipid metabolism	0.59 ± 0.03	0.68 ± 0.04	-
	Steroid biosynthesis	0.00019 ± 0.00004	0.0002 ± 0.0001	-
	Steroid hormone biosynthesis	0.02 ± 0.01	0.05 ± 0.01	0.041
	Synthesis and degradation of ketone bodies	0.041 ± 0.005	0.04 ± 0.01	-
Metabolism of Cofactors and Vitamins	**Biotin metabolism**	0.29 ± 0.01	0.31 ± 0.01	-
	Folate biosynthesis	0.66 ± 0.02	0.72 ± 0.02	-
	Lipoic acid metabolism	0.03 ± 0.01	0.06 ± 0.01	0.044
	Nicotinate and nicotinamide metabolism	0.83 ± 0.01	0.83 ± 0.01	-
	One carbon pool by folate	1.2 ± 0.01	1.22 ± 0.01	-
	Pantothenate and CoA biosynthesis	1.25 ± 0.02	1.22 ± 0.02	-
	Porphyrin and chlorophyll metabolism	2.41 ± 0.06	2.13 ± 0.07	0.038
	Retinol metabolism	0.029 ± 0.004	0.04 ± 0.01	-

	Riboflavin metabolism	0.37 ± 0.01	0.42 ± 0.01	-
	Thiamine metabolism	1.04 ± 0.02	1.01 ± 0.02	-
	Ubiquinone and other terpenoid-quinone biosynthesis	0.22 ± 0.02	0.29 ± 0.03	-
	Vitamin B6 metabolism	0.369 ± 0.005	0.38 ± 0.01	-
Metabolism of Other Amino Acids	**beta-Alanine metabolism**	0.37 ± 0.01	0.36 ± 0.01	-
	Cyanoamino acid metabolism	0.61 ± 0.01	0.64 ± 0.02	-
	D-Alanine metabolism	0.184 ± 0.003	0.18 ± 0.004	-
	D-Arginine and D-ornithine metabolism	0.004 ± 0.001	0.004 ± 0.001	-
	D-Glutamine and D-glutamate metabolism	0.273 ± 0.002	0.285 ± 0.003	0.041
	Glutathione metabolism	0.32 ± 0.01	0.35 ± 0.01	-
	Phosphonate and phosphinate metabolism	0.1 ± 0.01	0.11 ± 0.01	-
	Selenocompound metabolism	0.69 ± 0.01	0.7 ± 0.01	-
	Taurine and hypotaurine metabolism	0.191 ± 0.004	0.21 ± 0.01	0.036
Metabolism of Terpenoids and Polyketides	**Biosynthesis of ansamycins**	0.32 ± 0.01	0.28 ± 0.01	0.039
	Biosynthesis of siderophore group nonribosomal peptides	0.024 ± 0.004	0.035 ± 0.003	-
	Biosynthesis of vancomycin group antibiotics	0.107 ± 0.003	0.124 ± 0.004	0.039
	Carotenoid biosynthesis	0.002 ± 0.001	0.003 ± 0.001	-
	Geraniol degradation	0.026 ± 0.005	0.05 ± 0.01	0.045
	Limonene and pinene degradation	0.13 ± 0.01	0.12 ± 0.01	-
	Polyketide sugar unit biosynthesis	0.4 ± 0.01	0.44 ± 0.01	0.044
	Prenyltransferases	0.52 ± 0.01	0.56 ± 0.01	0.044
	Terpenoid backbone biosynthesis	1.11 ± 0.01	1.1 ± 0.02	-
	Tetracycline biosynthesis	0.34 ± 0.01	0.31 ± 0.01	0.044
	Zeatin biosynthesis	0.094 ± 0.002	0.106 ± 0.003	0.049
Nucleotide Metabolism	**Purine metabolism**	4.38 ± 0.02	4.3 ± 0.03	0.04
	Pyrimidine metabolism	3.69 ± 0.02	3.6 ± 0.03	-
	1.1.1-Trichloro-2.2-bis(4-chlorophenyl)ethane (DDT) degradation	0.000009 ± 0.000006	0.0000004 ± 0.0000003	-
	Aminobenzoate degradation	0.13 ± 0.01	0.16 ± 0.01	-
	Atrazine degradation	0.02 ± 0.003	0.016 ± 0.002	-
	Benzoate degradation	0.38 ± 0.01	0.36 ± 0.01	-
	Bisphenol degradation	0.207 ± 0.005	0.201 ± 0.003	-
	Caprolactam degradation	0.037 ± 0.003	0.025 ± 0.003	-
	Chloroalkane and chloroalkene degradation	0.41 ± 0.01	0.37 ± 0.01	0.05
	Chlorocyclohexane and chlorobenzene degradation	0.011 ± 0.001	0.009 ± 0.001	-
	Dioxin degradation	0.159 ± 0.004	0.14 ± 0.01	0.048

Xenobiotics Biodegradation and Metabolism	**Drug metabolism - cytochrome P450**	0.024 ± 0.003	0.029 ± 0.003	-
	Drug metabolism - other enzymes	0.661 ± 0.005	0.66 ± 0.004	-
	Ethylbenzene degradation	0.04 ± 0.01	0.055 ± 0.004	-
	Fluorobenzoate degradation	0.0008 ± 0.0003	0.0007 ± 0.0002	-
	Metabolism of xenobiotics by cytochrome P450	0.0237 ± 0.0031	0.029 ± 0.003	-
	Naphthalene degradation	0.25 ± 0.01	0.25 ± 0.01	-
	Nitrotoluene degradation	0.22 ± 0.01	0.17 ± 0.01	0.044
	Polycyclic aromatic hydrocarbon degradation	0.18 ± 0.01	0.2 ± 0.01	-
	Styrene degradation	0.031 ± 0.003	0.028 ± 0.004	-
	Toluene degradation	0.12 ± 0.01	0.13 ± 0.01	-
	Xylene degradation	0.156 ± 0.004	0.14 ± 0.01	0.04
Amino Acid Metabolism	**Unclass_Amino acid metabolism**	0.45 ± 0.01	0.41 ± 0.01	-
Biosynthesis of Other Secondary Metabolites	**Unclass_Biosynthesis and biodegradation of secondary metabolites**	0.138 ± 0.003	0.13 ± 0.003	-
Carbohydrate Metabolism	**Unclass_Carbohydrate metabolism**	0.52 ± 0.01	0.49 ± 0.01	-
Energy Metabolism	**Unclass_Energy metabolism**	1.69 ± 0.01	1.7 ± 0.01	-
Glycan Biosynthesis and Metabolism	**Unclass_Glycan biosynthesis and metabolism**	0.016 ± 0.003	0.031 ± 0.004	-
Lipid Metabolism	**Unclass_Lipid metabolism**	0.29 ± 0.01	0.26 ± 0.01	-
Metabolism of Cofactors and Vitamins	**Unclass_Metabolism of cofactors and vitamins**	0.15 ± 0.01	0.164 ± 0.005	-
Nucleotide Metabolism	**Unclass_Nucleotide metabolism**	0.09 ± 0.005	0.07 ± 0.01	-
Others	**Unclass_Others**	2 ± 0.03	1.9 ± 0.03	-

**Comparisons between functional abundances was analyzed by White's non parametrics t-test and corrected by Benjamini–Hochberg for multiple testing. Across rows (*) indicate means that are significantly different (P < 0.05)*

PAPER 3

Role of microbiota function during early life on child's neurodevelopment

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Abstract

There are critical periods during pregnancy and early life when child's neurodevelopment can be altered, where different factors including hormones, stress, genetics, and diet have an important role. Novel studies are indicating that also gut microbiota and maternal obesity can influence child's neurodevelopment. Scope and approach: This review summarises the current concepts related to microbiota-gut-brain axis, including microbiota modulation of the eating behaviour, child's cognitive function and brain structure, microbiota analysis techniques and neurodevelopment assessment in children. Further, we propose and present knowledge about potential mechanisms of action and ways to intervene for disease prevention and treatments, opening up an exciting area with important medical and industrial applications. Key findings and conclusions: This novel and fast developing research area is indicating that gut micro- biota in association with body weight might have an important impact on foetal and child neuro- development. However, the exact mechanisms are not known and further research in the field is warranted. Within the MyNewGut Project we aim to analyse the impact of microbiota in association with body weight on cognitive and behaviour development in children. We will study the phylogeny and function of the gut microbial communities in overweight, obese and gestational diabetes pregnancies and in their progeny, in association with infants and children's cognitive and behavioural outcomes. As well, the impact of gut microbiome on brain structure and function during childhood will be evaluated. Results from this study will shed light on the impact of maternal and offspring gut microbiome and body weight on child's neurodevelopment, brain structure and function, and will suggest potential mechanisms for intervention.

1. Introduction

There are vulnerable periods during early life when neurodevelopment can be altered by factors of diverse nature. Environmental factors such as diet, hormones, stress and genetic factors, among others, are candidates to affect offspring neurodevelopment in utero or during the postnatal period. Maternal obesity has demonstrated to have an impact on infant neurodevelopment (Torres-Espinola et al., 2015), and although the causal pathway still remains to be elucidated, an integrated mechanism involving inflammation has been proposed (van der Burg et al., 2015).

Gut microbiota also seems to have an impact on infant neurodevelopment (Keunen, van Elburg, van Bel, & Benders, 2014). Gut-brain axis is not a new concept (Track, 1980), and it can be defined as the crosstalk between gastrointestinal motor and sensory components and central nervous system and the return response to the intestine (Jones, Dilley Jb Fau - Drossman, Drossman D Fau - Crowell, & Crowell, 2006). However, extensive reports in the

literature have also associated gut microbiota with neurodevelopment and mental health, and given the importance of the gut microbiota in modulating health, the microbiota-gut-brain axis has been recently adopted (Collins, Surette, & Bercik, 2012; O'Mahony, Hyland, Dinan, & Cryan, 2011; Rhee, Pothoulakis, & Mayer, 2009). Besides, obesity can alter the composition of the gut microbiome (dysbiosis), although it is not yet clear if the alteration in the composition of gut bacteria occurs as a result of an obesogenic diet or if it is a causal factor in the development of obesity (Harley & Karp, 2012).

Obesity has become a worldwide epidemic impacting negatively the health of millions of people. The increase in the prevalence of obesity in young adults runs in parallel to an increase in the prevalence of obesity and diabetes during pregnancy, and given the adverse effects that these conditions have on both the mother and offspring's health, maternal obesity has become a very important topic of study (Haslam & James, 2005).

The 'normal' physiology during pregnancy differs between obese and normal-weight women. It is well known that obesity is associated, among others factors, with increased insulin resistance, and the same applies in pregnancy. Thus, early in pregnancy, obese mothers are more insulin resistant than lean women. These factors can lead to potential adverse effects in implantation and placentation processes (O'Reilly & Reynolds, 2013), and alter growth, development and metabolism of the foetus and even impact offspring neurodevelopment (Camprubi Robles et al., 2015; Torres-Espinola et al., 2015). Moreover, a maternal obesogenic condition has been related to dysbiosis, potentially altering the offspring's gut microbiota composition (Collado, Isolauri E Fau - Laitinen, Laitinen K Fau - Salminen, & Salminen, 2010). Gut microbiota solely or in combination with maternal obesity and others factors could have an impact on offspring's neurodevelopment (Borre et al., 2014) and also increase the risk of becoming obese during infancy or into adulthood (Gohir, Ratcliffe, & Sloboda, 2015) (Fig. 1). Until now, studies focused on the origins of obesity were oriented toward dietary excesses or host genes (Hollopeter, Erickson, & Palmiter, 1998). Experimental studies indicate an important function of the gut microbiota in promoting obesity (Delzenne & Cani, 2011). Some phyla and classes of bacteria are able to metabolise nutrients more efficiently than others, increasing the amount of energy usable for the host and increasing the absorption of calories from the diet, contributing to fat deposition (Turnbaugh, Backhed, Fulton, & Gordon, 2008a,b). Higher concentration of *Bacteroidetes* has been associated with a lean phenotype in several studies while *Firmicutes* have been found in higher amounts in obese subjects (Turnbaugh et al., 2006). These results were further confirmed in pregnant women (Santacruz, Collado, et al., 2010). It is necessary to identify the active bacteria that cause dysbiosis in the intestinal microbial community in order to design therapeutic strategies for long term protection against obesity and to prevent potential impairment in offspring's neurodevelopment. In recent years, improved high performance technologies and cultivation-independent methods have made it possible to characterize the composition of microbial community more accurately. This fact has enabled to link obesity with the intestinal microbial composition, demonstrating that a diversity of organismal assemblages can yield a core microbiome at a functional level, and that deviations from this core are associated with different physiological states (Santacruz, Collado Mc Fau - Garcia-Valdes, et al., 2010; Turnbaugh, Backhed, Fulton, & Gordon, 2008a,b; Turnbaugh et al., 2009).

Several studies have shown that microbiota disruption in children, as result of the type of delivery (i.e. colonization of the neonate is disrupted by C-section delivery (Blustein et al., 2013)) and by low-dose antibiotic exposure during maturation (Cox et al., 2014; Vrieze et al., 2014) can alter host metabolism and adiposity. However, the potential relationship between maternal and offspring microbiota composition and neurodevelopmental outcomes needs to be elucidated.

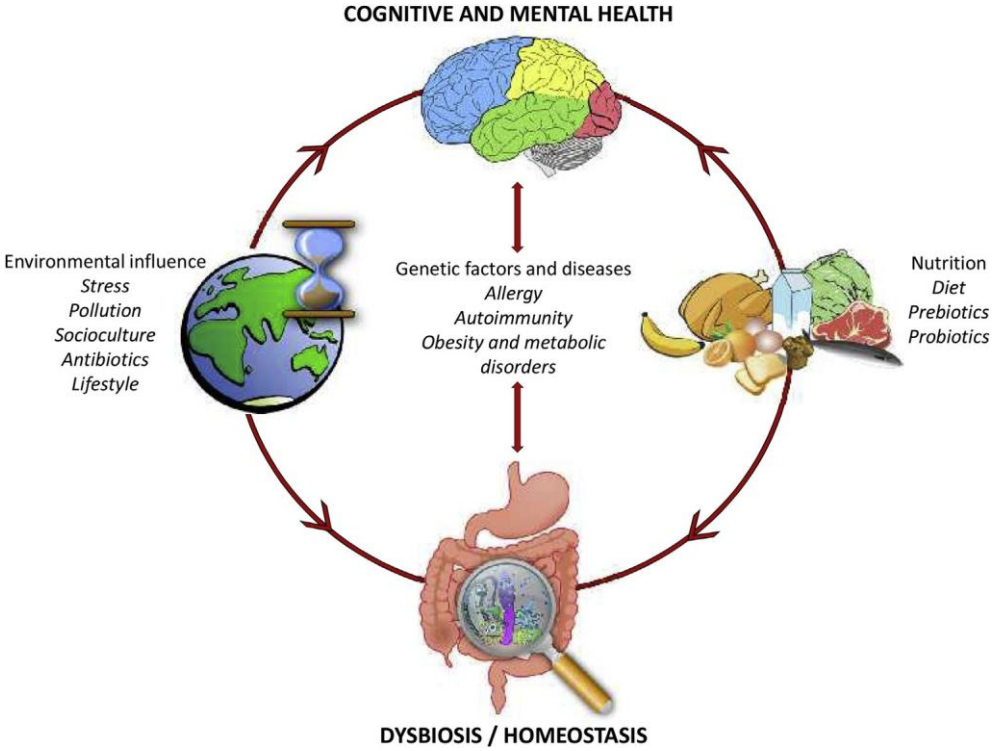

Fig. 1. Microbiota-gut-brain axis. Bidirectional crosstalk between gut microbiota and cognitive and mental health. Dysbiosis, diet and others external factors as mediators of obesity and related diseases and as key modulators of the bidirectional signalling pathways between the gut and brain that underlie neurodevelopmental disorders.

A dysbiosis of the intestinal microbiota may induce physiological and immunological alterations favouring inflammation. This inflammation will contribute to intestinal barrier loss, causing translocation of pathogenic bacteria components to the systemic circulation from the intestinal mucosa. These components activate the innate immune system, which produces pro-inflammatory cytokines, leading to an impairment of the central nervous system (CNS) function, such as visceral pain, and alterations in neurochemistry, cognition, behaviour and stress response (Borre et al., 2014).

Recent studies reveal the importance of gut-brain axis in the development of various mental diseases and cognitive development (Foster & Neufeld, 2013). Again, this topic becomes even more interesting if, in addition, we consider the potential negative effects of maternal obesity on their offspring's cognitive and behavioural development, but also considering that obesity implies changes in the gut microbiota. Maternal obesity has been linked to a reduced offspring's cognitive development in early life stages (Casas et al., 2013), an increased risk of childhood autism and a decreased progress in language development (Reynolds, Inder, Neil, Pineda, & Rogers, 2014).

Our research group is conducting a study within the MyNewGut Project that aims to analyse the impact of the microbiota in relation to obesity on cognitive and behaviour development in the offspring. This approach will be achieved studying the microbiome phylogeny and function of the gut microbial communities, thorough a holistic (or eco-systems biology), and infants' cognitive and behavioural outcomes during childhood. Results from this study will shed light on the impact of maternal and offspring gut microbiome on the infant neurodevelopment and behaviour, and on brain structure and function, and will suggest potential mechanisms of intervention to prevent adverse health effects on their offspring. This review will present the state of the art concepts related to this topic, including microbiota modulation of the eating behaviour, the microbiome impact on the infant cognitive function and behaviour and on brain structure and function, microbiota analysis techniques and neurodevelopment methodological assessment and functional changes. Further, we will propose and present knowledge about the potential mechanisms of action and ways to intervene for disease prevention and treatments, opening up an exciting area with important medical and industrial applications.

2. Microbiota during pregnancy: implications for neurological disorders

Critical periods for neural development during the intrauterine period are well known. Recent experimental evidence suggests a link between gut microbiota and brain function and behaviour (Sommer & Backhed, 2013). The establishment of the human gut microbiota could begin early in utero and then shaped by compositional and functional changes from birth and during the first years of life after postnatal environmental expositions (Rautava, Luoto, Salminen, & Isolauri, 2012). Pregnancy is a condition characterized by important physiological and immunological changes. In addition, during pregnancy alterations in vaginal and gut microbiota profile occur (Aagaard et al., 2012; Koren et al., 2012). Changes performed in the microbiota profile could have an impact on the maternal metabolic profile and also could contribute to the immune and metabolic health in their progeny (Laker, Wlodek, Connelly, & Yan, 2013). However, the extent for which microbial changes during the intrauterine life could impact child neurodevelopment needs to be confirmed.

Recently, the role of gut microbiota during pregnancy has gathered a considerable attention in the scientific community. Through pregnancy, changes in the gut microbiota composition have been observed from the first trimester of gestation (Koren et al., 2012). As we further explain, during the third trimester of a healthy pregnancy, the gut microbiota shows signs of inflammation, adiposity and insulin insensibility in a similar way as in obese women (Koren et al., 2012).

Until recently, the placenta has been considered a sterile organ. Nowadays, we know about the existence of the placental microbiome (Aagaard et al., 2014; DiGiulio Daniel et al., 2010; Lee Si, Romero, Lee Seung, & Yoon Bo, 2010; Romero et al., 2014). Placental microbiota composition has been related to preterm birth, which highlights the importance of the internal crosstalk between the microbiota and the pregnant woman (Aagaard et al., 2014). Also, changes in placental microbiota profile have been observed in association with low birth weight in term pregnancies (Zheng et al., 2015), which potentially could rise the risk of neurodevelopmental impairment. Infants born preterm are at increased risk of developing motor and cognitive impairments and a wide range of neurodevelopmental disabilities (Jarjour, 2015). Recently, placental microbiota profile has been associated with pre-eclampsia development during pregnancy (Amarasekara, Jayasekara, Senanayake, & Dissanayake, 2015); meanwhile significant associations between pre-eclampsia and autism spectrum disorder and developmental delay have been found (Walker et al., 2015). However, much remains to be clarified as the implications of prenatal microbial exposure are currently unknown and their relation with disease development and neurodevelopment is a promising and an emerging area of study.

The origin of the bacteria colonising the placenta is unknown. Placental microbiome consists of non-pathogenic bacteria from the phylum *Proteobacteria*, *Firmicutes*, *Bacteriodetes*, *Fusobacteria* and *Tenericutes* (Aagaard et al., 2014).

Placental microbial community composition and its function might have relevant implications for maternal and foetal health with important clinical applications. The use of probiotics might be an important tool in relation to microbiota manipulation. However, the effects of the bacterial profile modification by probiotic supplementation during pregnancy and the effects on placental microbiome modulation are still unknown and further studies are necessary in order to design appropriate recommendations for the general population. Placental dysbiosis during pregnancy as occurs with excessive weight gain during pregnancy (Antony et al., 2015) could have an important role in the early colonization and in the establishment of the human gut microbiota in early stages of life. Dysbiosis at a critical moment might also have an impact on the future health, behaviour and cognitive functions.

Early microbiota establishment and neurodevelopment share similar critical developmental windows vulnerable to damage (Borre et al., 2014). As little is known about these relationships and the implications for health at the population level are important, future studies on this topic are further warranted to confirm or refute these hypotheses and to shed light on the underlying associated neural, immunologic, endocrine, metabolic mechanism or pathways to facilitate potential ways of intervention.

3. Postnatal period: crucial for gut microbiota imprinting

Neonatal period is an important stage for the colonization and shaping of the gut microbiota, which might influence the lifelong health. Until recently, it was believed that the foetus was sterile, but new studies show the presence of a bacterial community in the amniotic fluid and in the placenta (Wassenaar & Panigrahi, 2014). At birth, the gut of the baby is colonized within a few hours by contact with the maternal vagina or the skin microbiota (Caesarean) and with the environment (Di Renzo, Maulik, &

Treadway, 2014). The first few weeks of life are very important for the gut colonization, because this stage will be influenced by the type of feeding, gestational age, hospitalization and antibiotics treatment, among other factors (Penders et al., 2006).

Human milk contains an abundance of bioactive components that contribute to improve cognitive function over formula-fed infants (Anderson & Burggren, 2014). The milk fat globule membrane (MFGM), composed for constituents typical of biological membranes like phospholipids, cholesterol or glycolipids, has gained interest for their potential positive health effects (Benoit et al., 2010). Their components have been shown to be essential for brain development. It has been found that supplementing infant formulas with MFGM favours reducing the gap in cognitive performance between breastfed and formula-fed infants at 12 months of age (Timby, Domellöf, Hernell, Lönnerdal, & Domellöf, 2014). Another function of the MFGM is to provide antimicrobial effects, such as mucin to decrease the adherence of Yersinia enterolytica to intestinal membranes or xanthine oxidase, which inhibits bacterial growth in the presence of a reducing substrate, generating hydrogen peroxide (Hancock et al., 2002). It has been found that formula-fed infants have a greater weight gain between 3 and 12 months of age than breastfed babies, presenting an increased risk of obesity, hypertension, type 1 and 2 diabetes (Timby et al., 2014). Further, the microbiota community of formula-fed infants presents different facultative anaerobes *Bacteroides* and Clostridium, which are not present in breastfed-only infants (Guaraldi & Salvatori, 2012). Although it was believed that *Bifidobacterium* was in a greater proportion in formula-fed infants than in breastfed infants, studies have raised questions about the actual levels (Gomez-Llorente et al., 2013).

A study about microbial colonization patterns in mouse confirms that the use of polyamines in formula-fed BALB/cOlaHsd mouse minimises differences with breast-fed BALB/cOlaHsd mouse. The effects of polyamines can improve the barrier function and modulate the immune system development, regulating the levels of *A. muciniphila* and *Bifidobacterium spp*. Nevertheless, these findings need to be studied in humans (Gomez-Gallego et al., 2014).

Although infant milk formulas have evolved greatly during the last years, a formula that provides exactly the same benefits than the breast milk has not yet been obtained. It is necessary to continue studying the composition and the positive effects of breast milk versus milk infant formula in order to better understand the beneficial role of breast milk on offspring's health and neurodevelopment and to improve the outcomes in the formula fed infants.

4.Microbiota-gut-brain axis

Microbiota-gut-brain axis is an extensively reviewed novel concept (Cryan & Dinan, 2012; Forsythe, Kunze Wa Fau - Bienenstock, & Bienenstock, 2012; Montiel-Castro, Gonzalez-Cervantes, Bravo-Ruiseco, & Pacheco-Lopez, 2013; Rhee, Pothoulakis, & Mayer, 2009). It involves gut microbiota as a new player in the gut-brain axis with marked effects on both components. Traditionally, studies have been centred in the link between the brain and gut function in the modulation of satiety and appetite. The microbiota-gut-brain axis can be defined as the bidirectional crosstalk between the development and composition of the microbiota in the intestinal mucosa and the CNS of the host including cognitive and vegetative functions.

It is well known that gut microbiota composition can be modulated by stress factors and the associated activity of the hypothalamus-pituitary-adrenal (HPA) axis (Tannock Gw Fau - Savage & Savage, 1974). It has been suggested that early stress in life and chronic stress in adulthood can have long-term effects on microbiota composition (Cryan & Dinan, 2012). Besides, an increased bacterial translocation in chronic depression, an stress-related behaviour, has been observed (Maes, Kubera, Leunis, & Berk, 2012). However, up to date, the mechanisms by which the CNS can modify microbiota composition remains to be fully understood. Literature highlighting the importance of the involvement of gut microbiota in different psychiatric diseases such as autism (Toh & Allen-Vercoe, 2015), schizophrenia (Dinan, Borre, & Cryan, 2014), hyperactivity (Pärtty, Kalliomaki, Wacklin, Salminen, & Isolauri, 2015) or depression (Dash, Clarke, Berk, & Jacka, 2015) has considerably increased in recent years.

Gut microbiota can trigger a variety of mechanism affecting the immune, nervous, endocrine and metabolic system of the host. Components of the microbiota-gut-brain axis includes the intestinal microbiota, the CNS, endocrine and immune systems, the HPA axis, the sympathetic-parasympathetic autonomic nervous system (ANS), and the enteric nervous system (ENS) (Collins & Bercik, 2013; Cong, Henderson, Graf, & McGrath, 2015; De Palma, Collins, Bercik, & Verdu, 2014).

Communication between the components of the microbiota-gut-brain axis is bidirectional, enabling top-down signalling from the brain to the gastrointestinal (GI) track and bottom-up from the gut to affect brain function (Cong, Henderson, Graf, & McGrath, 2015). One of the evidences of the cross-talk between gut microbiota and the brain is the fact that gut microbiota is able to regulate the ENS development and the stress-related behaviour by affecting the production of neurotransmitters; appetite, through regulation of gut hormones; and cognitive function such as learning, memory and decision making processes (reviewed in Mu, Yang, & Zhu, 2016) as well as brown adipose tissue (BAT) function (Geurts et al., 2015). Thus, the bottom-up regulation of the CNS by the microbiota can be achieved through neural, endocrine, metabolic and immunological pathways. The neural pathway can operate through the ENS that controls gastrointestinal functions, and vagal afferent nerves that convey sensory information from viscera to the CNS (Wang & Kasper, 2014). On the contrary, it is well recognized how the nervous system affects gut microbiota composition and function as well as colonic functions, as for example in situations that involve stress exposure or traumatic events (reviewed in Mu, Yang, & Zhu, 2016).

The way this bidirectional communication occurs is by afferent fibres, which arise from the gut and projects toward cortical areas such as cerebral, anterior and posterior cingulate, insular, and amygdala cortices, as well as effector fibres, projecting to the smooth muscle of the gut, going on the opposite direction (Montiel-Castro et al., 2013).

Given the important implications in mental and systemic health for an individual, the study of the long-term consequences of alteration in gut microbiota composition in early life on brain and behaviour needs further attention. In this respect, painful events and maternal separation experienced early in life may alter the GI function and the gut microbiome through the gut-brain axis with a potential impact on health in adulthood (Cong et al., 2015). It is important to mention that the influence of the maternal microbiota on the cognitive development of the offspring has not been thoroughly studied, and only independent studies have intended to link maternal and offspring microbiota transference with its effect on offspring cognitive development (Cong et al., 2015; Jasarevic, Howerton, Howard, & Bale, 2015; van der Burg et al., 2015). Additionally, others factors might have an impact on the microbiota-gut-brain axis functioning including genetic, dietary factors (diet, probiotics, prebiotics) and external factors such as social factors, environment or lifestyle. It is an emerging and promising area of study opening up exciting potential mechanisms of intervention early in life. However, before interventional approaches can be

applied, further studies are needed as the precise mechanisms of the microbiome-gut-brain axis interactions needs to be fully unravelled.

5. Maternal obesity, inflammation and neurodevelopment

It is well established that maternal obesity is associated with a pro-inflammatory milieu during pregnancy and in the placenta, and that inflammation is increased in pregnancies complicated by obesity (Saben et al., 2013, 2014). Classical understanding of inflammation is a normal physiologic response to an infectious threat or tissue injury, which leads to tissue repair, resolution, and restoration of the homeostatic balance. The chronic inflammatory state of obesity, however, has different consequences resulting in chronic tissue malfunction and a shift of the normal 'balance' to adapt to a new physiological or metabolic conditions (Medzhitov, 2008). Experiments on mice have shown that obesity during pregnancy augments inflammation through an increase in macrophage activation and elevation of cytokine gene expression, providing potential links between maternal obesity and placental inflammation and early programming of offspring (Jasoni, Sanders, & Kim, 2014).

Changes in microbiota composition or dysbiosis have been related to a broad range of inflammatory diseases, including obesity, allergies, asthma, inflammatory bowel diseases and associated non-communicable diseases (West et al., 2015). A reduced exposure to microbes in developed countries has been proposed, at least in part, as potential contributors to the increased arise in chronic low-grade inflammation diseases and impaired immune regulation (Rook, 2013; Wold, 1998). Microbiota can have effects on the immune system as there is a group of bacteria within the confined space created between the mucus layer and the intestinal epithelial surface that appears to be monitored both by dendritic cells and by epithelial Toll Like Receptors (TLRs) (Duerkop, Vaishnava, & Hooper, 2009; Forsythe & Bienenstock, 2010). This mechanisms of bacterial detection provokes the activation of endocrine (cortisol), immune (cytokines) and neural (vagus and enteric nervous system) pathways, that directly affect brain function (Cryan & Dinan, 2012).

Recent studies are suggesting that an inflammatory in utero environment is also associated with neurodevelopmental impairments in the offspring (Jelske W van der Burg et al., 2015). The mechanisms of child's neurodevelopment are a complex combination of genetic, environmental, and intrauterine programming, and the effects of a prenatal obesogenic environment on foetal brain development and future mental health are still largely unknown. Based on animal and basic research results, different hypothesis of how maternal obesity could influence foetal, neonatal, and later developmental outcomes by increasing the risk of systemic and brain inflammation have been summarised by a recent review (van der Burg et al., 2015) (see Fig. 2). Nevertheless, the causal pathways by which maternal obesity contribute to foetal adverse neurodevelopmental outcomes remains to be elucidated (Van Lieshout, Taylor, & Boyle, 2011; Van Lieshout, 2013).

It is concluded that a better control of maternal obesity and the associated dysbiosis could lead to important benefits for the cognitive and psychiatric functioning of the child (Brown, DeCoffe, Molcan, & Gibson, 2012).

6. Microbiome and eating behaviour in association with brain function

Unhealthy eating can lead to several diseases including obesity, diabetes, heart disease and cancer, among others. New approaches for managing diseases related to impaired nutrition, such as obesity and metabolic syndrome, and to brain function that can influence eating behaviour, involve gut microbiota composition and function. This new microbial approach could change the way these disorders may be investigated and prevented.

Recent studies are focussing on the capability of the gut microbiota to modify metabolites produced by the host or by themselves, which in addition to their involvement in metabolic diseases, these compounds have been shown to be crucial for neuronal development (Gasperotti et al., 2015; Ridaura & Belkaid, 2015). For instance, certain microbial-derived metabolites from dietary polyphenol compounds such as the so called polyphenol microbial metabolites of low molecular weight, could have a role in the gut microbiota-brain connection (Gasperotti et al., 2015). Also certain short-chain fatty acids from microbial production can affect the production of serotonin, an important hormone involved in the regulation of diverse physiological functions besides of playing an important role in behaviour (Ridaura & Belkaid, 2015). The fact that nutrition during early life periods has an impact on neurodevelopment is well known, however, information regarding gut microbiota in connection to eating behaviour and their effect in human brain is scarce or almost inexistent.

The effect of diet on the gut microbiota composition is well established and changes can be measured within 24 h of changing diet (David et al., 2014; Flint, 2012; Smits, Bouter, de Vos, Borody, & Nieuwdorp, 2013; Wu et al., 2011). Thus, changes in microbiota composition are easily reversible.

As diet affects microbiota composition, the other way round, bacteria can alter eating behaviour and food preferences of the host. A positive feedback loop between the host's dietary preferences and appetite and the gut microbiome has been recently proposed (Norris, Molina, & Gewirtz, 2013). Gut microbiota can influence host behaviour through the so called microbiome-gut-brain axis (Bercik, Collins, & Verdu, 2012; Collins, et al., 2012; Rhee, Pothoulakis, & Mayer, 2009) and evidence suggests that the vagus nerve, which plays a key role in this communication, regulates eating behaviour and body weight (Camilleri et al., 2008; Sarr et al., 2012). Besides alterations of the microbiome-gut-brain axes, the mechanisms proposed include microbes production of toxins that may alter mood with the aim to increase eating by the host, receptor alterations of diverse nature with different impacts on eating behaviour, or influences on the reward satiety pathways (reviewed in (Alcock, Maley, & Aktipis, 2014)). This in fact occurs during pregnancy in women under excessive stress, leading to HPA axis dysfunction that could impact the eating behaviour of the offspring in the long-term lifespan (Spencer & Sominsky, 2014) (Fig. 3). Intestinal gut constitutes an important source of dopamine and serotonin, which are important modulators of mood and behaviour (Kim & Camilleri, 2000). Certain strains of bacteria have been shown to produce such neurotransmitters involved in mood and behaviour as well as others involved in satiety and hunger, highlighting the gut microbiota as a complex endocrine organ (Clarke et al., 2014). The hypothesis of the origin of food cravings as a result of the specialization of processing of specific metabolic compounds of the gut microbes is gaining support, more than the conventional hypothesis of the cravings for food as a result of the deficiency in nutrients in the host (Alcock et al., 2014).

It has been suggested that a lower diversity in the gut microbiota could be associated with unhealthy eating behaviour (affecting satiety and food preferences), and a higher obesity risk (Alcock et al., 2014). Excess in energy income is predicted to cause a reduction in microbial diversity, therefore impacting satiety and favouring obesity (Alcock et al., 2014). The HPA axis has been indicated to play a role at the basis of the mechanisms of eating behaviour, and might be affected by factors such as insulin resistance and changes in lipids profiles which are emerging to be

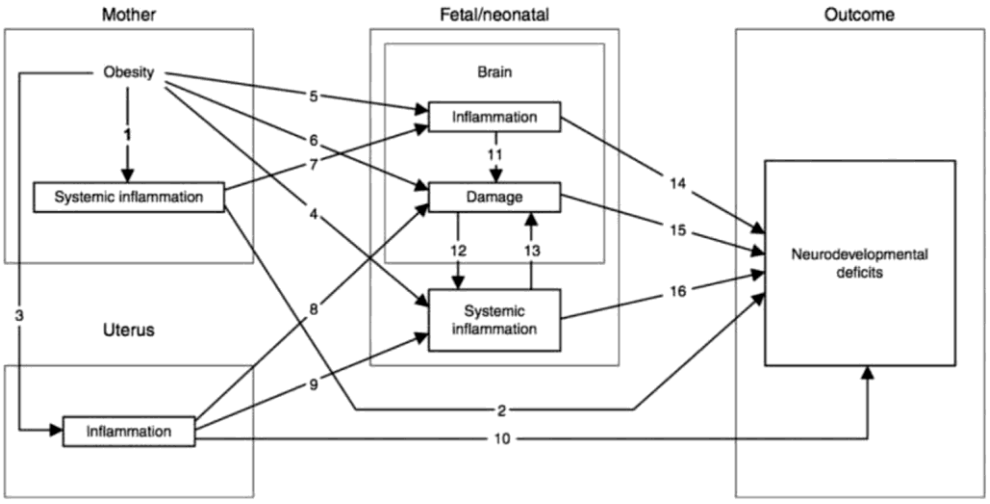

Fig. 2. Proposed networks outlining inter-relationships between maternal obesity, inflammation, and neurodevelopmental deficits. Numbers along arrows indicate relationships between: 1) maternal obesity and maternal systemic inflammation, 2) maternal systemic inflammation and longterm neurodevelopmental deficits, 3) maternal obesity and in- trauterine inflammation, 4) maternal obesity and systemic inflammation in the offspring, 5) maternal obesity and neuroinflammation in the offspring, 6) maternal obesity and fetal/ neonatal brain damage, 7) maternal systemic inflammation and fetal/neonatal brain inflammation, 8) intrauterine inflammation and fetal/neonatal brain damage, 9) intrauterine inflammation and fetal/neonatal systemic inflammation, 10) intrauterine inflammation and neurodevelopmental deficits, 11) fetal/neonatal inflammation and fetal/neonatal brain damage, 12) fetal/neonatal brain damage and fetal/neonatal systemic inflammation, 13) fetal/neonatal systemic inflammation and fetal/neonatal brain damage, 14) fetal/neonatal brain inflammation and clinical neurodevelopmental deficits, 15) fetal/neonatal brain damage and neurodevelopmental deficits, 16) fetal/neonatal systemic inflammation and neurodevelopmental deficits. (Reprinted with permission from Macmillan Publishers Ltd: Pediatric Research, van der Burg et al., 2015).

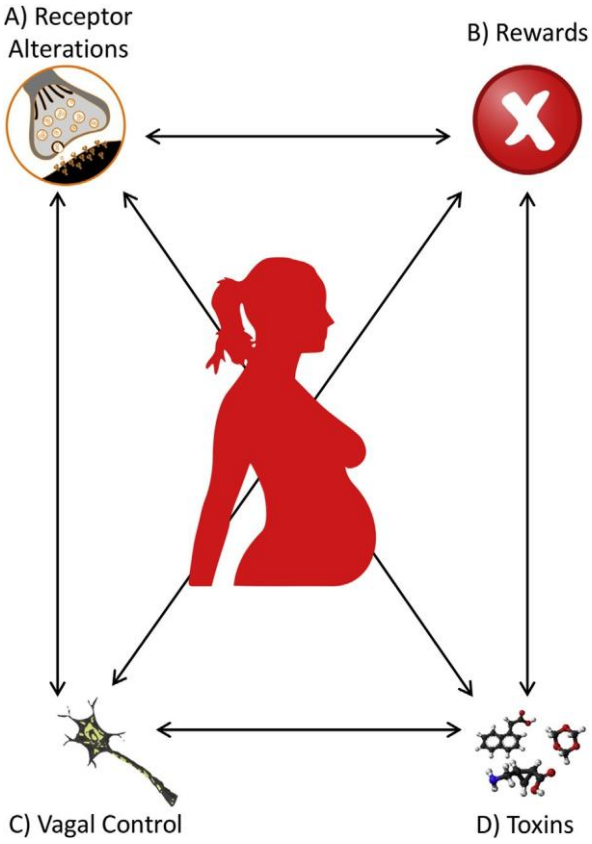

Fig. 3. Potential mechanisms through which bacteria may control the eating behaviour of the mother. A, Changes to receptors including taste receptors (Depoortere, 2015); B, Manipulation of reward pathways (Spencer & Sominsky, 2014); C, Hijacking of neurotransmission via the vagus nerve, which is the main neural axis between the gut and the brain (Bercik et al., 2011); D, Production of toxins that alter mood (Sekirov et al., 2010).

influenced by the gut microbiome composition and function (Dinan, Stilling, Stanton, & Cryan, 2015).

As mentioned previously, apart from maternal diet, pregnancy itself has an influence on maternal gut microbiota composition (O. Koren et al., 2012). Of relevance is how a shift in maternal gut microbiota during pregnancy could impact maternal eating behaviour during a vulnerable period per se prone to developing eating disorders (Knoph Berg et al., 2011). Also, it is important to consider how this change in the maternal gut microbiota in pregnancy could have an impact on the microbiome-gut-brain axes in relation to eating behaviour in the future children. A better understanding of the impact of microbiota on eating behaviour will help food industry to impact society in promoting healthier food choices by altering gut microbial populations. As we will discuss further, this could be achieved, among others, by the use of probiotics, food intake manipulation, faecal transplants, or the use of antibiotics to selectively remove targeted undesirable bacteria. New research is being conducted with the aim to investigate whether eating behaviour and food choices affect gut microbiome structure by applying metabolomics and proteomics analyses. Whether changes in the gut composition may propagate to the full metabolic capacities of the microbiome and the brain in relation to eating behaviour could be achieved by exploring the gut community at a global scale. Furthermore, efforts are being made to establish the extent to which microbiome function is modifiable by nutritional interventions in order to prevent diet and brain related disorders.

A recent study has shown that some probiotic strains administered to the mother during the prenatal stage have different ability to transfer from the mother to the child (Dotterud et al., 2015). Once the efficacy of the microbiome function manipulation on the eating behaviour by interventions have been tested in the general population, the applicability towards vulnerable populations such as obese and pregnant women and the evaluation of such intervention on the impact on their offspring's microbiome-gut-brain axis and eating behaviour at long term could be evaluated and future recommendations and interventions could be developed.

In our previous study, we demonstrated that the obese adolescents show a higher grade of impulsivity and more difficulties for decision making (Delgado-Rico et al., 2012). These aspects are very much linked to alterations of eating behaviour; if these changes are already programed during early life, even from prenatal time, and especially in children born to obese mothers, need further research.

7. Fatty acids and intestinal microbiota

The importance of fatty acids in brain development and function in the foetus and later in postnatal life is well established (Campoy et al. 2015; Demmelmair & Koletzko, 2015). Foetuses and infants have a limited ability to synthesise omega 3 long-chain polyunsaturated fatty acids (n-3 LC-PUFAs) de novo, so the prenatal fatty acids are obtained from maternal stores and diet, and postnatally from breast milk or infant formulas (Campoy et al. 2015). It has been reported repeatedly that breastfed children attain higher Intelligence Quotient (IQ) scores than non-breastfed children, probably because of the amount of fatty acids present (Anjos et al., 2013).

Recent studies by Stanton and co-workers showed that intestinal bacteria can convert linoleic and linolenic acids to bioactive isomers of conjugated linoleic acid (CLA) and conjugated a-linolenic acid (Wall et al., 2010). Some authors found that CLAs are a potent naturally occurring ligand and activator of some nuclear receptors, that might play key roles in the regulation of metabolism, e.g. peroxisome proliferator-activated receptor-gamma (PPARg) or PPARa, but there are still huge discrepancies between animal and human studies (Druart et al., 2014). Particularly, it has been shown that there exists a bi-directional relationship between DHA and microbiota. DHA may alter the gut microbial populations, and some microbial such as *Bifidobacterium* species may improve the tissue distribution of DHA especially into the brain (Tabbaa, Golubic, Roizen, & Bernstein, 2013). Some bacterial strains (*Bifidobacterium breve*, *Lactobacillus paracasei*) managed by oral way, have demonstrated to increase the activity of liver desaturases in rats, which resulted in increased amounts of arachidonic acid derived from linoleic acid (Barrett, et al., 2012; Rosberg-Cody, et al., 2011).

Recent years have seen the discovery of two types of lipid mediators derived from docosahexaenoic acid (DHA), resolvins and protectins, which modulate the inflammatory response, and not merely by decreasing cytokine production and dampening inflammation, but by actively promoting the resolution of inflammation (El-Sharkawy et al., 2010).

The capacity of the gut microbiota to ferment otherwise indigestible polysaccharides and provision of short-chain fatty acids (SCFAs), allow to obtain acetate, propionate and butyrate, which are the main energy sources for the intestinal epithelium and liver. These SCFAs are involved in the modulation of the immune response by reducing intestinal permeability (Ferreira et al., 2012), and control the regulation of the colon acting on the anti-inflammatory function through the activation of T cells (Tilg & Moschen, 2014). Besides, it has been found that propionate can protect from allergic airway inflammation, regulates dendritic cells function and bone marrow haematopoiesis (Tilg & Moschen, 2014). Furthermore, propionate and butyrate are able to promote the activation of gluconeogenesis. This, together with the enrichment of microbial genes involved in carbohydrate metabolism could promote adiposity and obesity (Blaut, 2014). The reduction of butyrate levels by the microbiota contributes to the development of insulin resistance. Children with Type 2 Diabetes Mellitus (T2DM) present a lower proportion of butyrate-producing *Clostridiales* (*Roseburia* and Faecalibacterium *prausnitzii*), and greater proportions of Clostridiales that do not produce butyrate (Karlsson et al., 2012). *F. prausnitzii* and *Roseburia spp* are thought to protect against bacterial translocation either directly (being mucosal-associated) or through their ability to produce butyrate, and to reduce adipose inflammation that take part in insulin-resistance (Marteau, 2013). Of relevance is that butyrate has direct effects on epigenetic regulation of gene expression, as a histone deacetylase inhibitor, influencing susceptibility to T2DM (Davie, 2003). In fact, a summary of major findings from metagenome-wide association studies (MGWAS) in patients with T2DM is that the butyrate-producing Roseburia intestinalis, *Faecalibacterium prausnitzii* concentrations and genes involved in vitamin synthesis (e.g. riboflavin) are lower in T2DM. While *Lactobacillus gasseri*, *Streptococcus mutans*, *Proteobacteria*, certain *Clostridiales* and the expression of microbiota genes involved in oxidative stress are higher in T2DM (Tilg & Moschen, 2014).

Evidence indicates that the gut microbiota may greatly influence many neurophysiological parameters, including cognitive functions such as learning, memory and decision making processes (Montiel-Castro et al., 2013). The gut microbiota has been associated with changes in gut peptide expression and release, and nutrient receptor expression, as well as changes in the peripheral and CNS (Carabotti, Scirocco, Maselli, & Severi, 2015). Patients with gastrointestinal diseases, such as inflammatory bowel disease, exhibit an aberrant gut microbiota sometimes associated with psychiatric disorders (Delhanty, van der Lely, 2014). In agreement, probiotic treatment (with *Bifidobacterium longum* str. NCC3001) in mice with moderate colitis has been shown to alter brain behaviour, normalizing anxiety-like behaviour (Collins et al., 2012). Moreover, administration of *Bifidobacterium* species together with n-3 PUFAs in animal model resulted in higher concentrations of EPA in liver tissue and DHA in brain tissue compared to concentrations achieved without bacterial administration. Thus, the gut microbiota is able to influence fatty acid concentrations in tissues other than the gut (Tabbaa et al., 2013).

8. Methodologies for assessing neurodevelopment and brain structure and function in infants and children

The existence of critical periods involved in the neuropsychological development and the gradual and sequential maturation and development of each brain domain should be taken into account when assessing cognitive function in infants and children.

Different methodologies available for neurodevelopmental assessment and brain structure and function in infants and children are summarised in Table 1. These non-invasive methods can help to explore the potential connection between gut microbiome and neurodevelopmental disorders in these vulnerable populations. The strengths and weaknesses of some of these methods have been reviewed elsewhere (Lee & Chamberlain, 2007).

Conventionally, the most common assessment used for global neurodevelopment impairment before 2 years of age is the Bayley Scales of Infant Development (Bayley M, 2006), which explores mental, motor and socioemotional development. However, in the neonate and during early infancy (<4 months), the clinical assessment involves the "General movements test" for predicting abnormal motor development. "Head Control" and "Roll Over" are neurological developmental milestones acquired by the infant that can be also assessed (Koshiba, et al., 2015). Finally, the Ages and Stages Questionnaire (ASQ) is a commonly used tool for developmental delay screening. The ASQ is a parent-completed questionnaire organized by age, from 1 month up to 5 years and a half, divided into domains of communication, gross motor, fine motor, problem solving, and personal-social domain (Squires et al., 2009).

Cognitive function is a complex process that is not easy to measure even when using the appropriate tests. It consists of different brain domains, which are measured using different

Table 1
Methodologies for assessing neurodevelopment and brain structure and function in infants and children.

Some of the most commonly used neuropsychological tests: (assessing: Psychomotor development, Intelligence, Mental performance, and Behaviour maturation)
Bayley Scales of Infant Development (version III)
General movements
ASQ
Kauffman ABC and K-Bit
McArthur
MSCA
Wechsler Intelligence Scale for Children (WISC)
Wechsler Preschool and Primary Scale of Intelligence (WPPSI)
NEPSY
Rey Auditory Verbal Learning Test
Stroop task
Trail-Making Test (TMT) or Trails A & B
Symbol Digit Modalities Test (SDMT)
Hooper Visual Organization Test (HVOT)
NUTRIMENTHE Neuropsychological Battery (Anjos et al., 2013)
BENCI (Cruz-Quintana, Perez-García, Roldan-Vílchez, Fernandez Lopez, & Perez-Marfil, 2013)
Children Behaviour Check List (CBCL) (Achenbach & Rescorla, 2001)
Electrophysiological recording:
EEG
EEG/ERP
Some tests and techniques to evaluate visual acuity and retinal development & function:
Stycar Rolling Balls ("Detection acuity")
Teller acuity cards ("Resolution acuity" e preferential looking procedure)
BUST-D symbol test ("Recognition acuity")
SSAS - Sheridan Gardiner (S-G) single letters ("Recognition acuity")
LH single symbols and line tests ("Recognition acuity")
HVOT visual acuity test ("Recognition acuity")
Visual Evoked Potentials (VEP)
▪ Sweep VEP
▪ Transient flash VEP
▪ Pattern-reversal stimuli VEP
▪ Steady-state VEP
Scotopic ERG
Some hearing tests and electrophysiological recording for auditory acuity:
Audiometry (2-5 yrs: behaviour modification; >5 cooperation)
Reaction to noise in infant
Rinne and Weber tests (Conductive e Auditory acuity)
Tympanometry (middle ear air pressure and abnormalities)
Brain-stem evoked audiometry (Hearing acuity)
Neuroimaging brain structure and function:
aMRI
fMRI
MEG
MRS
US

ASQ: Ages and Stages Questionnaire; MSCA: McCarthy Scales of Children's Abilities; NEPSY: A Developmental NEuroPSYchological Assessment; BENCI: Battery for Neuropsychological Evaluation of Children; EEG: Electroencephalogram; ERP: Event-related potentials; BUST-D: A recognition acuity test where the stimulus has to be recognized; SSAS: Sonksen-Silver acuity system; LH: A recognition acuity test where the stimulus has to be recognized; HVOT: Single letters that are presented to the child using the Electronic Visual Acuity System; ERG: Electroretinogram;; aMRI: anatomical magnetic resonance imaging; fMRI: functional magnetic resonance imaging; MEG: magneto encephalography; MRS: Magnetic resonance spectroscopy; US: Cranial ultrasound.

assessment methods. When designing a study for assessing the influence of a stressor on cognitive function it is germane to include a battery of neuropsychological tests that covers a variety of cognitive domains. Within the European Project NUTRIMENTHE, we have designed and completed neuropsychological battery for cognitive assessment by domains in European children (Anjos et al., 2013). The neuropsychological battery comprises the evaluation of learning and memory, attention, processing speed, executive, motor and perceptual functions.

The brain structure and function in children can be assessed by using different neuroimaging techniques. Anatomical magnetic resonance imaging (aMRI) scan permits to measure anatomical regions of the brain, including volumes, cortical thickness and white and grey matter microstructure in the brain as a whole or by regions. Electroencephalogram (EEG), evoked potentials (EP) and event-related potentials (ERPs) can measure objectively brain electrical functions in response to stimuli (Nelson & Luciana, 2001). The functional magnetic resonance imaging (fMRI) can be used for measuring brain activity showing which part of the brain is involved in a particular cognitive process. These methodologies have been successfully used in children (Anjos et al., 2013; Rosales, Reznick, & Zeisel, 2009). They have the advantage of being linked with the moment of the onset of the stimuli or insult and to determine which parts of the brain are activated related to specific cognitive tasks.

The magnetic resonance spectroscopy (MRS) has been used for determining the concentration of brain metabolites such as N-acetyl aspartate, choline, creatine and lactate, metabolites potentially involved in the microbiota-gut-brain axis. The cranial ultrasound (US) is another neuroimaging technic for neurological assessment.

Multivariable outcome prediction models aiming to identify the combination of factor, including clinical and environmental parameters, strongly associated to cognitive impairment in infancy and adulthood are being currently developed (Linsell, Malouf, Morris, Kurinczuk, & Marlow, 2015). The combination and integration of different psychophysiological and functional neuroimaging techniques allow to obtaining the maximum potential compared to the application of one single technique. Multidimensional diagnosis to reveal neuropathological mechanism for a precise and early preventive intervention is also encouraged (Koshiba, Kakei, et al., 2015).

9. Microbiota analysis and functional changes

Until recently, the study about resident microbial community in the human intestinal tract was done through culture-dependent methods (Lagier, Million, Hugon, Armougom, & Raoult, 2012). Among these methods the most commonly used were: fatty acid analysis, bacteriophage typing, polyacrylamide gel electrophoresis of soluble proteins, serotyping and phenotypic fingerprinting analysis (Rogers & Bruce, 2010).

One of the main problems that such methods provide, is that most of the microbiota present in the human intestine is anaerobic, which requires special conditions for cultivation and which could be difficult to obtain (Sekirov, Russell, Antunes, & Finlay, 2010).

Recent years, with the improved high performance technologies and culture-independent genomic methods, have made it possible to characterize the composition of the microbial ecology, with greater sensitivity, precision and less time consuming than the classical culture-dependent technologies. The success of these new methods is based on the identification from nucleic acids in live or dead cells, not depending on the use of growing bacteria. This allows the identification of individual bacterial species in the complexity of bacterial microbiota and gets information on bacterial evolutionary relationship (Weinstock, 2012).

The culture-independent methods are based on the quantitative polymerase chain reaction (q-PCR) analysis, PCR-based DNA profiling techniques, DNA microarray, flow cytometry, FISH (Fluorescence In Situ Hybridization), insertion sequencing, and particularly next-generation DNA sequencing (McCartney, 2002; Zoetendal, Collier, Koike, Mackie, & Gaskins, 2004) (see Table 2).

DNA sequencing was a method that caused a rapid change in the development of vaccines, medical treatments and diagnostic methods (Sanger, Nicklen, & Coulson, 1977). This allowed progress to what is today known as the NGS (Next-Generation Sequencing) technologies, which are divided into two groups: PCR-based technologies and single molecule sequencing (SMS) (Zhang et al., 2011). The 16S sequencing approach is the most widely used technique but it presents several limitations. In the process of DNA extraction depending on the cell lysis method used, it may lead to variation in different taxa, showing considerable variation between studies (Lee et al., 2012). Regarding the precision, it depends on the proportions of the 16S gene sequences that reflect the proportion of bacteria in the original sample, which will be influenced by the type of PCR primer, amplification bias and copy number variation. The use of this technique for the study of gut microbiota presents the disadvantage of sequencing a small amount of human DNA including DNA from Archaea, Fungi and viruses that can also be found in the stool samples (Morgan & Huttenhower, 2014). Despite their limitations, it is considered the gold standard method today for the identification of taxonomy of bacteria down to the level of species. The resulted sequences are compared with a database to identify microorganisms by sequencing the full-length 16S rRNA gene. In order to delineate the taxonomic rank of one specie a sequence deviation range of 0.5–1% is used in addition to a 97% cut-off point to define operational taxonomic units (Camarinha-Silva et al., 2014). Further, bioinformatics tools are limited because the same species are identified if they have >97% identity in the 16S ribosomal RNA gene. However, the genomes of the same species can have large differences in the DNA sequences outside the 16S ribosomal RNA genes. Importantly, often they have different sets of groups of genes that regulate the production of specialized metabolites (e.g. antibiotics, virulence factors, siderophores, and so on) (Drancourt et al., 2000).

These findings are changing the approach of "who" to "what", meaning that the interest is shifting from the microbial composition to the microbial functionality. Emerging methods for characterize the functional capacities of the microbiome have been denominated with 'omics' technologies. These techniques include metagenomics, metatranscriptomics, metaproteomics, and metabolomics (Gong & Yang, 2012) (summarised in Table 3):

9.1. Metagenomics

Shotgun metagenomics sequencing assesses the diversity and metabolic functions of a specific microbial community. The taxonomic identification of the sequences is performed based on the nucleotide composition or homology. The advantage of this technique is that it resolves the problems of metagenomics based on 16S rDNA, particularly in some less abundant bacterial populations, as *Bifidobacterium* for instance. Some of the disadvantages of this technique include the high proportion of sequences to which it cannot assign a taxonomic identification or function (due to the lack of identity in reference databases), the extraction method of DNA or the length of the sequence, the coverage and the error rate

Table 2
Techniques used to characterize the gut microbiota.

Technique	Description	Advantages	Disadvantages
Culture	Isolation of bacteria on selective media	Cheap, semi-quantitative	Labour intensive,<30% of gut microbiota have been cultured to date
QPCR	Amplification and quantification of 16S rRNA. Reaction mixture contains a compound that fluorescences when it binds to double-stranded DNA	Phylogenetic identification, quantitative, fast	PCR bias, unable to identify unknown species
DGGE/TGGE	Gel separation of 16S rRNA amplicons using denaturant/temperature	Fast, semi-quantitative, bands can be excised for further analysis	No phylogenetic identification, PCR bias
T-RFLP	Fluorescently labelled primers are amplified and then restriction enzymes are used to digest the 16S rRNA amplicon. Digested fragments separated by gel electrophoresis	Fast, semi-quantitative, cheap	No phylogenetic identification, PCR bias, low resolution
FISH	Fluorescently labelled oligonucleotide probes hybridize complementary target 16S rRNA sequences. When hybridization occurs, fluorescence can be enumerated using flow cytometry	Phylogenetic identification, semi-quantitative, no PCR bias	Dependent on probe sequences unable to identify unknown species
DNA microarrays	Fluorescence labelled oligonucleotide probes hybridize with complementary nucleotide sequences. Fluorescence detected with a laser	Phylogenetic identification, semi-quantitative, fast	Cross hybridization, PCR bias, species present in low levels can be difficult to detect
Cloned 16S rRNA gene sequencing	Cloning of full-length 16S rRNA amplicon, Sanger sequencing and capillary electrophoresis	Phylogenetic identification, quantitative	PCR bias, expensive, laborious
Direct sequencing of 16S rRNA amplicons	Massive parallel sequencing of partial 16S rRNA amplicons for example, 454 Pyrosequencing® (Roche Diagnostics GMBH Ltd, Mannheim, Germany) (amplicon immobilized on beads, amplified by emulsion PCR, addition of luciferase results in a chemoluminescence signal)	Phylogenetic identification, quantitative, fast, identification of known bacteria	PCR bias, expensive, laborious
Microbiome shotgun sequencing	Massive parallel sequencing of the whole genome (e.g. 454 pyrosequencing® or Illumina®, San Diego, CA, USA)	Phylogenetic identification, quantitative	Expensive, analysis of data is computationally intense

DGGE, denaturing gradient gel electrophoresis; FISH, fluorescence In situ hybridization; qPCR, quantitative PCR; TGGE, temperature gradient gel electrophoresis; T-RFLP, terminal restriction fragment length polymorphism.
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Table 3
'Omics' tools. Advantages and disadvantages to study the intestinal microbiota.

Meta-omics	Molecule	Knowledge	Limits	Clinical implications
Phylogeny	16S rDNA	Bacterial composition and diversity	- No information on bacterial functions - Except Archaea	- Composition dysbiosis - Healthy or disease specific species
Metagenomics	Chromosomal genomic DNA	- High resolution Microbiota profiling - Genes contents from uncultivated microbes	No information on microbial expressed functions	- Functional dysbiosis - Healthy or disease specific microbial genes - Toward diagnostics functional based studies
Metatranscriptomics	Messenger RNA/cDNA	- High resolution gene expression profiling - Differential microbial gene expression various physiological/environmental conditions	- Poor stability of bacterial mRNA - Representatively unknown/Multiple purification steps needed - No unique protocol	- Functional Dysbiosis - Microbial activity kinetics - Expressed genes at specific time and location - Specific monitoring of active bacteria
Metaproteomics	Proteins/Peptides	- High resolution protein mapping and profiling - Differential microbial proteins production under various physiological/environmental conditions	- Many unknown proteins in databases - Heterogeneous stability - No unique protocol	- Function confirmed: genome annotation improvement - Eucaryotes-procaryotes analogs identification - Biomarkers
Metabolomics	Metabolites	- Microbial and host Metabolic profiling - Easy to perform on very low amount of material faeces/serum/urine	- Many unknown metabolites in databases - Strict identification of compound tedious - No unique protocol - Combination of host and microbial molecules	- New pathways confirmed or identified - High throughput metabolomic screening of biomarkers - Easy translation to clinical setting

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(which influences the bioinformatic processing) (Thomas, Gilbert, & Meyer, 2012).

9.2. Metatranscriptomics

The introduction of RNA-seq allowed the massive sequencing of the cDNA generated from all RNA transcripts in the sample. This creates technical reports about the functional and metabolic potential of the microbiota, based on the active transcription of the cell. In this type of analysis, information about the direction of transcription is preserved, which is important for the analysis of non-coding and antisense RNA and for defining operons in bacteria. The main problem of metatranscriptomics analysis in the gut microbiota is the host DNA, because it can interfere the result, consuming a major number of readings and reducing the coverage (Wang, Gerstein, & Snyder, 2009).

9.3. Metaproteomics

This technique permits untargeted identification of proteins with high performance from complex environment. For this purpose, the step of cell lysis and the enzymatic digestion of all reachable proteins in a sample is crucial. The resulting peptide fragments are separated by liquid chromatography and subjected to tandem mass spectrometry (MS/MS). The peptides mass and the spectrums are quantified and compared with databases of reference proteins. The advantage of this method is that no gel separation is needed or de novo protein sequencing (Dill, Young, Carey, & VerBerkmoes, 2010).

9.4. Metabolomics

Spectroscopic techniques are used to analyse all metabolites of a sample (untargeted) or measure certain metabolites of interest (targeted) of metabolic profiles from different cellular/tissue origin (urine, serum or fecal water). Mass spectrometry (MS) discriminates metabolites according to their mass and charge (m/z), often followed by other separation techniques such as gas chromatography or liquid chromatography. Identification of the metabolites is carried out using existing databases ratio values m/z as METLIN (Smith et al., 2005). The nuclear magnetic resonance spectroscopy identifies chemical structures based on the chemical shift (the resonance frequencies of atomic nuclei in relation to a reference standard) followed by radiofrequency pulses. The main advantages are that it needs a minimal sample preparation and the identification of chemical compounds and establishing metabolic interaction clarifies in more detail. The main drawback is the low number of identified compounds, limiting databases, use of different bioinformatic tools according to the platform, the need to use different extraction systems chemicals compounds and loss of volatiles in samples stored.

All techniques described above, are enhanced with the development of the era of 'omics' and bioinformatics, in which many programs (CANOCO, lefse, R, PROPHANE, among others) allow to obtain maximum structural and functional information of the microbial community.

10. Emerging therapeutic applications

The emerging knowledge about the influence and the important implications that the microbiota-gut-brain axis could have on our mental and systemic health has open up a number of potential therapeutics tools to be applied in order to revert or prevent the adverse consequences associated with an imbalance in this axis. The maternal microbiota has been considered as a potential cause

for adverse perinatal outcomes. Understanding the crosstalk between maternal and foetal microbiota in relation to the microbiota-gut-brain axis and the associated systemic and mental health consequences will permit the onset of new diagnostics and therapeutics in maternal-foetal medicine. Hereby we summarise the therapies that have been recently proposed or have been used with relatively success in recent years.

10.1. Probiotics

The World Health Organization's 2001 definition of probiotics is "live micro-organisms which, when administered in adequate amounts, confer a health benefit on the host" (Hotel & Cordoba, 2001). The microbiota colonization of the maternal intestine influences offspring's metabolic and immune system development (Josef Neu, 2015). However, the contribution of the specific gut bacteria, along with the life-style, to the maintenance of microbial equilibrium is being investigated to improve our understanding of the etiological factors underlying specific disorders and to develop more efficient strategies for their management (Collado, Rautava, Isolauri, & Salminen, 2014).

For instance, some probiotics have shown to improve insulin sensitivity (*Lactobacillus acidophilus NCFM*) and glucose tolerance (*Bifidobacterium spp*) (Delzenne, Neyrinck, Backhed, & Cani, 2011; Kootte et al., 2012) and could be potentially used as a therapeutic tool to modulate gut microbiota and thus control eating behaviour and obesity risk. Quorum sensing has been suggested as one route that microbes can use to manipulate host eating behaviour in favour of their own growth (Alcock et al., 2014). Thus, treatments that increase bacterial diversity might prevent some populations of bacteria from achieving a quorum which is used by many gut bacteria to regulate density-dependent conditional strategies, including tissue invasion and colonization and changes in growth.

Further, the positive influence of probiotics has been seen on the reduction of the adipose tissue mass (*Lactobacillus gasseri SBT2055*), hyperinsulinemia (*Lactobacillus acidophilus NCFM*), cholesterol (*Lactobacillus bulgaricus*) and leptin levels (*Lactobacillus casei IMVB-7280*), but only in studies from animals and adults (Delzenne et al., 2011; Kootte et al., 2012; Savcheniuk, Virchenko, Falalieieva, Beregova, Babenko, Spivak et al, 2013; Vitetta, 2011). In children, the influence of probiotics has been studied by maternal and/or early infant supplementation. Maternal dietary intervention using specific probiotics can modulate the infant gut microbiota through breast milk intake or through placenta, (Balasubramanian & Patole, 2015).

The most commonly used probiotics are *Lactobacilli, Bifidobacterium,* and *Enteroccoci*, which are also part of the intestinal microflora (Fan, Chen,Yu, Si and Liu, 2006). Several studies have shown how the use of probiotics during pregnancy prevents various postnatal disorders (Pelucchi et al., 2012). A recent study in pregnant mothers and infants more than 13 months old, showed that the prenatal administration followed by postnatal administration of *Lactobacillus* alone and *Lactobacillus* with *Bifidobacterium* protects against atopic dermatitis (Panduru, Panduru, Salav astru, & Tiplica, 2015). However, to gain efficacy, probiotics must be administered with no discontinuity.

Concern about the safety of the use of probiotics in pregnant women, preterm neonates and infants exists – we must be cautious, as the long-term effects are still unknown (J. Neu, 2011). Thus, several studies about the safety for the use of probiotics during pregnancy and lactation have been performed (Elias, Bozzo, & Einarson, 2011; Madden, 2015). The studies included in the meta-analysis compared Lactobacillus spp alone or in combination with *Bifidobacterium spp* versus placebo group. There was no increase in the incidence of miscarriages or malformations, and no significant difference in birth weight, gestational age, or the incidence of Caesarean section (Elias et al., 2011). Even more, there is evidence that probiotics administration to mice with autism-like symptoms appear to improve their behaviours as it improves their dysbiosis (Hsiao et al., 2013). Up to date, there are no similar studies in humans; however, clinical trials in adults and infant populations have been proposed (Critchfield, van Hemert, Ash, Mulder, & Ashwood, 2011; Gilbert, Krajmalnik-Brown, Porazinska, Weiss, & Knight, 2013). Despite of the findings, few studies have been carried out to date in relation to the use of probiotics during pregnancy and the possible influence on diseases prevention of the offspring, and no studies in relation to neurodevelopmental disorders.

10.2. Prebiotics

The use of non-digestible oligosaccharides has been seen as a safer alternative to the use of probiotics during pregnancy to enhance the positive effects on the health of the mother and the neonate. Prebiotics could increase the growth or activity of beneficial bacteria, hence improving a maternal and neonatal healthy microenvironment. Additionally, consumption of prebiotics during pregnancy may also increase the production of bacterially derived metabolites, alleviating or diminishing disease risk and preventing certain psychiatric disorders (Thum et al., 2012). The use of single or combined prebiotics during pregnancy needs further research as the desirable beneficial effects on the neonate might not be transferred from the mother (Shadid et al., 2007; Taghizadeh & Asemi, 2014).

10.3. Synbiotics

The simultaneous use of probiotics and prebiotics in pregnant women and their infants has been recently used as a therapy for the prevention of allergies (Kukkonen et al., 2008). Current research is focussing in the effect of the use of synbiotic food on the maternal glycaemic status (Taghizadeh & Asemi, 2014) and on blood lipids profile (Taghizadeh et al., 2014). Studies investigating the complex mechanisms underlying the functional effects of using synbiotics are further warranted, as prenatal and perinatal periods are an excellent opportunity to impact health early in life through maternal interventions.

10.4. Faecal microbiota transplants (FMT)

Together with changes in diet and lifestyle, microbiota transplantation by faeces implants has been proposed as another mechanism to alter microbiota composition and hence impact food preferences and behaviour (Alcock et al., 2014). The term faecal microbiota transplantation is used to describe the transfer of stool from a healthy donor to a patient, by different methods, such as enema, colonoscopy or via the GI tract including pills or a more sophisticated technique such as endoscopy among others.

The use of pills containing bacteria is currently being tested in clinical trials (Youngster et al., 2014). Initially, faecal microbiota transplantation (FMT) was used in the treatment of Clostridium difficile infection (CDI) (Kassam, Lee, Yuan, & Hunt, 2013). Interest has been raised about the FMT application in metabolic syndrome, autoimmunity, autism, all of which have been related to altered microbiota or dysbiosis (Bowman, Broussard, & Surawicz, 2015; Brandt & Aroniadis, 2013; Smits et al., 2013). Currently, in addition to CDI, FMT is being assessed in the treatment of inflammatory bowel disease (IBD), irritable bowel syndrome (IBS), chronic constipation and neurological diseases such as multiple sclerosis and Parkinson's disease and hematologic disease such as idiopathic thrombocytopenic purpura (Bowman et al., 2015).

FMT from healthy donors to hosts with metabolic syndrome have shown efficacy in reducing insulin resistance (Vrieze et al., 2012), although the underlying mechanisms remains unclear. However, as others factors are impacting the microbiota profile, the effect of this treatment could be easily reversible. If there are FMT long-term sequelae or if the microbiota transplant remains after several months of treatment are questions which remain unresolved (Hamilton, Weingarden, Sadowsky, & Khoruts, 2012). The safety of this therapy is currently being questioned. The inconsistency in findings in clinical trials has been suggested to be explained by a methodological approach more than conceptual, thus, the source of stools and the laboratory procedures might affect the quality of the faecal microbiota and hence the safety and the response to the FMT treatment (Cui, Xu, & Zhang, 2015). Therefore, methodology for using FMT must be previously validated. Additionally, other alternatives to faecal transplants for microbiome-directed therapeutics are being proposed.

10.5. Others therapies

Alternatives therapies include, among others, the use of culture collections of bacteria and gastric bypass surgery. Culture collections may provide a safer and more precise alternative to faecal transplant for therapeutic clinical applications (Borody, Paramsothy, & Agrawal, 2013). Gastric bypass surgery induces changes in the microbiota and alters food preferences and satiety (Bueter et al., 2011; Kong et al., 2013; Mathes & Spector, 2012).

11Conclusions and future perspectives

Rapid advances in sequencing and analytical technologies have discovered the enormous diversity of microorganisms that inhabits ours guts. Microbiota has the potential to impact health status of a more complex living individual and in a broad extent. However the precise mechanisms through which bacteria milieu communicates with human systems and alters its function, including the brain, need to be fully elucidated in order to provide effective and safer therapies during vulnerable stages in life, such as pregnancy and postnatal periods of life.

Inflammatory pathways have been revealed as a central key by which microorganism might impact health and increase disease risk. New research is being focussed on the connections between microbiota, physiology and brain as an emerging area with promising therapies to impact health with potentially beneficial immediate and long term effects.

Until recently, it was believed that the foetal environment was sterile. Placenta harbours its own microbiome, and bacterial communities in the foetus are similar to the digestive tract and to the oral cavity of the mother. There is also a shift in the microbiota community from the first trimester to the third trimester of pregnancy (Koren et al., 2012). It has been suggested that maternal dysbiosis at a critical moment might also have a programming effect on metabolic and mental diseases in the long-term impacting on health, behaviour and cognitive functions of the offspring. Changes in placental microbiome profile have been related to adverse outcomes, which potentially might help to shape the neonatal microbiome and impact neurodevelopment and reprogramming metabolism early in life with unknown consequences. Now we know that placental microbiome varies in association with excess maternal gestational weight gain (Antony et al., 2015), however, the effects on the early exposure of a particular microbiota profile on the foetal neurodevelopment and in infancy and beyond has not yet been fully explored. So, further studies involving populations vulnerable of neurodevelopmental impairment early in life, such as obese pregnant women, gestational diabetic women, women with gestational weight gain excess, and other medical conditions, are necessary to clarify the mechanisms triggering adverse outcomes in their offspring and for the development of futures therapies and preventive strategies.

In order to be able to apply the correct therapy, a healthy microbiota is yet to be fully defined. New hypotheses are emerging about the potential of the intestinal microbial community to affect food preferences of the host, based on the metabolic compounds that the gut microbes are specialized. Intestinal microbiota not only regulates the processing of nutrients ingested, but it has the ability to promote diseases that could be prevented, or influence the development and well-being of the brain. Thus, for a more reliable and successful approach for obesity and related diseases treatment and prevention, it is necessary to take into account not only the composition of the microbial community, but also its function and its network connections with our brain and intestine.

Some therapies to impact gut microbiota during pregnancy or in infants are being proposed as for example the use of probiotics. However, caution must be paid to the benefits attributed to probiotics as up to date only a few strains have been investigated in relation to the potential health benefits; so, other species solely and in combination with others should be investigated. Safer therapies with validated and scientific proven benefits for both the mother and offspring to maintain long-term brain and metabolic health, are urgently needed.

In conclusion, more research in the field is warranted in order to unravel the functioning of the intricate microbiota-gut-brain axis network. Next, there is also an urgent need to search for effective and appropriated strategies and therapies to be applied specifically in pregnant women and their infants for preventing and treating obesity and related metabolic and neurodevelopmental diseases.

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PAPER 4

Probiotic, Prebiotic, and Brain Development

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Abstract

Recently, a number of studies have demonstrated the existence of a link between the emotional and cognitive centres of the brain and peripheral functions through the bi-directional interaction between the central nervous system and the enteric nervous system. Therefore, the use of bacteria as therapeutics has attracted much interest. Recent research has found that there are a variety of mechanisms by which bacteria can signal to the brain and influence several processes in relation to neurotransmission, neurogenesis, and behaviour. Data derived from both in vitro experiments and in vivo clinical trials have supported some of these new health implications. While recent molecular advancement has provided strong indications to support and justify the role of the gut microbiota on the gut–brain axis, it is still not clear whether manipulations through probiotics and prebiotics administration could be beneficial in the treatment of neurological problems. The understanding of the gut microbiota and its activities is essential for the generation of future personalized healthcare strategies. Here, we explore and summarize the potential beneficial effects of probiotics and prebiotics in the neurodevelopmental process and in the prevention and treatment of certain neurological human diseases, highlighting current and future perspectives in this topic.

1. Introduction

The micro-organisms that inhabit the human gastrointestinal tract (GI) have been implicated in the development and functioning of a number of basic physiological processes, such as digestion, immunity, and the maintenance of homeostasis. The GI microbiota may also play a role in multiple diseases, ranging from inflammation to obesity [1,2]. Recently, many studies have shown that gut microbiota play a very important role in the development and function of the central nervous system (CNS) through specific channels, such as metabolic, neuroendocrine, and immune pathways [3]. In particular, these researchers have found bi-directional communication between the brain and the gut microbiota, denominated the microbiota–gut–brain axis [4–6].

Although the molecular mechanisms by which the gut microbiota communicate with the brain are not yet clear, the link between both components is currently attributed to immune signals and the vagus nerve. Cellular components produced by gut microbiota, such as lipopolysaccharide (LPS), peptidoglycan, and flagellin, are recognized by pattern-recognition receptors (PRRs), such as Toll-like receptors (TLRs), NOD-like receptors (NLRs), or RIG-1-like receptors (RLRs), on epithelial and immune

cells, producing cytokines, hormones, and other molecular signals, which will act as neurotransmitters within the CNS [7]. Several studies have found that, in the densely innervated gut, the vagus nerve is involved in the bi-directional communication of the microbiota–gut–brain axis [8,9], while others have shown vagus-independent effects [10,11]. Either way, a supplementing nutrition therapy with specific probiotic commensals and prebiotics can alter the excitability of enteric nervous system (ENS) sensory neurons [12–14]. Prebiotics-induced growth of probiotic members within the *Bifidobacterium* and Lactobacillus genera show multiple beneficial effects on host immunity and physiology [15]. Moreover, strong effects of Bifidobacterium and Lactobacillus spp. on the brain–gut axis have been reported [16].

This review summarizes current knowledge on the influence of the establisment of the gut microbiota in critical neurodevelopmental windows, and discusses recent findings on the interactions between the gut microbiota and the host's brain–gut axis communications. In addition, current research on the effects of the administration of probiotics and prebiotics in specific neurological disorders is reviewed. Finally, recommendations for future research on this topic are also discussed.

2. Establishment of Intestinal Microbiota during Early Neurodevelopmental Windows

Gut microbiota establish a beneficial cohabitation with the host that will prime for health later in life [17]. The assembly of the gut microbiota occurs during the first three years of life, starting from birth, where there is a rapid rate of colonization and expansion of gut bacteria dominated by *Actinobacteria* and Proteobacteria that shifts towards one dominated by Firmicutes and Bacteroidetes, increasing compositional diversity and stability while maturing into an adult-like state [18]. This process coincides in time with the intense synaptogenesis and pruning in the cerebral cortex during early life [18–20], ending in adolescence [21]. Therefore, perturbations of gut microbiota colonization and maturation by environmental factors may influence brain development. The dynamics of the microbial ecosystem's maturation during this critical period of CNS development is influenced by several environmental factors, such as mother-to-child bacterial transfer, mode of delivery, and type of feeding. The mother-to-child transfer of commensal bacteria in the uterus has been shown to influence an infant's immune system development [22,23]. Until recently, the idea that foetuses were sterile in the uterus and that the microbial colonization of the new-born started during and after birth had been widely accepted [24]. However, nowadays this belief has been challenged by evidence of microbes in placenta and other tissues surrounding the foetus, such as umbilical cord blood after vaginal and caesarean birth [25–27]. Several studies have analysed the meconium of new-born babies and showed the presence of bacterial populations, including *Enterococcus*, *Lactococcus*, *Escherichia*, *Leuconostoc*, and *Streptococcus*, though at low levels, concluding that gut colonization occurs mainly after birth [28]. Based on these findings, prenatal probiotic intervention has been shown to modulate the expression of TLR-related genes in the placenta and foetal GI tract and to reduce atopic dermatitis [29,30]. Thus, prenatal and postnatal maternal oral probiotic therapy may represent an effective method of intervention to prevent pathologies such as allergy [31], atopic diseases [32], and neurodevelopmental disorders, reviewed below. Still, the origin of the microbiota colonizing the placenta is unknown and results have to be carefully interpreted, because, in samples with low microbial biomass, such as those from placenta, the risk of contamination is high when using high-throughput sequencing methods based on DNA amplification [29]. Further studies are needed to discern whether foetuses have contact with bacteria before birth or are colonized during and after parturition.

Regardless of mother-to-child transmission within the intrauterine environment, two different modes of maternal–infant transmission during delivery have been proposed: (a) horizontal, in which microbes are taken up from the environment for infants born by caesarean section; and (b) vertical, in which vaginal microbes are transferred during parturition to the infants [33]. Infants delivered by Caesarean section are more likely to suffer several diseases, such as asthma, obesity, or allergies,

in adulthood [34]. Interestingly, a study carried out by Jasarevic et al. using a mouse model of early prenatal stress found that changes in the vaginal microbiome were associated with shifts in the abundance of Lactobacillus in the expression of maternal stress proteins related to vaginal immunity, in offspring metabolic profiles related to energy balance, and in the amino acid profiles of the developing brain [35].

The third strong environmental factor that influences an infant's gut microbial development as well as neurodevelopment is the type of feeding. In recent years, several studies have reported that breastfeeding and particularly full breastfeeding has beneficial effects on child neuropsychological development [36]. Human milk is the optimal feeding source, since it provides all the nutrition factors that an infant needs for healthy development. Human milk is not sterile, and, during breastfeeding, bacteria from mother's skin and mammary gland via maternal dendritic cells and macrophages [37] are transferred to the baby [38]. Breast-fed infants tend to contain a more uniform population of gut microbes dominated by *Bifidobacterium* and *Lactobacillus* [39], whereas formula-fed infants exhibit higher proportions of *Bacteroides*, *Clostridium*, *Streptococcus*, *Enterobacteria*, and *Veillonella* spp. [21]. Bacteria belonging to the *Bifidobacterium* genus present in human milk are early colonizers that characterize the gut microbial composition of healthy breast-fed new-born's [40] with beneficial functions for the host, such as the acceleration of the maturation of the immune response, the limitation of excessive inflammation, the improvement of the intestinal permeability, and an increase of acetate production [41]. In mice, *B. infantis* produces antidepressant-like effects and normalizes peripheral pro-inflammatory cytokine and tryptophan concentrations, both of which have been implicated in depression [42–44]. Moreover, breastfeeding has an additional role in the establishment of an infant's gut microbiota, since it contains bioactive molecules that are increasingly recognized as drivers of microbiota development and overall gut health [45]. Among the nutrients present in human milk, oligosaccharides constitute the third-most abundant class of molecules in terms of concentration after lactose and lipids. Nowadays, more than 200 different structures have been identified as human milk oligosaccharides (HMOs) [35]. HMOs can act as prebiotics, stimulating the growth of specific bacterial groups such as *Staphylococci* [46] and *Bifidobacteria* [47].

These results suggest that postnatal neurodevelopment and gut microbiota establishment co-occur, suggesting the intriguing possibility of a bi-directional regulation of each other's maturation [48]. Further studies are needed in order to clarify whether those differences in bacterial acquisition during early life lead to neurodevelopmental differences in infants.

3. Gut Microbiota–Brain Axis

The brain and the gut reciprocally influence each other by constant communication (Figure 1). The brain–gut–microbiota axis includes the CNS, the endocrine-immune system, the hypothalamus–pituitary–adrenal (HPA) axis, the autonomic nervous system, the ENS, and the gut microbiota [49]. This bi-directional communication enables signalling from the brain to influence motor, sensory, and secretory modalities of the GI tract, and conversely, signalling from the gut to affect brain function, most notably the hypothalamus and amygdala that are implicated in stress [50–52].

Though communication between brain and gut was realized in the middle of the nineteenth century [53], gut microorganisms had not been considered important for the development and function of the CNS or for brain diseases until recently, expanding the term to microbiome–gut–brain axis [54]. In humans, evidence of microbiome–gut–brain axis interactions have been obtained from the association of shifts in gut microbiota composition with central nervous disorders (i.e., autism spectrum disorder (ASD) and anxiety and depressive behaviours) and functional gastrointestinal disorders [54]. Most of the data demonstrating the role of the microbiota in the gut–brain axis have been obtained from germ-free animals [55]. Mice fed with prebiotics showed diminished stressor-induced anxiety-like

behavior [56]. In a mouse model of ASD, Buffington et al. showed that a maternal high-fat diet reduced the number of oxytocin immunoreactive neurons in the hypothalamus and induced dysbiosis that was restored by a commensal *Lactobacillus reuteri* strain [57]. In a mouse model of Parkinson's disease, Sampson et al. highlighted a negative interaction in the microbiome–gut–brain axis because the absence of gut bcateria decreased aggregated misfolded α-synuclein levels and reduced the severity of the animals' abnormal movements. The authors showed that short chain fatty acids (SCFA), such as acetate, propionate, and butyrate, the end products of anaerobic fermentation of dietary fibre and starch, promoted a microglia-mediated immune response and increased α-synuclein aggregation, causing movement abnormalities [58]. Butyrate can cross the blood-brain barrier (BBB) and produce a dose-dependent increase in neuronal and glial nuclear histone H3 acetylation in mice due to its potential to inhibit histone deacetylation [59]. Another metabolite whose levels in the host are influenced by gut microbiota is tryptophan, the amino acid precursor of the neurotransmitter serotonin, and kynurenine, the main breakdown product of tryptophan catabolism [60]. Kynurenine intake during gestation and postnatal development, a time frame in which the maternal and offspring microbiota undergo major compositional and functional remodelling, produced neurochemical and cognitive deficits later in adulthood [61]. The prenatal inhibition of kynurenine synthesis modified hippocampal neuron morphology and changed neocortical and cerebellar protein expression that persisted into adulthood. In germ-free and in antibiotic-induced microbiota-depleted mice, despite increased circulating tryptophan levels, serotonin and kynurenin availabilities were decreased, suggesting that gut microbiota modulated kynurenin metabolism [62]. Distinct gut microbial species affect host physiology, producing diverse neuromolecules involved in mood regulation. *Lactobacillus* and *Bifidobacterium* spp. generate gamma-aminobutyric acid (GABA). *Candida*, *Streptococcus*, *Escherichia*, and *Enterococcus* spp. synthesise serotonin while *Bacillus* spp. produces dopamine [63].

Gut microbiota also influence the regulation of BBB integrity. The BBB is an active interface between systemic circulation and the CNS that maintains brain homeostasis by preventing the entry of potentially toxic or harmful substances and regulates the transport of nutrients and the removal of metabolites [64]. Braniste et al. [65] showed that the transplantation of gut microbiota into germ-free mice normalized BBB permeability and upregulated the expression of tight junction proteins. Therefore, gut microbiota have a key role in regulating BBB permeability, suggesting that the maternal gut microbiome influences an offspring's BBB integrity. Together with the results discussed in the previous section, these findings open an intriguing question on the mechanism by which a mother's gut microbiota cooperate in regulating BBB integrity and ultimately brain function development.

Gut microbiota have direct effects on the immune system, which constitutes another route of communication between gut microbes and the brain. The signalling molecules of the immune system, cytokines and chemokines, access the brain from the periphery via the vagus nerve or directly via the circumventricular organs [66]. The administration of rifaximin (a non-systemic, broad-spectrum antibiotic) to stressed rats increased the abundance of *Lactobacillus* in the ileum and the expression of the tight junction protein occludin while decreasing the expression of pro-inflammatory interleukin 17, interleukin 6, and tumour necrosis factor α mRNA [67].

Since many of the above effects have been observed during early life, it is plausible that an environmentally induced dysbiosis of infants' microbiota (e.g., mode of birth, maternal transmission of a suboptimal microbiota, antibiotics) may generate altered patterns of microbial metabolites with detrimental effects in human CNS development. Further research is needed to unravel these mechanisms and develop probiotics or prebiotics therapies that shape gut microbial composition and metabolism to ultimately modulate CNS development.

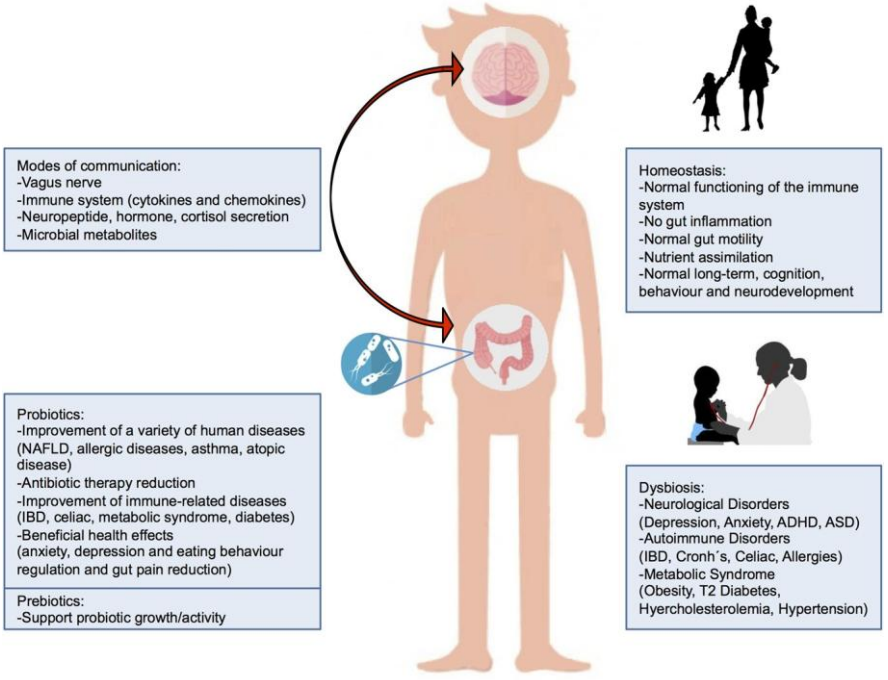

Figure 1. The gut microbiota–brain axis. The central part of the figure shows the bidirectional influence between the brain and gut microbiota. The left side of this figure shows modes of communication in the bidirectional crosstalk between gut microbiota and the brain and the possible influences of prebiotics and probiotics on human diseases. The right side of the figure shows the consequences of gut dysbiosis/homeostasis. Intestinal dysbiosis can adversely influence gut physiology, leading to inappropriate brain–gut axis signalling and associated consequences for CNS functions and disease states. Abbreviations: Non-Alcoholic Fatty Liver Disease (NAFLD), Inflammatory Bowel Disease (IBD), Attention deficit hyperactivity disorder (ASD).

4. Probiotics

In 2001, the Food and Agriculture Organization of the United Nations and the World Health Organization (FAO/WHO) proposed the following definition of probiotics: "live micro-organisms which, when administered in adequate amounts, confer a health benefit on the host" [68], which was reaffirmed in 2014 [69]. Probiotics, comprised by strains of *Lactobacilli*, *Bifidobacteria* and *Saccharomycetes* have been suggested to play a role in fighting human diseases, such as non-alcoholic fatty liver disease (NAFLD), allergy diseases, and asthma. They also promote protection against atopic disease in the infant during pregnancy and breastfeeding [32,70,71]. In addition, probiotics also reduce the duration of antibiotic therapy, and reduce symptom severity in immune-related diseases, such as inflammatory bowel diseases (IBDs), celiac disease, metabolic syndrome and diabetes [72,73].

The search for probiotics that can affect cognitive functions, known as psychobiotics, has increased in recent years (Table 1). Psychobiotics are defined as live organisms that, when ingested in adequate amounts, produce beneficial health effects to patients suffering from psychiatric illness [74]. Depression is currently a major psychiatric disorder in developed countries, and is characterized by a low mood or loss of interest and anxiety affecting appetite and sleep. Messaoudi et al. [75,76] reported a double-blind, placebo-controlled, randomized study where a multispecies probiotic containing *Lactobacillus helveticus* R0052 and *Bifidobacterium longum* R0175 (PF) was administered to healthy women for 30 days. This treatment resulted in a decrease in the global scores of the hospital anxiety and depression scale (HADs) and the global severity index of the Hopkins symptoms checklist (HSCL-90) due to the decrease of the sub-scores of somatization, depression, and anger–hostility spheres. In a cohort of 124 healthy humans, Benton et al. reported that the consumption of *Lactobacillus casei*-containing yogurt improved the self-reported mood of those whose mood was initially poor [77].

Similarly, Steenbergen et al. reported a significantly reduced overall cognitive reactivity to depression, in particular aggressive and ruminative thoughts, in forty healthy young adults that consumed either a probiotic supplement or placebo for 4 weeks [78]. Recently, Akkasheh et al. showed that the consumption of a probiotic supplement significantly decreased Beck Depression Inventory (BDI) scores, indicating overall improved symptoms, including mood, in 40 patients diagnosed with depression [79]. Conversely, Marcos et al. reported that probiotics decreased, respectively, levels of stress and anxiety assessed using the state-trait anxiety inventory (STAI) that remained unchanged in subjects under academic examination stress [80]. In a recent study carried out by Romijn et al. [81], administering a multispecies probiotic containing *L. helveticus* and *B. longum* in 79 participants that were not taking psychotropic medications at that moment and with at least moderate scores on self-report mood measures, found no evidence that the probiotic formulation was effective in treating low mood or in moderating the levels of inflammatory and other biomarkers. Improved cognitive function (neuropsychological and cognitive fatigue) was reported by Chung et al., which tested a *L. helveticus*-fermented milk in healthy 60–75 year olds, though no effects on stress or geriatric depression symptoms were observed [82].

Probiotics affect mood by their ability to modulate pain in the gut. A recent study reported that the administration of *Lactobacillus reuteri* DSM 17938 in the treatment of children with functional abdominal pain (FAP) and irritable bowel syndrome (IBS) is associated with a possible reduction of the intensity of pain [83]. In 35 patients suffering from chronic fatigue syndrome, Rao et al. showed that while the consumption of the probiotic improved anxiety scores, it had no effect on depressive symptoms [84]. Giannetti et al. also reported that a probiotic mixture of *B. infantis* M-63, *B. breve* M-16V, and *B longum* BB536 was associated with improvement in children with IBS, but not in children with functional dyspepsia (FD) [85]. In healthy women without gastrointestinal or psychiatric symptoms, the consumption of a fermented milk product containing *B. animalis* subsp. *lactis*, *Streptococcus thermophilus*, *Lactobacillus bulgaricus*, and *L. lactis* subsp. *lactis* resulted in robust alterations in activity in the brain regions that control the central processing of emotions and sensations, as observed by functional magnetic resonance imaging [86].

Table 1. Studies evaluating probiotics supplementation on central nervous system (CNS) disorders.

Study (Reference)	Cohort Population	Probiotic Used	Key Findings
Messaoudi et al. (2011) [75,76]	55 healthy human volunteers plus 25 subjects with urinary free cortisol (UFC) levels less than 50 ng/mL (less stressed subjects), 10 subjects received the probiotic and 15 placebo.	*Lactobacillus helveticus* R0052 and *Bifidobacterium longum* R0175 (PF)	Beneficial effects on anxiety and depression related behaviors in healthy human volunteers and volunteers with lower levels of cortisol
Benton et al. (2007) [77]	124 healthy adults volunteers were randomly allocated to a group that consumed, on a daily basis, a probiotic-containing milk drink or a placebo	*Lactobacillus casei* Shirota	The consumption of a probiotic-containing yoghurt improved the mood of those whose mood was initially poor. However, there was not an increased frequency of defaecation.
Steenbergen et al. (2015) [78]	40 healthy young adults were randomly assigned to receive a 4-week intervention of either placebo or multispecies probiotics in a triple-blind intervention assessment design.	*Bifidobacterium bifidum* W23, *Bifidobacterium lactis* W52, *Lactobacillus acidophilus* W37, *Lactobacillus brevis* W63, *Lactobacillus casei* W56, *Lactobacillus salivarius* W24, and *Lactococcus lactis* (W19 and W58)	Participants who received multispecies probiotics showed a significantly reduced overall cognitive reactivity to sad mood, which was largely accounted for by reduced rumination and aggressive thoughts.
Akkasheh et al. (2016) [79]	40 patients with a diagnosis of major depressive disorder (MDD) whose age ranged between 20 and 55 years were randomized.	*Lactobacillus acidophilus*, *Lactobacillus casei*, and *Bifidobacterium bifidum*.	Patients who received probiotic supplements had significantly decreased Beck Depression Inventory total scores
Marcos et al. (2004) [80]	136 university students were randomized.	*Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus salivarius* subsp. *thermophilus* plus *Lactobacillus casei* DN-114001	There was no significant treatment effect on anxiety.
Romijn et al. (2017) [81]	79 participants not currently taking psychotropic medications with at least moderate scores on self-report mood measures. Participants were preparation or placebo.	*Lactobacillus helveticus* and *Bifidobacterium longum*	No significant difference was found between the probiotic and
Jadrešin et al. (2017) [83]	55 children with age between 4 and 18 years old, diagnosed as functional abdominal pain (FAP) or irritable bowel syndrome (IBS) were randomly allocated.	*Lactobacillus reuteri* DSM 17938	Administration of L. reuteri DSM 17938 was associated with a possible reduction of the intensity of pain and significantly more days without pain in children with FAP and IBS
Giannetti et al. (2016) [85]	48 children with IBS aged between 8 and 17.9 years and 25 with functional dyspepsia (FD) with age between 8 and 16.6 years were randomized.	*Bifidobacterium infantis* M-63, *breve* M-16V, and *longum* BB536	In children with IBS a mixture of Bifidobacteria is associated with improvement in abdominal pain (AP) and quality of life (QoL).
Kałużna-Czaplińska et al. (2012) [87]	22 autistic children.	*Lactobacillus acidophilus*	The probiotic supplementation let to a significant decrease in D-arabinitol (DA) and the ratio of D-/L-arabinitol (DA/LA) and to a significant improvement in ability of concentration and carrying out orders
West et al. (2013) [88]	33 ASD children.	Delpro® (*Lactocillus acidophilus*, *Lactobacillus casei*, *Lactobacillus delbruecki*, *Bifidobacteria longum*, *Bifidobacteria bifidum*)	88% reported a decrease in total autism treatment evaluation checklist (ATEC) score, an improvement of ASD symptoms. Participants also had significant improvements in all ATEC domains (speech/language/communication, sociability, sensory/cognitive awareness, and health/physical/behavior)

Table 1. Cont.

Study (Reference)	Cohort Population	Probiotic Used	Key Findings
Tomova et al. (2015) [89]	10 children with autism, 9 siblings and 10 healthy children.	"Children Dophilus" containing three strains of *Lactobacillus* (60%), two strains of *Bifidobacterium* (25%), and one strain of *Streptococcus* (15%)	Probiotic diet supplementation normalized the *Bacteroidetes*/*Firmicutes* ratio, *Desulfovibrio* spp. and the amount of *Bifidobacterium* spp. in feces of autistic children. No significant difference was found to reduce symptom severity in patients with autism.
Santocchi et al. (2016) [90]	100 preschoolers with ASD on the basis of a symptom severity index specific to gastrointestinal (GI) disorders. Patients with and without GI disorders were blind randomized to regular diet with probiotics or with placebo	"Vivomixx®" (one strain of Streptococcus thermophilus DSM 24731, three strains of *Bifidobacterium Bifidobacterium breve* DSM 24732, *B. longum* DSM 24736, *B. infantis* DSM 24737), and four strains of Lactobacillus (*Lactobacillus acidophilus* DSM 24735, *Lactobacillus plantarum* DSM 24730, *Lactobacillus paracasei* DSM24733, *Lactobacillus delbrueckii* subsp. *bulgaricus* DSM 24734))	Ongoing study
Dickerson et al. (2014) [91] and Tomasik et al. (2015) [92]	32 patients healthy and 33 patients with schizophrenia meeting DSM-IV criteria and with at least moderately severe psychotic symptoms	*Lactobacillus rhamnosus* strain GG and *Bifidobacterium animalis* subsp. *lactis* strain Bb12	No significant difference was found to reduce symptom severity in patients with schizophrenia. Probiotic regulate immune and intestinal epithelial cells through the IL-17 family of cytokines

Probiotics have been tested to normalize gut microbial composition and metabolism, enhance gut barrier, and relieve patients suffering from ASD. In 2012, Kaluzna-Czaplinska and Blaszczyk reported that the administration of *Lactobacillus acidophilus* in 22 ASD subjects decreased D-arabinitol concentration and the ratio of D-arabinitol to L-arabinitol in urine, and improved their ability to follow directions, as demonstrated through a comparison with data collected before the treatment [87]. Another study reported that a combination of *Lactobacillus acidophilus*, *L. casei*, *Lactobacillus delbrueckii*, *B. longum* and *Bifidobacterium bifidum*, formulated with the imunomodulator Del-Immune V (Lactobacillus rhamnosus V lysate), decreased the severity of ASD symptoms and improved GI symptoms in 33 children [88]. Moreover, a recent study of "Children Dophilus" (a combination of three species of *Lactobacillus*, two species of *Bifidobacterium* and one strain of *Streptococcus*) in 10 ASD children showed higher GI dysfunction in ASD children and siblings and a very strong association of the amount of *Desulfovibrio* spp. with the severity of autism. After the intervention, the *Bacteroidetes/Firmicutes* ratio, *Desulfovibrio* spp., and the amount of *Bifidobacterium* spp. were normalized in faeces of autistic children [89]. However, the effects of treatments with probiotics on children with ASD need to be evaluated through rigorous, controlled trials. In a recent clinical study currently in progress, Santocchi et al. are providing a multispecies probiotic (one strain of *S. thermophilus* DSM 24731, three strains of *Bifidobacterium* (*B. breve* DSM 24732, *B. longum* DSM 24736, and *B. infantis* DSM 24737), and four strains of Lactobacillus (L. acidophilus DSM 24735, *Lactobacillus plantarum* DSM 24730, *Lactobacillus paracasei* DSM 24733, and *L. delbrueckii* subsp. *bulgaricus* DSM 24734) to a group of 100 pre-schoolers with ASD. This study will try to provide new insights to clinical and neurophysiological patterns in response to a probiotic mixture in ASD patients [90].

Probiotics are also tested in the treatment of schizophrenia and bipolar disorder. One of the first trials of probiotic compounds in schizophrenia used a combined probiotic of *L. rhammosus* strain GG and *B. animals* subsp. *Lactis strain* Bb12. The results showed no significant difference in psychiatric symptom severity between probiotic and placebo supplementation [91]. However, other studies have found that probiotic supplementation significantly alters the levels of several serum proteins, including the von Willebrand factor and the brain-derived neurotrophic factor, and lowered the level of antibodies to the fungus *Candida albicans* [92,93].

Despite that the majority of the studies found positive results on symptoms in these neurological disorders, future studies are needed to identify potential probiotics for the effective modulation of these disorders as well as to define probiotics risk in therapeutic interventions. Gut microbial studies that use 16S rRNA gene sequencing to characterize bacteria must consider that highly similar bacteria (higher than 97% sequence identity) can have large differences in genomic sequences and profound differences in growth and metabolism. Hence, it is important to characterize probiotics to the strain level and apply next-generation sequencing techniques to analyse the functions encoded by their genome [94]. Therefore, the effects of one probiotic strain should not be generalized to others without confirmation in separate studies.

5. Prebiotics

Although the concept of a prebiotic was first defined in 1995 by Gibson, the current definition of a prebiotic is the one proposed by the International Scientific Association for Probiotics and Prebiotics (ISAPP): a substrate that is selectively utilized by host micro-organisms and confers a health benefit [95]. The group of substances recognized for their ability to influence gastrointestinal health comprise certain non-digestible oligosaccharides (NDOs), soluble fermentable fibres, and HMOs. NDOs are low molecular weight carbohydrates in nature that are intermediates between simple sugars and polysaccharides. The use of NDOs as prebiotics has rapidly increased because the enrichment of a diet with NDOs provides the opportunity to improve the gut microbial ecosystem, including bacterial populations, biochemical profiles, and physiological effects [96]. Fibre influences satiety by the following two mechanisms. One is by increasing the chewing time of fibre-rich foods, which promotes saliva and gastric acid production and increases gastric distension, triggering afferent vagal signals of

fullness contributing to this end. The other mechanism is by slowing gastric emptying and decreasing the rate of glucose absorption in the small intestine. Consequently, the insulin response may also be attenuated; this is sometimes correlated with satiation and satiety [97]. Various hormones (i.e., ghrelin, the polypeptide YY, and the glucagon-like peptide) have been related to satiety, and are sent to the brain, where they regulate food intake and overall energy balance [98].

Though prebiotic therapies potentially could be beneficial for children with a genetic pre-disposition to develop ASD or attention deficit hyperactivity disorder because of their selective enhancement of *Lactobacilli* and *Bifidobacteria* growth [99], a small number of studies has examined the effect of these prebiotics on disorders related to CNS (Table 2). Inductive evidence that prebiotics modulated emotional satisfaction was provided by Hume et al., who investigated the effect of oligofructose-enriched inulin/d administration versus a placebo (maltodextrin) in a randomized, double-blind, placebo-controlled trial with 42 children (who were aged 7–12 and were overweight and obese) [100]. Prebiotic supplementation improved subjective appetite ratings, reducing energy intake in older but not in younger children.

In a cohort of healthy male and female subjects (n = 45), Schmidt et al. tested the intake of fructo-oligosaccharides (FOS) and Bimuno®-galactooligosaccharides (B-GOS), and reported that only B-GOS reduced the waking-cortisol response [101]. Exaggerated waking cortisol is a biomarker of emotional disturbances, such as depression [102]. Besides this, the subjects also provided measures of vigilance, or attention to negative stimuli, which is also a behavioral marker of anxiety and depression [103]. B-GOS attenuated vigilance, suggesting a reduction in anxiety and depression [104]. Van den berg et al. found no evidence that the use of short-chain galacto-oligosaccharides/long-chain fructo-oligosaccharides/pectin-derived acidic oligosaccharides in preterm infants at 24 months improves neurodevelopmental outcomes [105]. LeCouffe et al. studied the effect of an enteral supplementation of a prebiotic mixture (neutral and acidic oligosaccharides) in the neonatal period and found no effect on neurodevelopment [106], though lower *Bifidobacteria* counts are associated with serious neonatal infections and lower neurodevelopmental outcomes.

More studies are required to determine whether prebiotics exert a beneficial effect on neurodevelopmental disorders in infants, and to understand the mechanism of action, by stimulating certain bacterial taxa or bacterial activities within gut microbiota. Efficacy, safety, and dosing schedules should be established for each prebiotic product in long-term follow-up studies.

Table 2. Studies evaluating prebiotics and synbiotics supplementation on CNS disorders.

Study (Reference)	Cohort Population	Prebiotic Used	Key Findings
Prebiotics			
Hume et al. (2017) [100]	42 boys and girls, ages 7–12 years, with a body mass index (BMI) of ≥85th percentile	Oligofructose-enriched inulin/d	Prebiotic supplementation in children with overweight and obesity significantly increased feelings of fullness and reduced prospective food consumption in older but not in younger children
Schmidt et al. (2105) [101]	45 adults healthy volunteers	FOS and Bimuno®-galactooligosaccharides, B-GOS	B-GOS reduced waking-cortisol response and decreased attentional vigilance to negative versus positive information
van den Berg et al. (2016) [105]	77 preterm infants (gestational age <32 weeks and/or birth weight <1500 g), admitted to the level-III neonatal intensive care unit (NICU)	scGOS/lcFOS/pAOS	Neurodevelopmental outcomes were not different in the scGOS/lcFOS/pAOS and placebo group. Infections, lower bifidobacteria counts, and higher serum cytokine levels during the neonatal period were associated with lower neurodevelopmental outcomes at 24 months of age
LeCouffe et al. (2014) [106]	93 Infants, with a gestational age (GA) of less than 32 weeks and/or birth weight of less than 1500 g, participed in the study (prebiotic mixture group (n = 48) and placebo group (n = 45)	80% scGOS/lcFOS and 20% pAO	Short-term enteral supplementation of a prebiotic mixture in the neonatal period had no effect on neurodevelopmental outcome in preterm infants in the first year of life
Synbiotics			
Malaguarnera et al. (2007) [107]	60 cirrhotic patients (30 with synbiotics and 30 with placebo)	Bifidobacterium longum plus fructo-oligosaccharides	Patients with minimal hepatic encephalopathy (MHE) treated with Bifidobacterium + FOS, showed an improvement and a recovery of neuropsychological activities related to short-term memory, attention and computing ability, language, orientation ability, and cognitive activities
Firmansyah et al. (2011) [108]	393 healthy 12 month-old toddlers	The probiotic Bifidobacterium longum BL999 (ATCC: BAA 999) and Lactobacillus rhamonosus, LPR (CGMCC 1.3724), the prebiotics inulin (30%) and fructo-oligosaccharide (70%), and the LCPUFA, arachidonic acid (AA) and docosahexaenoic acid (DHA)	Changes in cognitive and adaptive behaviour scores between 12 and 16 months were higher but not significantly different in the synbiotics group compared with the control group

6. Synbiotics

The term synbiotic was primarily stated considering the benefits of a product that combines prebiotics and probiotics and in which the prebiotic compounds selectively favour the probiotic strains [109]. Several studies have shown positive synergistic effects for synbiotics on obesity, diabetes, non-alcoholic fatty liver disease, necrotizing enterocolitis in very low birth weight infants, and in the treatment of hepatic encephalopathy [110–114]. Despite these findings, few studies have tested the potential benefits of synbiotics on neurodevelopmental disorders (Table 2). Malaguarnera et al. reported that *B. longum* plus FOS improved cognitive function in the treatment of minimal hepatic encephalopathy (MHE) [107]. Firmansyah et al. provided milk containing synbiotics (BL999, LPR, and prebiotics) and LCPUFA to 393 healthy toddlers at 12 months-old for 12 months. The authors reported that the change in cognitive and adaptive behaviour scores between 12 and 16 months was higher but not significantly different in the synbiotics group compared with the control group [108]. Future work is needed to determine whether synbiotics may contribute to relieve neurological diseases and to explore the benefits of new potential synbiotics during critical time windows in an infant's CNS development and susceptibility to neurological disorders.

7. Future Perspectives

During the last decade, numerous in vivo and in vitro studies have explored the influence of probiotics and prebiotics in host physiology [115]. Their results showed that gut microbiota may modulate inflammation, adiposity, satiety, energy expenditure, and glucose metabolism. Most efforts have focused on studying the mechanisms by which certain probiotics regulate the colonization of and protect against pathogens through the activation of the mucosal immune system and competition for limited nutrients [116,117]. Alternate approaches such as recombinant probiotics expressing therapeutic biomolecules, faecal microbiota transplantation and phage therapy, need be explored for the manipulation of the gut ecosystem. A proof of concept was the experiment performed by Paton et al., where they created a recombinant probiotic by introducing glycosyltransferase genes from *Neisseria meningitidis* or *Campylobacter jejuni* in a harmless *Escherichia coli* strain (CWG308) to treat and prevent the diarrheal disease caused by enterotoxigenic *E. coli* strains [118]. The same group also developed a recombinant probiotic for the treatment and prevention of cholera [119]. A recent study showed that microbiota transfer therapy improves ASD symptoms in children, which persists for at least 8 weeks after the treatment ends [120]. And finally, phage therapy has become an interesting strategy to treat bacterial infections due to the rise of antibioticresistant microbial strains. The only approved phage therapy clinical trial in the human gut was carried out in 120 patients with diarrhoea caused by E. coli, who were infected by a coliphage mix. The treatment failed to solve diarrhoea, although no adverse effects of phage infection were observed [121]. Customized phage cocktails could be an alternative for future therapies. These phages would directly target pre-identified bacterial pathogens though the main drawback would be the high interindividual variation of the gut microbiome and legislative approval [122,123].

In conclusion, this review summarized the accumulating evidence on the modulation of gut microbial composition and metabolism as a potential strategy for neurological disorders and CNS development. Despite this wealth of information, the effect of probiotics and prebiotics is still largely unexplored, and numerous gaps and inconsistencies exist when the studies are compared. Differences in quantity of dose, type of strain, type of prebiotic, assessment of gut microbiota, duration of intervention, standardization of neurological measurements, variety and complexity of neurological symptoms, study design, and cohort size make it difficult to confirm evidence of efficacy. To this end, double-blind placebo in vivo studies that exploit the power of the latest robust high-throughput multi-omic technologies are required to identify the molecular mechanisms of the gut's microbial modulation of neurological disorders and CNS development and ultimately to design effective probiotic and prebiotic therapies.

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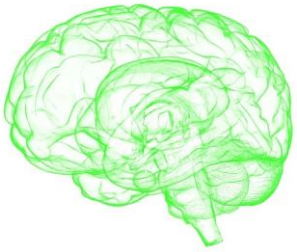

Intestinal bacteria are associated with the cognitive development of children at 6 months of age

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ABSTRACT

Compelling evidence suggest that gut microorganisms influence neurodevelopment in mice. To test this hypothesis in humans, we conducted a longitudinal study in full-term healthy infants where cognitive function assessed with Bayley III was associated with gut microbial composition, structure and metabolism. Children were categorized according to their Bayley scores within each domain into two groups, above and below the median (50th percentile). Composite cognitive scale (CCS) was the only test in which both study groups, divided according to the mean, showed significant differences in gut microbial composition.
Higher evenness ($p<0.004$), Shannon ($p<0.011$) and Simpson ($p<0.021$) diversity and reduced dominance ($p<0.021$) values in gut microbiota characterized the gut microbiota of infants with above median CCS. Principal coordinate analysis based on weighted UniFrac metrics of β-diversity showed that the gut microbiota of infants clustered by CCS ($p<0.014$), indicating significant phylogenetic dissimilarities in the microbial profile of highly abundant taxa. Taxa within *Lactococcus* and *Lachnospiraceae_Incertae_Sedis* were significantly enriched in infants with below the median CCS. Conversely, taxa within *Bacteroides* showed a higher abundance in children with above the median CCS.
Metaproteomic analyses suggested mechanisms that might underlie microbial effects on infant neurodevelopment. In main COG category, proteins involved in intracellular trafficking were more abundant in children with below the median CCS while those involved in Carbohydrate transport were enriched in children with above the median CCS. In children with below the median CSS, there was increased abundance of aspartate carbamoyl transferase and dihydroorotase. Interestingly, in children with above the median CSS histidine ammonia lyase was significantly enriched, an enzyme involved in histamine metabolism. In conclusion, our forecoming study will show an association between gut microbiota and infant cognitive performance where regulation of histidine metabolism by gut microbiota in early life may underlie this relationship.

INTRODUCTION

The first year of life is the critical period when the complex microbial ecosystem is assembled de novo that is influenced by host-extrinsic factors (antibiotic exposure, feeding pattern), host-intrinsic factors (genetics, immune system, maternal pre-pregnancy weight) and environmental factors (maternal vertical transmission, type of delivery) (1).

The colonization by the gut microbiota simultaneously occurs with the dynamic phase of postnatal brain development, including cell differentiation, axon myelination and synaptogenesis, and the rapid emergence of infant cognitive functions (2). Subsequently, the diversity of commensal species within less-heterogeneous communities increases with age as well as the metabolic landscape of gut microbial metabolic pathways/the repertoire of microbiota-derived molecules (3) that have been shown to interact/modulate/contribute brain development and function/influence the fine maturation of the brain with long lasting effects (4-8). Dysbiosis of the intestinal microbiota produce changes in neuromodulators/neurotransmitters and brain volumes that impact central nervous system (CNS) function and social behaviour in rodents (9-12). In humans, associations of commensal gut bacteria with autism (13), temperament (14), cognition (15) and depression (16) have been shown. Based on these emerging evidences, a link between gut microbial colonization and ecosystem metabolic performance with infant CNS function may exist. To test this hypothesis, we conducted a longitudinal study in full-term healthy infants where cognitive function assessed with Bayley III scales of infant development was associated with gut microbial composition, structure and metabolic performance.

RESULTS AND DISCUSSION

Cognitive abilities of subjects

Our analysis focused on BSID-III scales of infant development of full term healthy infants of 6-months of age. Children were categorized into two groups, above and below the median (50^{th} percentile), according to their scores in the seven individual BSID-III scales (gross motor, fine motor, composite motor, receptive language, expressive language, composite language and composite cognitive). Characteristics of the study population are shown in Supporting Information Table S1.

Potential confounders relevant to infant neurodevelopment

Potential confounders relevant to cognitive function assessed by BSID-III scales were examined to determine whether any identified associations between gut microbiota and infant neurodevelopment could be influenced by other aspects of perinatal condition. Our analyses showed that birth head circumference, type of feeding, maternal IQ and pre-pregnancy body mass index, gestational age, type of delivery and number of siblings influenced BSID-III scales of infant development, confounding effects that were adjusted for in downstream statistical analyses (Supporting Information Table S2).

Microbial composition and functionality

After quality filtering, 4,435,206 read sequences rendered a gut microbial profile consisting of 665 species-level bacterial operational taxonomic units (OTUs) that narrowed to 88 distinct genera belonging to 39 families after high confidence phylogenetic annotation. In agreement with previous reports (17, 18), community membership was dominated by *Firmicutes* (502), followed by *Bacteroidetes* (104), *Proteobacteria* (32), *Actinobacteria* (23) and *Fusobacteria* (4). The most abundant genera (60.7% of total reads) were *unclassified_Lachnospiraceae*, *unclassified_Enterobacteriaceae*, *Lachnospiraceae_ incertae_sedis*, *Bacteroides* and *Enterococcus* (60.7% of total reads). Phylotype occurrence showed high inter-sample variation in infants' gut microbiota. Only four OTUs, assigned to *Lachnospiraceae_incertae _sedis*, *Enterococcus* and *Pluralibacter*, were present in all samples and accounted for 17.8% of all sequence reads. Twenty-four OTUs were highly abundant (>1% of all sequence reads) but only fifteen of them were highly prevalent (>90% of samples). These frequent and highly abundant OTUs belonged to *Firmicutes*, *Bacteroidetes* and *Proteobacteria*. Our metaproteomics analyses characterized 4,424 bacterial protein groups that were phylogenetically assigned and unambiguously quantified. These protein groups were assigned to 131 genera belonging to 64 families. Due to the functional redundancy of orthologous proteins in our metaproteomics dataset, 4,424 protein groups narrowed to 658 COG functions. The mean number of protein groups per sample was 742 ± 87. Overall distribution exhibited a rather even pattern across the samples where the most abundant secondary COGs belonged to 'Metabolism' category: 'Carbohydrate Transport and Metabolism', 'Amino Acid Transport and Metabolism', 'Energy Production and Conversion' and 'Inorganic Ion Transport and Metabolism'. This result is consistent with previous reports on the functional profile of protein groups expressed by gut microbiota (19-23).

Gut microbial diversity associated with composite cognitive outcomes

We first tested whether measures of gut microbial community structure differed between infants

categorized as above and below the median in the seven individual BSID-III scales. The unique BSID-III scale with significant differences in gut microbial community structure between infant groups was composite cognitive scale. Principal coordinate analysis based on weighted UniFrac metrics of β-diversity showed that the gut microbiota of infants clustered by composite cognitive performance (p<0.014), indicating overall dissimilarities in the microbial profile of highly abundant taxa (Figure 1a). We observed increased α-diversity but reduced β-diversity as a function of composite cognitive performance in infants (Figure 1b). Higher evenness (p<0.004), Shannon (p<0.011) and Simpson (p<0.021) diversity and reduced dominance (p<0.021) characterized the gut microbiota of infants with above median cognitive composite scores (Figure 1c).

Bray-Curtis dissimilarity metrics showed that the gut microbiota was more conserved among infants with above the median composite cognitive scores (Figure 1d). The gut microbiota of infants with above the median composite cognitive scores was characterized by a significant enrichment in *Bacteroides* and *Bacteroidaceae* while a higher abundance of *Lactococcus* and *Lachnospiracea_incertae_sedis* characterized that of infants with below the median composite cognitive scores (Figure 1e). At OTU-level analysis, OTU-119 and OTU-309 corresponding to *Bacteroides* and *Actinomyces* were significantly increased in the gut microbiota of 57% and 78% of the infants with above the median composite cognitive scores, respectively (Figure 1f). They shared ≥97% sequence identity with isolated strains of *Bacteroides clarus* and *Actinomyces odontolyticus*.

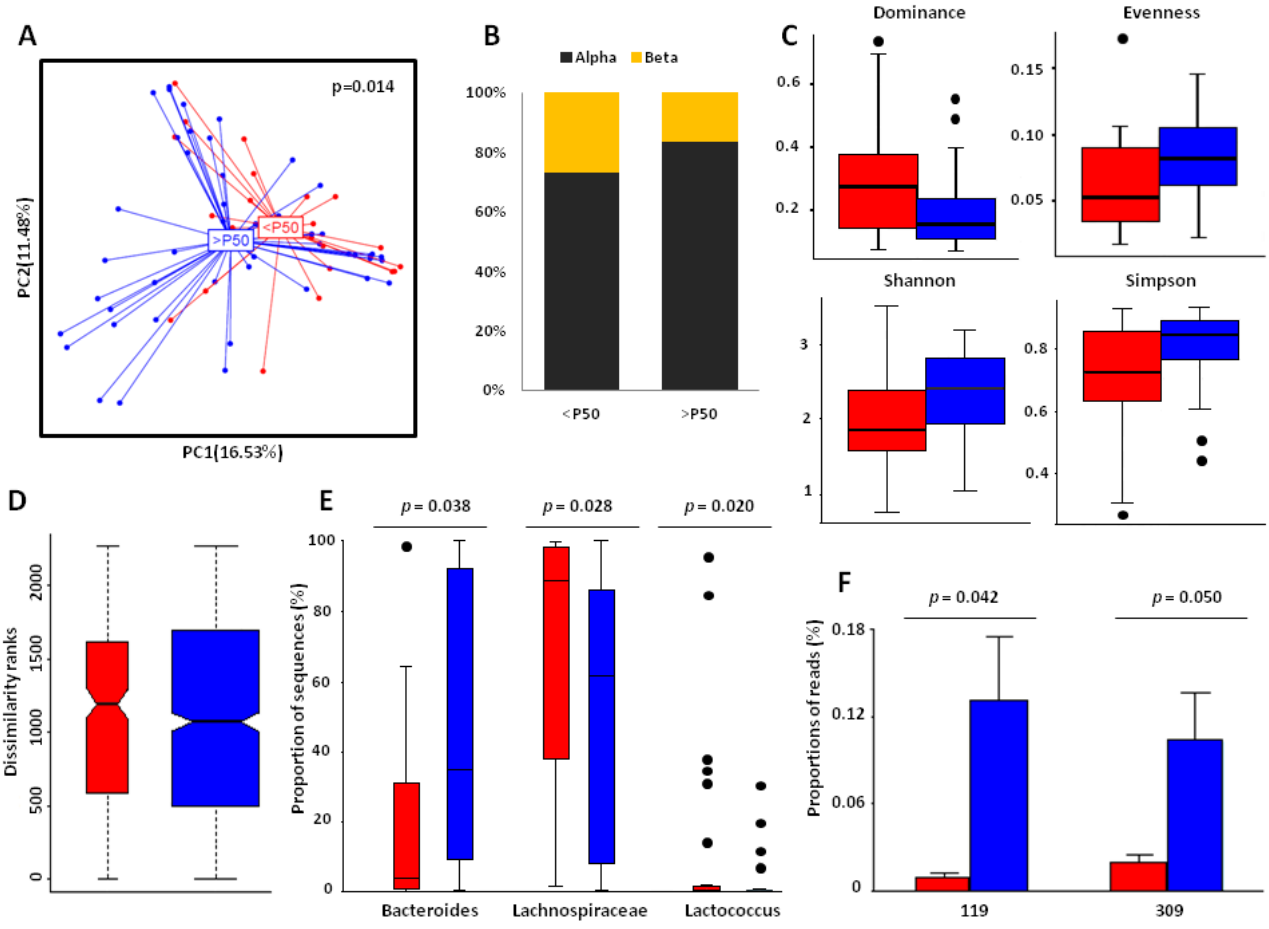

Figure 1. Gut microbial communities of infants with below (red) and above (blue) the median Composite Cognitive (A) Scatterplot from principal coordinate analysis using weighted Unifrac metrics. p value for PERMANOVA test with 999 permutations. (B) a-diversity and b-diversity determined by Rao's diversity at phylum level.(C) Boxplot of Alpha-diversity indices (Dominance, Evenness, Shannon and Simpson). Alpha diversity indexes are composite indexes reflecting abundance and consistency. (D) β-diversity (Bray-Curtis dissimilarity metrics) at phylum level. (E) Differentially abundant microbial genera of infants with scores above and below the median. (F) OTUs significantly (p<0.05) increased in the gut microbiota of 57% (OTU 119) and 78% (OTU 309) of the infants with above the median composite cognitive scores.

Twelve OTUs were enriched in the gut microbiota of the infants with below the median composite cognitive scores. Only OTU-30 and OTU-43 were highly frequent (73% and 50% of the individuals) and were closely related to isolated strains of *Lactococcus lactis* and *Ruminococcus gnavus*, respectively (≥99% sequence identity).

Co-occurrence networks showed significant positive and negative correlations between taxa (Figure 2). *Lachnospiracea_incertae_sedis* was the hub of a positively-correlated module with *unclassified_Bacteria*, *unclassified_Firmicutes* and *unclassified_Lachnospiraceae* (Figure 2A). OTU-309 positively correlated with OTU-30 and OTU-32, both belonging to *Streptococcus* (Figure 2B).

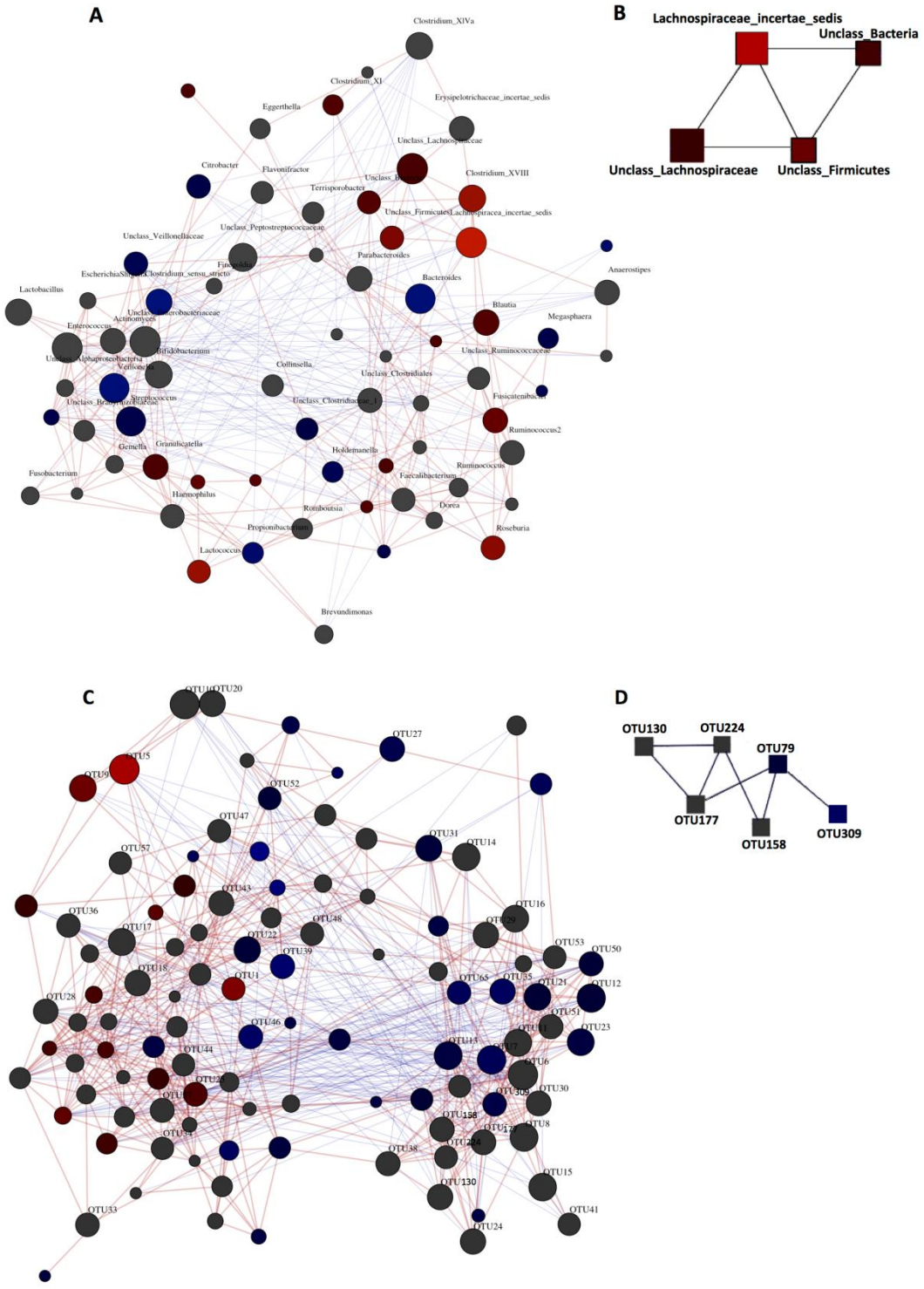

Figure 2. Co-occurrence network of gut microbiota in infants with above the median (blue) and below the median (red) CCS at genera (A,B) and OTU levels (C,D). Significant modules involving signature taxa are depicted (B,D). To reduce noise in OTU network visualization, the data were thresholded such that only OTUs measured above background (>0.1% total reads) in 50% of samples are shown.

Enrichment analysis identifies cognition-specific functional signatures in the gut microbiota

Group differences were observed in COG category between infants with above and below the median CCS (Figure 3). The gut microbiota of infants with below the median CSS had an increased abundance of proteins involved in 'Intracellular Trafficking Secretion and Vesicular Transport' (p<0.027) (Figure 3A). At COG function level, significant abundances were observed in dihydroorotase (PyrC, p<0.007) and aspartate carbamoyl transferase (PyrB, p<0.007), enzymes involved in the first steps of pyrimidine biosynthesis along with carbamoylphosphate synthetase, the subunit DdpA of ABC-type permease (p<0.024) involved in di- and tri-peptide transport, a GTP cyclohydrolase (QueFC, p<0.05) involved in folate biosynthesis and ribosomal protein L11 (p<0.05) in infants with below the median CSS.

An interesting finding was that histidine ammonia lyase (HutH), an enzyme that catalyzes the non-oxidative deamination of L-histidine in histidine catabolism, was significantly more abundant in the gut microbiota of infants with above the median CSS (p<0.05). Histidine is the precursor of histamine, a neurotransmitter that has been long regarded as a modulator of cognition (24).

CONCLUSIONS

Our forecoming study will show an association between gut microbiota and infant cognitive performance where regulation of histidine metabolism by gut microbiota in early life may underlie this relationship. Results may have implications for developmental disorders characterized by cognitive delay.

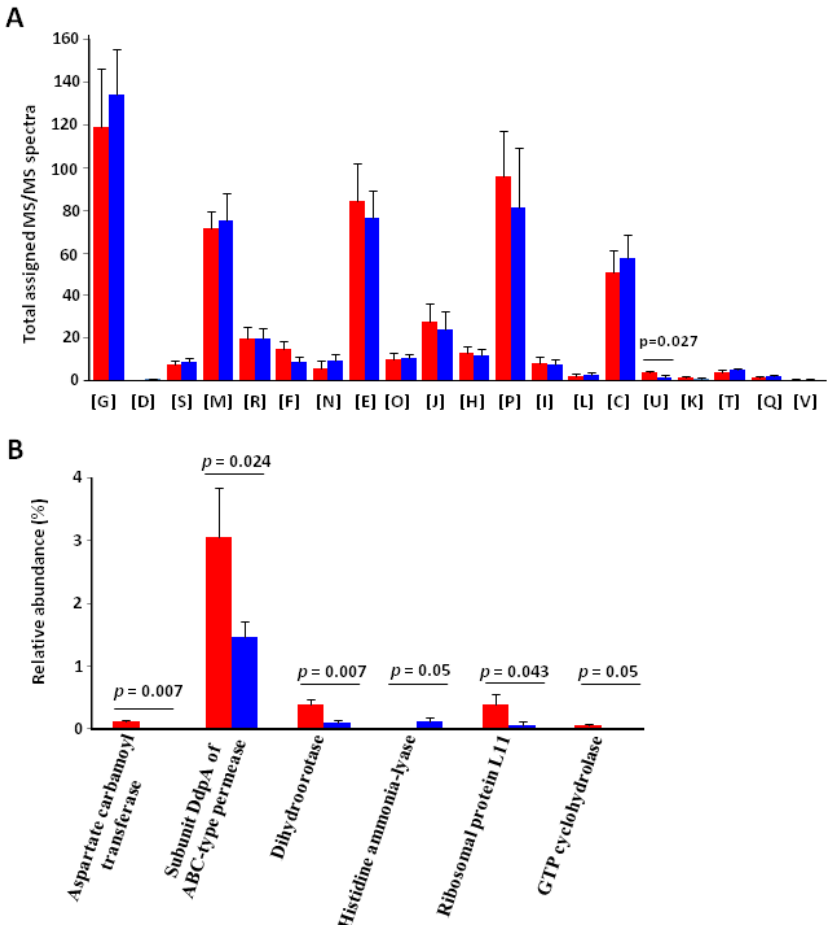

Figure 3. Distribution of COGs in infants above (blue) and below (red) the median CCS. (A) Significant abundances at tier 2 of COG category. (B) Significant abundances at COG function level.

EXPERIMENTAL PROCEDURES

Subjects, experimental design and ethical guidelines

In the present study, full-term healthy infants aged 6-months, who did not present any intestinal disorders and had not taken antibiotics, were chosen from the panel of infants that belonged to PREOBE study cohort (25). In this period of life, the transition from weaning to solid food consumption occurs. Characteristics of the study population are shown in Supporting Information Table S1. In this project, pregnant women were recruited between 2007 and 2012 at San Cecilio and Mother-Infant University Hospitals in Granada, Spain. The study exclusion criteria for mothers were: simultaneous participation in any other research study, any kind of drug treatment, diagnosed diseases (e.g., pre-gestational diabetes, hypertension or preeclampsia, intrauterine growth retardation, maternal infection, hypo/hyperthyroidism, hepatic or renal disease) and vegan diet. Fresh stools were collected at 6-months after delivery and were immediately stored at -80ºC, until processing. The study included anthropometric measurements, health questionnaires and medical assessments of the child. This project followed the ethical standards recognized by the Declaration of Helsinki (reviewed in Hong-Kong 1989 and in Edinburgh 2000) and the EEC Good Clinical Practice recommendations (document 111/3976/88 1990), and current Spanish legislation regulating clinical research in humans (Royal Decree 561/1993). The study was explained to the participants before starting, and the parents signed an informed consent.

Assessments of infant neurodevelopmental outcome

The Bayley Scales of Infant Development, Third Edition (BSID-III), were used for assessed of infants´ neurodevelopment at 6 and 18 months of age. All infants of this study were examined by the same trained psychologist (FJTE). This neurobattery is specifically designed for children for 1-42 months of age. The infant evaluation by BSID-III is performed across three principal domains: Cognitive skills, Language and Motor development. The language scale explores two branches of the development, the receptive and expressive language. The motor scale permits the examination of both developmental skills, fine and gross motricity. A composite score of the language and motor domain is obtained. The scaled score and composite score was calculated for each scale and was adjusted for each child and age (days), using the correction manual tables (26).

DNA extraction from stool samples

Genomic DNA was extracted from faecal bacteria of 6-month (n: 68) old infants as previously described (21). Briefly, faecal samples were resuspended in 1 ml of TN150 buffer (10 mM Tris-HCl pH 8.0 and 150 mM NaCl). Zirconium glass beads (0.3 g) and 150 ml of buffered phenol were added and bacteria were disrupted with a mini bead beater set to 5000 rpm at 48C for 15s (Biospec Products, USA). After centrifugation, genomic DNA was purified from the supernatant using phenol-chloroform extraction. Quality was checked by agarose gel electrophore- sis and quantified with Quant-iT PicoGreen dsDNA assay kit (Invitrogen, Darmstadt, Germany).

16S rRNA gene sequencing and data processing

Genomic DNA from faecal bacteria was used as templates for 16S rRNA gene amplification using 27F and 338R universal primers and two consecutive PCR reactions to integrate Illumina multiplexing sequences as previously described (27). The library was prepared by pooling equimolar ratios of amplicons and was sequenced using an Illumina MiSeq platform (Genetic Service, University of Granada). Reads were demultiplexed and sorted, and paired ends were matched to give 240nt reads. Data set was filtered and OTUs were defined at 99% similarity with MOTHUR programs unique.seqs and pre.cluster (28). Taxonomic classifications of OTUs were assigned using the naïve Bayesian algorithm CLASSIFIER of Ribosomal Database Project (29). OTUs were considered unassigned when confidence value score was lower than 0.8, and were annotated using upper taxonomic ranks.

Protein extraction, separation, identification and data processing

Protein extraction was performed from faecal bacteria of 6- months (n: 14) old infants as previously described (21). Faecal samples (0.5 g) were thawed and diluted in 1mL of 0.05% L-cysteine phos- phate saline buffer solution (PBS) under anaerobic conditions. After differential centrifugation, faecal bacteria were disrupted by mechanical lysis in BugBuster Protein Extraction Reagent (Novagen) for 30 min at room temperature, followed by soni- cation

for 2.5 min on ice. Protein extracts were centrifuged for 10 min at 12.000 rpm to separate cell debris. Protein concen- trations were determined with the Bradford assay(30). For 1-DE analysis, two 75-lg protein samples (technical replicates denoted by a or b) were precipitated with five- fold volumes of ice-cold acetone and separated on a 12% acrylamide separating gel with the Laemmli buffer system (31).

After electrophoresis, protein bands were stained with Coomassie Brilliant Blue G-250. Entire protein lanes were indi- vidually cut into one band prior to performing in-gel tryptic digestion. Peptide lysates were desalted using C18 ZipTip prior to MS analysis. Peptides were analysed by nano-HPLC system Advion NanoMate and Orbitrap Fusion mass spec- trometer (Thermo Fisher Scientific). The peptides were eluted over 115 min with a gradient of 2 to 55% solvent (acetonitrile,0.1% formic acid). MS scans were measured at a resolution of 120,000 in the scan range of 400–1600 m/z, MS2 in the Iontrap (rapid mode). Raw data files were searched with Proteome Discoverer (v1.4, Thermo Fisher Scientific) using the SequestHT algorithm against a database containing protein-coding entries of bacterial taxa selected via 16S rRNA gene sequencing. Only rank 1 peptides were considered to be identified with a threshold of FDR <1%. The mass spectro- metry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (32) partner repository with the dataset identifier PXD009056. Higher protein abundance is represented by a higher number of MS/MS spectra acquired from peptides of the respective protein. Thus, protein abundances were calculated based on normalized spectral abundances that allow relative comparison of protein abundances over different samples (21, 33, 34). 'PROteomics results Pruning & Homology group ANotation Engine' (PROPHANE) (35) was used to assign proteins to their taxonomic and functional groups using the functional annotation of COGs. For KEGG pathway reconstructions, a BLASTP v2.2.27 search of the original protein sequences against NCBInr to retrieve KEGG Orthology identifiers was performed (36). The use of a metaproteome-specific database containing fully sequenced genomes from closely related genera to the sam- ple's strains and other documented gut genera in the database of proteomes together with the specificity of the identification procedure resulted in a high proportion of taxo- nomic and functional annotation(37).

Statistical and data analysis

Statistical analyses were carried out using SPSS version v19.0 (IBM, IL) and R statistical package (38). Normal distribution of the data was assessed using the Shapiro-Wilk test and Levene test. Continuous and normal variables are displayed as mean and standard deviation (SD) and those not following a normal distribution by median and interquartile amplitude. Student t-test for continuous normal variables was performed. For variables not following a normal distribution, U Mann Whitney test was performed. Chi-square test and Fisher test was applied to determine the association between categorical or categorized variables. Each neuropsychological domain was divided by the median and was categorized, above and below the median value, in order to determine for each domain, the confusion variables that affect the model. The microbiota at 6 months was divided by each neuropsychological domain without adjusting and adjusting the confounding variables by means of the multivariate analyses of covariance (MANCOVA). The confounder introduced in the models, they are explained in the results. $P > 0.05$ was considered as minimum level of significance. For the response of composition and function of the microbiota, multivariate analysis of variance using distance matrices was performed, based on Bray-Curtis distance metrics. The matrices were partitioned in sources of variation with subject and characteristics of the study population as explanatory variables. Significance of the pseudo-F ratios was assessed by permutation test (999 permutations, using the adonis function from the R package vegan) (39) β-diversity for compositional data was calculated as Unifrac distance with GUnifrac package. Permanova analysis of the distance between different time points was calculated with adonis function from vegan package. Bray-Curtis dissimilarity measures were calculated with vegan package and anosim test was used to establish significant differences between the study groups. Statistical Analysis of Metagenomic Profiles v2.0 was used to compare the abundances of taxa, COG categories and subcategories between study groups (40). α-diversity indices were calculated with PAST software (41). Pearson's correlation network analysis and visualization were carried out using Calypso v8.20 (42). Network node parameters were calculated using Cytoscape v3.1.1 (43).

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SUPPORTING INFORMATION

Supplementary Table S1. General Characeristics of the studied population

Mother		
Maternal Age (y)		33.27 ± 3.92
Pre-conceptional maternal Height		1.63 ± 0.07
Preconceptional maternal Weight		72.92 ± 15.27
Preconceptional maternal BMI		27.4 ± 5.90
IQ mother (points)		107.13 ± 14.41
Maternal education level	Primary/Secondary	36(52.94%)
	University/Doctor	32(47.06%)
Smoking during pregnancy	Yes	4(5.88%)
	No	64(94.12%)
Alcohol consumption during pregnancy	Yes	66(97.06%)
	No	2(2.94%)
Mode delivery		
	Vaginal	51(75%)
	Cesarea	17(25%)
Newborn Infant		
Gestational Age (weeks)		39.5 ± 1.6
Birth weight (g)		3342.91 ± 416.58
Lenght at birth (cm)		50.54 ± 1.86
Röhrer Ponderal Index (kg/cm3)		25.89 ± 3.54
Breast Feeding		
	Yes	73(70.19%)
	No	31(29.81%)
Infant at 6 months (n=68)		
Height (cm)		68.4 ± 2.76
Weight (kg)		7.79 ± 0.93
BMI (kg/m2)		16.63 ± 1.52
Gender		
	Male	40(58.82%)
	Female	28(41.18%)

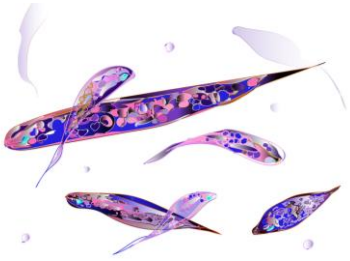
Values listed are total for the variable (percent of total value n) or Means ± SD.

Supplementary Table S2. Confounding variables.

BSDIII scales	Expressive language		Receptive language		Composite language		Fine motor		Gross motor		Composite motor		Composite cognitive	
Study groups	<p50	>p50	<p50	>p50	<p50	>p50	<p50	>p50	<p50	>p50	<p50	>p50	<p50	>p50
n	34	34	14	54	33	35	23	45	25	43	22	46	22	46
Birth HC (cm)	-		0.029*		-		0.042*		-		0.001*		-	
Maternal Age (y)	0.047*		-		-				-		0.005*		-	
Birth weight (g)	0.008*		-		-				-		-		-	
Infant type of feeding	0.039*		-		0.019*				-		-		-	
Prepregancy BMI	-		-		-		0.016*		-		-		-	
Maternal educational level	-		-		-		0.012*		-		-		-	
Gestational Age (wk)	-		-		-		0.019*		-		0.046*		0.001*	
Prepregancy BMI (group)	-		-		-		-		0.030*		-		-	
Maternal IQ (points)	-		-		-		-		0.003*		-		-	
Type of delivery	-		-		-		-		0.024*		0.049*		-	
Nº of siblings	-		-		-		-		-		0.050*		-	

** P-values for overall differences between children above and bellow of the median according to each Bayley test. Analysis of variance for normally distributed variables, Krukal-Wallis rank-sum test for non-normal continuous variables and Chi-square test for proportions.*

DISCUSSION



A major challenge in microbial ecology is to identify its functional members and understand how their functional and phylogenetic dynamics ultimately influence human physiology and health. Critically important are the initial stages of microbiota colonization and maturation in the gut because early dysbiosis has been demonstrated to affect future human health and brain development (80-82).

Few studies have been focused on the infant gut microbiota and historically relied on culture-based enumeration; more recently several studies are based on taxonomic profiling by 16S rRNA gene sequence analyses (83-85). Moreover, functional descriptions of gut microbial communities have been contingent on conventional genetic approaches with metagenomic annotation and *in silico* evidence from community membership that only provide a prediction of microbial metabolic response to environmental factors (86, 87). Metaproteomics can overcome these limitations because protein abundance and function are indicators of current metabolic activity and physiological adaptation, providing microbial ecologists with a robust framework that facilitate a closer understanding of the complex dynamics that drive ecosystem functional and compositional responses to environmental pressures (88).

Our hypothesis is that the metaproteome provides insight about the relative importance of its members in ecosystem functioning, their collective functional pattern and the network topology in relation to host physiology. Our goal was to address those questions in relation to gut microbial maturation, maternal health status and infant neurodevelopment during early life. To test this hypothesis, we conducted a longitudinal study in full-term healthy infants who did not present any intestinal disorders and had not taken antibiotics, selected from the panel of infants participating in the PREOBE Project (89). Faecal samples, dietary and life style questionnaires and data on cognitive function assessed by Bayley III were collected at 6- and 18-months of life.

We combined cultivation-independent 16S rRNA gene sequencing and metaproteomics to investigate the structure, composition and metabolic performance of gut microbiota.

The phylogenetic composition and categorical breakdown of identified species-level bacterial operational taxonomic units (OTUs) in our samples showed that 7,890,853 read sequences rendered a gut microbial profile consisting of 679 (OTUs) that narrowed to 89 distinct genera belonging to 40 families after high confidence phylogenetic annotation. We also identified 11,901 peptides of which 9,173 bacterial protein groups were assigned and unambiguously quantified. These protein groups were assigned to 134 genera belonging to 61 families. This is the highest number of distinct proteins groups identified in human gut metaproteomics studies published so far. The most striking result was the low correlation between 16S rRNA gene abundances and microbial source of protein groups, indicating discordance between microbial membership and biological activity. The log ratio of abundances between organism-origin of protein groups and 16S rRNA gene abundances showed deviations that were in a many cases of an order-of-magnitude (highly significant absolute fold change >10) either lower or higher than expected from 16S rRNA gene abundances of their corresponding taxa. The most significant deviation was the high relative proportion of protein groups identified for *Verrucomicrobia* versus its null detection by 16S rRNA gene sequencing.

In our data, the statistical analysis of variables showed that age was the unique variable to significantly explain a relevant proportion of total variation in microbiota composition and function (7% and 13.5%, respectively). β-diversity metrics of total (phylogeny of OTUs) and functional (phylogeny of proteins) gut microbial communities confirmed that samples clustered by age. We observed increased α-diversity but reduced β-diversity as a function of time, suggesting that both total and functional communities accumulated diversity into less heterogeneous structures. *Firmicutes* dominated the total community and its functional subset that enriched in *Bacteroidetes* and *Firmicutes* and depleted in *Proteobacteria* and *Actinobacteria* with time. In total gut microbiota, signature highly abundant genera (>1% mean relative abundance) at 6-months were *Enterococcus*, *Lactobacillus*, *Erysipelotrichaceae_Incertae_*Sedis, *unclassified_Veillonellaceae* and *unclassified_Enterobacteriaceae* while the 18-month's total gut microbiota was significantly enriched in obligate anaerobes from the genera *Bacteroides*, *Anaerostipes*, *Blautia*, *Fusicatenibacter*, *Lachnospiraceae_incertae_sedis*, *Roseburia*, *Ruminococcus2*, *Faecalibacterium* and *unclassified_Clostridiales*. The functional gut microbiota was characterized by few signature genera due to the high inter-individual variability. In 6-month's functional gut microbiota, signature highly abundant genera were *Bifidobacterium* and *Veillonella* while *Eubacterium* and *Faecalibacterium* were enriched at 18 months. In agreement with previous studies (8, 90, 91) , our findings reflected the shift of gut microbiota towards an

adult-like structure and composition as infants grew, possibly associated to physiological fitness to persist in increasingly lower oxygen levels.

When metabolic performance and functionality was compared between infant groups, we found that the 6 months' gut microbiota had an over-representation of COGs classified into the main COG category 'Cellular processes and signalling', distributed within 'Cell wall membrane envelope biogenesis', 'Cell motility', 'Intracellular Trafficking Secretion and Vesicular Transport' and 'Signal transduction mechanisms'. The 18-month metaproteome was enriched in COGs classified into the main COG category 'Metabolism', distributed within 'Lipid transport and metabolism' and 'Nucleotide transport and metabolism'.

The most interesting result of metaproteomics was that the majority of COG functions that differentiated the 6- and 18-months gut microbiota belonged to 'Metabolism' with relevant roles in polysaccharide catabolism, central carbon metabolism and fermentation. Our data demonstrated that, depending on the carbohydrate source and oxygen concentration, gut microbiota use distinct pathways of polysaccharide degradation, monosaccharide catabolism and central carbon metabolism that end in the production of the main non-gaseous products of microbial fermentation: lactate and the short-chain fatty acids (SCFA) acetate, propanoate and butyrate

In polysaccharide catabolism, we detected abundances of 24 GH COGs that belong to 20 GH families whose mean number, protein abundance and catalytic activities of GH increased with age. Consistent with the contribution of human and formula milk to infant diet, the microbiota of 6-months infants was enriched in β-galactosidase and arabinogalactan endo-1,4-β-galactanase, mostly expressed by *Actinobacteria*. Additionally, *Actinobacteria* and *Firmicutes* expressed an α-glucoside phosphotransferase IIC subunit, involved in the phosphorylative transport of glucose, glucosamine and n-acetylneuraminic acid while *Proteobacteria* expressed maltoporin involved in maltose and maltodextrin transport. In contrast, the higher diversity and complexity of dietary carbohydrates in 18-months' infants resulted in a significant enrichment in α-amylase, α-glucosidase and β-glucosidase that were expressed by multiple taxa within *Bacteroidetes* and *Firmicutes*. Endo-β-N-acetylglucosaminidase D involved in the hydrolysis of branched oligosaccharides was expressed only by *Bacteroidetes* while cellobiose phosphorylase involved in the phosphate-dependent hydrolysis of cellulose was assigned to *Firmicutes*. The determination of gut microbial β-galactosidase, α-glucosidase and β-glucosidase activities in 6- and 18-monts infants confirmed their enrichment in the metaproteomes. Taken together, these

results indicated that the gut microbiota used the proper upper glycolytic pathways depending on the availability of the carbohydrate source in a diet shifting from breast milk or formula to solid foods.

In central carbon metabolism, we observed differential abundances of enzymes involved in Embden-Meyerhoff-Parnas, the pentose phosphate and the Entner-Doudoroff pathways that convert monosaccharides into phosphoenolpyruvate between infant groups. The most striking result was that *Firmicutes* and to a minor extent in *Bacteroidetes*, *Proteobacteria* and *Verrumicrobia* employed a variation of the classical TCA cycle based on an unorthodox enzyme, acetate:succinate CoA-transferase (ASCT) to synthesize succinyl-CoA. Kwong et al. recently showed that ASCT genes were widespread in prokaryotic genomes and functionally replaced succinyl-CoA synthetase in TCA cycle of human microbial commensals (92). In a carbon-rich anaerobic gut ecosystem, this strategy may be a result of niche specialization where gut microbiota may use acetyl-CoA, the keystone molecule of central metabolism produced from monosaccharides, amino acids, fatty acids and other secondary metabolites, as driver of a reverse TCA cycle to maintain redox balance and obtain energy for growth.

Regarding microbial fermentation, our metaproteomics revealed the gut microbial age-associated maturation of fermentative strategies to harvest energy from diverse carbon resources in a shifting glycobiome environment. At 6-months, the enrichment in galactokinase, galactose mutarotase, gluconate/galactonate dehydratase, N-acetyl-glucosamine 6-phosphate 2-epimerase, 2-dehydro-3-deoxy-rhamnonate aldolase and fucose dehydrogenase suggested active catabolism of milk and mucin-derived monosaccharides by early gut microbiota. We did not detect lactoyl-CoA dehydratase in the acrylate pathway while lactate dehydrogenase was highly abundant and phylogenetically assigned to all phyla suggesting that fucose-derived lactate may be a central substrate for metabolic cross-feeding in early life. Acetate kinase that produces ATP and acetate as end product was also enriched, a strategy mainly used by *Bifidobacteria*, confirming their importance in providing acetate that reduces faecal pH and protects host epithelial cells from enterotoxins (93). With age, the introduction of solid foods generates a richer repertoire of released monosaccharides available for microbial metabolism. In SCFA metabolism, the enrichment in succinyl-CoA reductase, acetyl-CoA acyl-transferase, enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase, suggested that gut microbial metabolism shifted towards butyrate fermentation. The fact that these protein groups were mainly assigned to Clostridia support their important role in the metabolic welfare of colonocytes by releasing butyrate as a fermentation end-product (94). The enrichments in ASCT and succinyl-CoA reductase suggest a

link between succinate fermentation to butyrate and acetate production, as has been observed in Clostridium (95). Notably, our metaproteome revealed an alternate route for acetate synthesis in 18-months gut microbiota. We observed an enrichment in ethanolamine ammonia-lyase that catalyzes the adenosylcobalamin (AdoCbl)-dependent conversion of ethanolamine to acetaldehyde and ammonia (96). Ethanolamine is abundant in the human gut because the constant renewal of the intestinal epithelium daily releases 25% of enterocytes whose membranes are rich in phosphatidylethanolamine (97).

These data confirmed that distinct metabolic performances of the gut microbiota in infants of 6- and 18-months of age. The question is who are the truely biologically active taxa and how do they configure the gut microbial ecosystem? The analysis of microbial contributions to overall community activity indicated that the ecological network was remodelled as the functional gut microbiota of infants evolved with time. The topology of the co-occurrence networks of active taxa collapsed at family level determined by Pearson's correlation coefficient showed two mutually exclusive modules clustered by age. A low-connected *Bifidobacteriaceae*-centred guild of facultative anaerobes was succeeded by a rich club of obligate anaerobes densely interconnected around *Lachnospiraceae*, underpinning their pivotal roles in microbial ecosystem assemblies. Further analysis revealed the succession of metabolic functions between taxa in these mutually exclusive consortia, suggesting a high level of functional redundancy between taxa. These data supported that the maturation of the human microbiota during early life may be proposed as an example of ecological succession, in which communities undergo consecutive compositional and functional transitions in dominant taxa to establish physiological syntrophy among microbiota for niche adaptation (8, 98).

Taken together, the detailed reconstruction of the gut microbial carbon metabolism presented here, including the assignment of enzymes to microbial taxa, revealed alternate temporary microbial and metabolic configurations where community-wide metabolic relationships to harvest energy by fermentation of prevailing dietary and host-derived carbon substrates, mainly glycans, differentiated chronological states. Our data provide a proteomic catalogue of the functional maturation of early gut microbiota, which may constitute an important research tool for indicators of future healthy or diseases states and for the design of microbiota-targeted health-promoting strategies early in life.

Regarding colonization and maturation in infant gut in relation to maternal health status, experimental evidences suggest that maternal obesity and weight gain modify the composition

and metabolism of the microbiota in the gut and breast milk during pregnancy and lactation(99-101). Still, few studies have addressed how maternal obesity influences the gut microbial metabolic potential during early life (85, 91, 102). Thus, we set out to identify differences in the functions encoded by the microbiota of infants at 18 months of age born to pre-pregnancy normoweight and obese mothers. We used PICRUSt functional prediction to construct a community-level metabolic network of the microbiota and compare the abundance of pathways across infant groups.

On a compositional level, we observed that *Firmicutes* was significantly enriched in children born to normoweight mothers whereas *Bacteroidetes* was significantly enriched in children born to obese women, due mainly to a significant increase in *Lachnospiraceae* and *Clostridicaeae* 1 and *Bacteroidaceae* families. Our functional predictions with PICRUSt showed an overall enrichment in genes involved in metabolic pathways in the gut microbiota of infants born to obese mothers. Of note, abundances of all KO modules involved in lipopolysaccharide biosynthesis were increased. These functional annotations highlight the importance of sulphur metabolism in infants born to obese mothers. This pathway is involved in the degradation of sulphur-containing compounds, such as products from the fermentation of certain amino acids (cysteine and methionine) and taurine bile acid derivatives. According to this, we also found a significantly overrepresentation of genes involved in taurine and hypotaurine metabolism in children born to obese mothers. Despite infants born to obese mothers had higher abundances of *Bacteroides*, specialized in the degradation of dietary non-digestible carbohydrates as well as host carbohydrates including mucus, we found no enrichment in KO modules of polysaccharide degradation. In our study, the microbiome of infants born to normoweight mothers have a significantly higher proportion of genes involved in lipoic acid metabolism, an essential cofactor of pyruvate dehydrogenase, and pentose-phosphate pathway. Considering that the fermentation routes were present in the gut microbiota, our results suggest an increased ability to process the larger variety of monosaccharides in the diet by the gut microbiota of infants born to normoweight mothers. Taken together, we showed that gut microbial community structure is different in infants' gut microbiota depending on maternal pre-pregnancy body mass index with potentially distinct metabolic performances.

Compelling experimental evidence suggests a link between gut microbial colonization and ecosystem metabolic performance with central nervous system function. Consequently, we summarised in the review *"Role of microbiota function during early life on child's neurodevelopment"* and the review *"Probiotic, prebiotic and Brain development*", the current

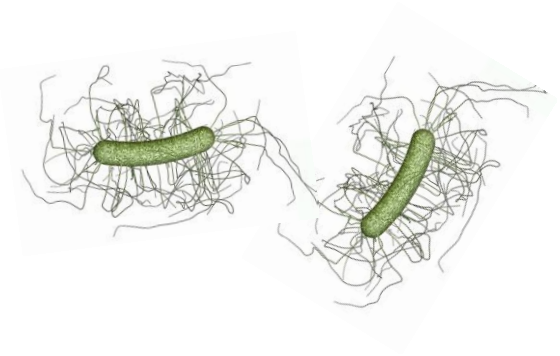
concepts related to microbiota-gut-brain axis, including microbiota modulation of the eating behaviour, child's cognitive function and brain structure, microbiota analysis techniques and neurodevelopment assessment in children, in order to use that knowledge in an original paper that link neurodevelopment and early gut microbiota.

To test this hypothesis, we compared gut microbial composition and function between infants with above and below the median scores in Bayley III scale of infant development. After correcting for confounding variables, we found a strong association between gut microbiota composition and neurodevelopment at 6 months of age. This is accordance with the finding that reconstitution of normal gut microbiota early in life normalized behavioural patterns associated to germ-free mice (103). In the Bayley III scales, composite cognitive domain was the only test in which both study groups, divided according to the mean, showed significant differences in α- and β-diversity. Higher evenness (p<0.004), Shannon (p<0.011) and Simpson (p<0.021) diversity and reduced dominance (p<0.021) characterized the gut microbiota of infants with above median cognitive composite scores Principal coordinate analysis based on weighted UniFrac metrics of β-diversity showed that the gut microbiota of infants clustered by composite cognitive performance (p<0.039), indicating significant phylogenetic dissimilarities in the microbial profile of highly abundant taxa. The gut microbiota of infants with above the median composite cognitive scores was characterized by an enrichment in *Bacteroides* and *Bacteroidaceae* while a higher abundance of *Lactococcus* and *Lachnospiracea_incertae_sedis* characterized that of infants with below the median composite cognitive scores.

Comparison of protein groups expressed by gut microbiota of infant groups identified significant differences in metabolic performances that might underlie microbial effects on neurodevelopment. In the main COG category, "Intracellular trafficking" was more abundant in the gut microbiota of infants with below the median scores, while "Carbohydrate transport" was enriched in that of infants above the median. In infants with below the median scores, we observed an enrichment in proteins involved in "ABC type dipeptide periplasm transport system", "ribosomalprotein L11", "aspartate carbamoyl transferase" and "dihydroorotase". In children with above the median scores, "histidine ammonia lyase" was significantly more abundant. Histidine ammonia lyase catalyzes the first reaction in histidine catabolism, the decarboxylation of histidine (104), the amino acid precursor of histamine, a neurotransmitter that has been historically associated with cognitive function (105). In fact, histidinemia is a metabolic disorder characterized by increased levels of histidine in body fluids that is associated with mental retardation and speech defects. Further experiments are required to demonstrate the

regulation of histidine and histamine levels as a mechanimsm by which host gut microbes influence infant cognition development.

CONCLUSIONS



Conclusion 1

Our metaproteomics data revealed that the gut microbiota harbours a distinctive subset of biologically active microorganisms, indicating considerable discordance between microbial composition and phylogenetic origin of proteins at all taxonomic levels and suggesting that using bacterial taxa or even metagenomics as input information to build predictive theoretical models of microbial activity may be highly misleading.

Los datos metaproteómicos obtenidos en el presente estudio han revelado que la microbiota intestinal alberga un subconjunto de microorganismos biológicamente activos, lo que indica discordancia considerable entre la composición microbiana y el origen filogenético de las proteínas en todos los niveles taxonómicos y sugiere que el uso de taxones bacterianos o incluso de metagenómica como información para crear modelos de actividad microbiana pueden ser muy engañosos.

Conclusion 2

The detailed reconstruction of the gut microbial carbon metabolism by metaproteomic analysis, including the assignment of enzymes to microbial taxa, revealed alternate temporary microbial and metabolic configurations where community-wide metabolic relationships to harvest energy by fermentation of prevailing dietary and host-derived carbon substrates, mainly glycans, differentiated chronological states in infant early life.

La reconstrucción detallada del metabolismo del carbono en la comunidad microbiana intestinal mediante análisis metaproteómicos, incluyendo la asignación de enzimas a taxones microbianos, demuestra un cambio temporal en la configuración microbiana y metabólica, donde la capacidad de obtener energía por parte de la comunidad se basa en la fermentación de la dieta y en los sustratos de carbono derivados del hospedador, principalmente glicanos, diferenciando estados cronológicos en los primeros años de vida.

Conclusion 3

Our results show that the maturation of the gut microbiota during the first 18 months of life is a non-random process where two mutually exclusive modules of functional families, built around *Bifidobacteriaceae (6 months)* and *Lachnospiraceae (18 months)*, which metabolically succeeded each other.

Los resultados obtenidos muestran que la maduración de la microbiota intestinal no es un proceso aleatorio, sino que dos módulos únicos de familias funcionales, construidas alrededor de *Bifidobacteriaceae* y *Lachnospiraceae* respectivamente, se sucedieron metabólicamente.

Conclusion 4

Mothers imprinted different gut microbiotas in their children depending on their pre-pregnancy weight, enriched in taxa within *Bacteroidetes* in infants born to obese mothers and in *Firmicutes* in infants born to normoweight mothers, with different predicted metabolic outcomes that may influence infants' development later in life.

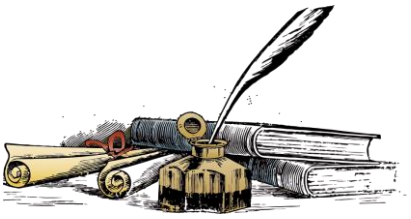
Las madres transmiten diferentes comunidades microbianas intestinales a sus hijos dependiendo del peso previo al embarazo; los niños nacidos de madres obesas presentan una mayor abundancia de bacterias pertenecientes al phylum *Bacteroidetes*, sin embargo, los nacidos de madres normopeso presentan mayor abundancia de bacterias pertenecientes al phylum *Firmicutes*. Estas diferencias pueden determinar potenciales resultados metabólicos diferentes y cambios en el crecimiento y desarrollo de los hijos durante la infancia y la niñez, con efectos desconocidos a largo plazo.

Conclusion 5

Our forecoming study will show an association between gut microbiota and infant cognitive performance where regulation of histidine metabolism by gut microbiota in early life may underlie this relationship.

Nuestro próximo estudio mostrará una asociación entre la microbiota intestinal y el rendimiento cognitivo infantil, donde la regulación del metabolismo de la histidina por la microbiota intestinal en los primeros años de vida puede ser la base de dicha relación.

References



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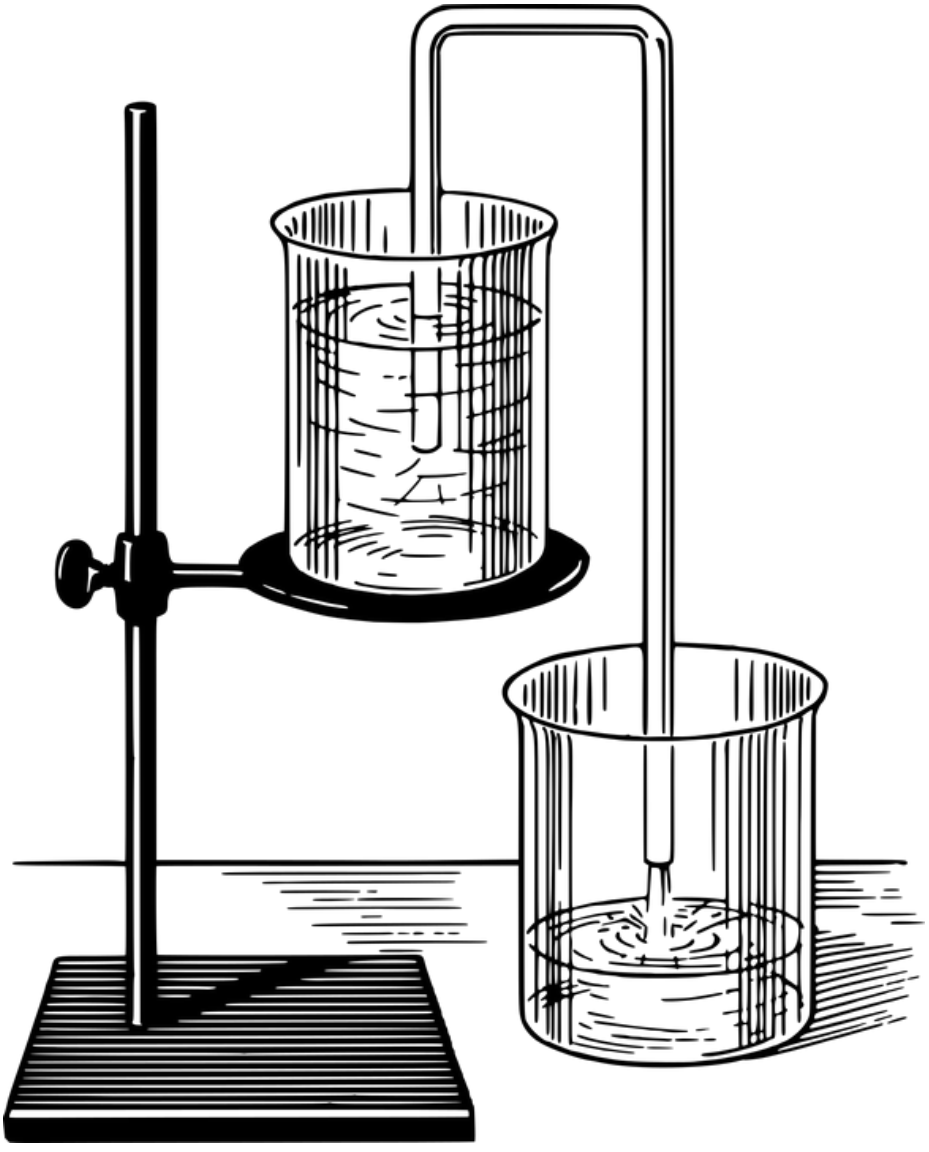
"*Cuando quieras emprender algo, habrá mucha gente que te dirá que no lo hagas, cuando vean que no pueden deternerte, te dirán cómo tienes que hacerlo y cuando finalmente vean que lo has logrado, dirán que siempre creyeron en ti*"

"*When you want to do something new, there will be many people who will tell you not to do it, when they see that they can't stop you, they will tell you how you have to do it, and finally when you have been successful, they will say that they always believed in you*"

John C. Maxwell

ANNEXES



Communications to International Meetings

Title of the work: Accuracy of a gut microbial model to predict infant cognitive development
Name of the conference: Gut Microbiota for Health World Summit 2018
Type of participation: Póster
City of event: Roma, Italia
Date of event: 10/03/2018
End date: 11/03/2018
Organising entity: Microbiota Intestinal y Salud de la Sociedad Europea de Neurogastroenterología y Motilidad
Tomás Cerdó; Alicia Ruíz; Francisco José Torres-Espínola; Ruy Jáuregui; Antonio Suarez; Cristina Campoy.

Title of the work: Gut microbial community composition and functionality associated with maternal pre-pregnancy body mass index
Name of the conference: Gut Microbiota for Health World Summit 2018
Type of participation: Póster
City of event: Roma, Italia
Date of event: 10/03/2018
End date: 11/03/2018
Organising entity: Microbiota Intestinal y Salud de la Sociedad Europea de Neurogastroenterología y Motilidad
Tomás Cerdó; Alicia Ruíz; Aucña Inmaculada; Ruy Jáuregui; Nico Jehmlich; Sven-Bastian Haange; Martin von Bergen; Antonio Suarez; Cristina Campoy.

Title of the work: Milk fat globule membrane and synbiotics modulate gut microbiota and reduce illness symptoms in infants
Name of the conference: Gut Microbiota for Health World Summit 2018
Type of participation: Póster
City of event: Roma, Italia
Date of event:10/03/2018
End date: 11/03/2018
Organising entity: Microbiota Intestinal y Salud de la Sociedad Europea de Neurogastroenterología y Motilidad
Tomás Cerdó; Alicia Ruíz; Ana Nieto-Ruíz; Miriam Arias; Estefanía Diéguez; Florian Hermann; María Teresa-Miranda; María Rodríguez-Palmero; Jesús Jiménez; Antonio Suarez; Cristina Campoy.

Title of the work: Effects of a new infant formula on the prevention of infections and stablishment of the gut microbiota in infants
Name of the conference: XI versión del Congreso Iberoamericano de Ingeniería de Alimentos (CIBIA2017): Alimentos procesados para la salud y el bienestar del siglo XXI: un pilar fundamental para el desarrollo de Enginomics
Type of participation: comunicación oral
City of event: Valparaíso, Chile
Date of event: 22/10/2017
End date: 25/10/2017
Organising entity: Instituto Chileno de Ingeniería para Alimentos A.G. (IChIA)
Tomás Cerdó; Alicia Ruiz; Ana Nieto-Ruiz; Miriam Arias; Estefanía Diéguez; Flo Hermann; María Teresa Miranda; M Rodríguez-Palmero; J Jiménez; Antonio Suárez; Cristina Campoy.

Title of the work: Health beneficial effects of infant formula supplementation with milk fat globule membranes
Name of the conference: XI versión del Congreso Iberoamericano de Ingeniería de Alimentos (CIBIA2017): Alimentos procesados para la salud y el bienestar del siglo XXI: un pilar fundamental para el desarrollo de Enginomics
Type of participation: Póster
City of event: Valparaíso, Chile
Date of event: 22/10/2017
End date: 25/10/2017
Organising entity: Instituto Chileno de Ingeniería para Alimentos A.G. (IChIA)
Tomás Cerdó; Alicia Ruiz; Ana Nieto-Ruiz; Antonio Suárez; Cristina Campoy.

Title of the work: Functionality and structure of neonatal gut microbiota community depend on pre-pregnancy maternal weight
Name of the conference: 10th World Congress: Life Course Health and Disease: Observations, experiments and interventions
Type of participation: Póster
City of event: Rötterdam
Date of event: 15/10/2017
End date: 18/10/2017
Organising entity: Developmental Origins of Health and Disease (DOHad)
Tomás Cerdó; Alicia Ruiz; Ruy Jáuregui; Antonio Suárez; Cristina Campoy.
Publication in: Journal of Developmental Origins of Health and Disease. 8, pp. 404. Cambridge University Press, 12/10/2017DOI:
https://doi.org/10.1017/S2040174417000848

Title of the work: Influences of maternal obesity on gut microbiome and brain structure and function
Name of the conference: 10th World Congress: Life Course Health and Disease: Observations, experiments and interventions
Type of participation: Póster
City of event: Rötterdam
Date of event: 15/10/2017
End date: 18/10/2017
Organising entity: Developmental Origins of Health and Disease (DOHad)
Tomás Cerdó; Alicia Ruiz; Ruy Jáuregui; Antonio Suárez; Cristina Campoy.
Publication in: Journal of Developmental Origins of Health and Disease. 8, pp. 404. Cambridge University Press, 12/10/2017DOI:
https://doi.org/10.1017/S2040174417000848

Title of the work: Body mass index determines gut microbial metabolism independently of diet-induced shifts in community structure
Name of the conference: 4th International Conference on Nutrition and Growth
Type of participation: Póster
City of event: Amsterdam
Date of event: 02/03/2017
End date: 04/03/2017
Organising entity: Nutrition and Growth
Alicia Ruíz; Tomás Cerdó; Ruy Jáuregui; Dietmar Pieper; Alfonso Clemente; Ascensión Marcos; Cristina Campoy; Manuel Ferrer; Antonio Suárez.

Title of the work: Pre-pregnancy maternal weight-dependent imprinting of neonatal gut microbial community and metabolism
Name of the conference: 4th International Conference on Nutrition and Growth
Type of participation: comunicación oral
City of event: Amsterdam
Date of event: 02/03/2017
End date: 04/03/2017
Organising entity: Nutrition and Growth
Tomás Cerdó; Alicia Ruíz; Ruy Jáuregui; Manuel Ferrer; Antonio Suárez; Cristina Campoy.

Title of the work: Evaluating the Relationship between Gut Microbiota and Neurodevelopmental Outcomes in Infants
Name of the conference: The power of programming 2016
Type of participation: Póster
City of event: Munich, Alemania
Date of event: 13/10/2016
End date: 15/10/2016
Organising entity: Early Nutrition Academy
Tomás Cerdó; Alicia Ruíz; Francisco José Torres Espínola; Luz García Valdés; Maite Segura Moreno; Ruy Jauregui; Manuel Pérez García; Antonio Suárez; Cristina Campoy

Title of the work: Gut microbiota composition and functioning in obese adolescents before and after one year of calorie restriction
Name of the conference: International Conference on Childhood Obesity & Child Development
Type of participation: comunicación oral
City of event: Atlanta, Estados Unidos de América
Date of event: 29/08/2016
End date: 30/08/2016
Organising entity: Conferenceseries
Cristina Campoy; Alicia Ruíz; Tomás Cerdó; Ascensión Marcos; Manuel Ferrer; Antonio Suarez.
Publication in: Journal of Obesity & Weight Loss Therapy. 6 - 6, pp. 59 - 59. 30/08/2016. Online: <http://dx.doi.org/10.4172/2165-7904.C1.034>. ISSN 2165-7904

Título del trabajo: Glycoside-hydrolase activity in the human gut microbiome
Name of the conference: ESPGHAN 49th ANNUAL MEETING
Type of participation: Póster
City of event: Atenas, Grecia
Date of event: 25/05/2016
End date: 28/05/2016
Organising entity: the European Society for Paediatric Gastroenterology, Hepatology and Nutrition (Espghan)
Alicia Ruíz; Tomás Cerdó; Ascensión Marcos; Manuel Ferrer; Cristina Campoy.

Title of the work: Functional dynamics of gut microbiome after dietary intervention in obese
Name of the conference: 12th European Nutrition Conference
Type of participation: Póster
City of event: Berlín, Alemania
Date of event: 20/10/2015
End date: 23/10/2015

Organising entity: Federation of European Nutrition Societies (FENS)
Antonio Suárez; Alicia Ruíz; Tomás Cerdó; Ruy Jauregui; Dietmar Pieper; Cristina Campoy; Manuel Ferrer.
Publication in : Annals of Nutrition and Metabolism. 67 - 1, pp. 288 - 289. KARGER, 20/10/2015. ISSN 0250-6807 DOI: 10.1159/000440895

Title of the work: Imprinting and functionality of the gut microbiota in infants born to obese and diabetic mothers and their relationship to the pattern of growth and neurodevelopment
Name of the conference: Summer School on nutrition
Type of participation: comunicación oral
City of event: Cambridge, East Anglia, Reino Unido
Date of event: 05/07/2015
End date: 10/07/2015
Organising entity: The European Society for Paediatric Gastroenterology Hepatology and Nutrition (ESPGHAN)
Tomás Cerdó; Alicia Ruíz; Ruy Jauregui; Antonio Suárez; Cristina Campoy.

Title of the work: Dietary intervention reshapes gut microbial functional community through body mass index determines its metabolic output
Name of the conference: The Human Microbiome
Type of participation: Póster
City of event: Heidelberg, Alemania
Date of event: 10/06/2015
End date: 12/06/2015
Organising entity: European Molecular Biology Laboratory (EMBL)
Tomás Cerdó; Ruy Jauregui; Dietmar Pieper; Alicia Ruíz; Alfonso Clemente; Manuel Ferrer; Cristina Campoy; Antonio Suárez.

Title of the work: It is not who they are but what they do
Name of the conference: The Gut Microbiota Throughout Life
Type of participation: Póster
City of event: Karlsruhe, Karlsruhe, Alemania
Date of event: 24/09/2014
End date: 26/09/2014
Organising entity: European Network for Gastrointestinal Health Research (ENGIHR)
Alicia Ruíz; Tomás Cerdó; Ester Hernández; Rafael Bargiela; María Suárez Díez; Anette Friedrichs; Ana Elena Pérez Cobas; María José Gosalbes; Henrik Knecht; Mónica Martínez Martínez; Jana Seifert; Martin Von Bergen; Alejeandro Artacho; Amparo Latorre; Stephan J.Ott; Andrés Moya; Vitor A.P Martins dos Santos; Natalia Chueca; Federico García; Manuel Ferrer; Cristina Campoy; Antonio Suárez.

Communications to National Congresses

Title of the work: La microbiota intestinal está asociada al rendimiento cognitivo infantil
Name of the conference: VII Jornadas del Instituto de Neurociencias Federico Olóriz
Type of participation: comunicación oral
City of event: Granada, Andalucía, España
Date of event: 15/03/2018
End date: 15/03/2018
Tomás Cerdó; Alicia Ruíz; Inmaculada Acuña; Francisco José Torres-Espínola; Hatim Azaryah; Antonio Suárez; Cristina Campoy

Title of the work: Imprinting and functionality of the gut microbiota in infants born to obese and diabetic mothers and their relationship to the pattern of growth and neurodevelopmental
Name of the conference: Encuentro para la excelencia de la investigación en salud pública
City of event: Maó, Illes Balears, España
Date of event: 20/09/2017
End date: 22/09/2017
Organising entity: CIBER EPIDEMIOLOGIA Y SALUD PUBLICA (CIBERESP)
Tomás Cerdó.

Title of the work: Pre-pregnancy mother´s body mass index associates with offspring cognitive outcomes and gut microbiota
Name of the conference: I Jornadas Científicas del Centro de Investigación Biomédica
City of event: Granada, Andalucía, España
Date of event: 21/06/2017
Organising entity: Centro de InvestigaciónBiomédia
Ciudad Organising entity: Granada
Tomás Cerdó; Alicia Ruíz; Francisco José Torres-Espínola; RuyJáuregui; Miguel Pérez-García; Antonio Suárez; Cristina Campoy.

Publication in: ISSN 2340-9894

Title of the work: Gut microbial glycosidases as translational biomarkers to assess host-microbe interactions in obesity
Name of the conference: VIII WorkShop SEPyP
Type of participation: comunicación oral
City of event: Santiago de Compostela, Galicia
Date of event: 23/02/2017
End date: 24/02/2017
Organising entity: Sociedad Española de Probióticos y Prebióticos
Alicia Ruíz; Tomás Cerdó; Ruy Jáuregui; Dietmar Pieper; Ascensión Marcos; Cristina Campoy; Manuel Ferrer; Antonio Suárez.
Publication in: ISBN 978-84-617-9657-1

Title of the work: Relación entre el IMC de la madre y la microbiota intestinal del hijo
Name of the conference: XVII Reunión de la Sociedad Española de Nutrición
Type of participation: comunicación oral
City of event: Santiago de Compostela, Galicia, España
Date of event: 02/11/2016
End date: 05/11/2016
Organising entity: SOCIEDAD ESPAÑOLA DE NUTRICION
Tomás Cerdó; Alicia Rúiz; Ruy Jáuregui; Antonio Suárez; Cristina Campoy.
Publication in: Nutrición Hospitalaria. 33-7, pp. 60. (España): 05/11/2016.
Online version: <http://dx.doi.org/10.20960/nh.678>. **ISSN 1699-5198**

Title of the work: Evolución de la microbiota intestinal del lactante durante los primeros 18 meses de vida
Name of the conference: I Jornadas de Jovenes Investigadores en Formación
Type of participation: comunicación oral
City of event: Granada, Andalucia
Date of event: 18/05/2016
End date: 20/05/2016
Organising entity: Escuela internacional de Postgrado UGR
Tomás Cerdó; Alicia Ruíz; Antonio Suárez; Cristina Campoy
Publication in conference proceedings: Actas de las I Jornadas de Investigadores en Formación. **ISBN** 978-84-16478-94-1. Depósito legal: GR 838-2

Title of the work: Microbiota intestinal y neurodesarrollo durante los primeros 18 meses de vida.
Name of the conference: VI Jornadas del Instituto de Neurociencias Federico Olóriz
Type of participation: Comunicación Oral

City of event: Granada, Andalucía

Date of event: 06/03/2016

End date: 06/03/2016

Organising entity: Instituto de Neurociencias Federico Olóriz

Tomás Cerdó; Alicia Ruíz; Ruy Jauregui; Francisco Torres Espínola; Luz García Valdés; Antonio Suárez; Cristina Campoy

Title of the work: It is not who they are but what they do

Name of the conference: Jornadas Doctorales Internacionales: "Avances en la investigación biomédica y biotecnológica"

Type of participation: comunicación oral

City of event: Jaén, Andalucía

Date of event: 04/12/2014

End date: 05/12/2014

Organising entity: Universidad de Jaén

Tomás Cerdó; Alicia Ruíz; Ester Hernández; Rafael Bargiela; María Suárez Díez; Anette Friedrichs; Ana Elena Pérez Cobas; María José Gosalbes; Henrik Knecht; Mónica Martínez Martínez; Jana Seifert; Martin Von Bergen; Alejandro Artacho; Amparo Latorre; Stephan J.Ott;Andrés Moya; Victor A.P Martins dos Santos; Natalia Chueca; Federico García; Manuel Ferrer; CristinaCampoy; Antonio Suárez

AWARD

XVII

Reunión de la Sociedad Española de Nutrición

XXII Congreso de la Sociedad de Nutrición y Dietética de Galicia

XXII Jornadas de Nutrición para enfermería

Reunión de Jóvenes Investigadores

VIII Curso de Nutrición y Coaching Nutricional

Impacto en Salud de los estilos de vida
Abordaje integral del niño al anciano

Santiago de Compostela

2016

3 - 5 Noviembre

Santiago de Compostela, 4 de noviembre de 2016

Por la presente otorgamos el premio a la

Mejor comunicación Jovenes investigadores:

Relation Between Mother´s BMI and Infant´s Gut Microbiota

realizada por:

Cerdó Ráez T, Ruí Rodríguez A, Jáuregui R, Suárez A, Campoy C

presentada durante la **XVII Reunión de la Sociedad Española de Nutrición** y del **XXII Congreso de la Sociedad de Nutrición y Dietética de Galicia**

y para que conste donde convenga firman la presente

Alberto Cepeda
Presidente del XVII Reunión de la SEÑ

María Rosaura Leis
Presidenta del XVII Reunión de la SEÑ