GUT MICROBIOTA CONTRIBUTES TO COGNITIVE PERFORMANCE IN INFANTS

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ABSTRACT

Gut microbiota has been related to infant neurodevelopment. Here, an association between infant composite cognition with gut microbiota composition was established as soon as 6 months. Higher diversity and evenness characterized microbial communities of infants with composite cognition above (Inf-aboveCC) versus below (Inf-belowCC) median values. Metaproteomic and metabolomic analyses established an association between microbial histidine ammonia lyase activity and infant histidine metabolome with cognition. Fecal transplantation from Inf-aboveCC versus Inf-belowCC donors into germ-free mice showed that memory, assessed by novel object recognition test, was a transmissible trait. Furthermore, Inf-aboveCC mice were enriched in species previously linked to cognition

belonging to *Bacteroides*, *Phaeicola* and *Bifidobacterium*. Finally, Inf-aboveCC mice showed differential faecal histidine, hippocampal urocanate and histidine-urocanate-glutamate ratios compared to Inf-aboveCC mice. Overall, these findings reveal a causative role of gut microbiota on infant cognition pointing at modulation of histidine metabolite levels as a potential underlying mechanism.

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INTRODUCTION

The first year of life is the critical period when the complex microbial ecosystem is *de novo* assembled (1). The colonization by the gut microbiota simultaneously occurs with the dynamic phase of postnatal brain development, including glia differentiation, axon myelination and synaptogenesis, and the rapid emergence of neurodevelopmental functions (2). The commensal microbiota communicates with the brain through immunological, endocrinological, neuroactive and metabolic pathways, leading to effects on mood and behaviour, suggesting the existence of a microbiota-gut brain axis (3). The role of this axis in early life neurodevelopment has been shown in rodent models where dysbiosis of the gut microbiota or its absence in germ-free (GF) mice changes neurotransmitters and brain volumes that impact central nervous system function and cognitive and social behaviours (4-6). Neurological alterations and altered behaviour of GF mice were partially rescued when mice were colonised with commensal gut microbiota at 3 but not at 10 weeks postweaning, suggesting that early life programming of brain circuits are needed for later-life behaviours (7) (8). Early in life, humans may experience changes in cognitive, behavioral, motor, and emotional outcomes that have been associated with disruptions in the gut microbial community (9) (10). Cognitive impairment in autism, important for the paediatric population, was associated to an altered gut microbial composition (11). Transfer of gut microbiota from subjects with autism increased repetitive behaviour, and decreased locomotion and social communication in GF mice (12). Furthermore, changes in the gut microbiota result in altered metabolomic profiles, impacting the availability and diversity of nutrients and metabolites that may interact with the central nervous system (12) (13) (14).

A relationship between gut microbiota and neurodevelopment exists but has not been empirically demonstrated. In this study, we tested a forward approach to compare gut

microbiota profiles of full-term healthy 6 months-old infants, performed metaproteomic and metabolomic analyses, determined causality by transplantation of infant gut microbiota to GF mice, and narrowed down to a few species that may modulate neurodevelopment. With this strategy, herein, we report gut microbial community and functional associations to infant cognitive performance that impacted memory functions when transplanted to GF mice. Determining the dynamics of the behavior-gut microbiota associations is important because it may provide novel microbiota-based therapies for infant neurodevelopmental disorders (15).

MATERIALS AND METHODS

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 (16). 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 months of age. All infants of this study were examined by the same trained psychologist. 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 (17). Infants were dichotomized into two groups, above and below the median (50^{th} percentile), according to their scores in each BSID-III neurodevelopmental scales.

DNA extraction from stool samples

Genomic DNA was extracted from fecal bacteria of 6-month (n = 69) old infants as previously described (18). Briefly, fecal 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 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 universal primers as previously described (19). 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 240 nt reads. Data set was filtered and operational taxonomic units (OTUs) were defined at 99% similarity with MOTHUR programs unique.seqs and pre.cluster (20). Taxonomic classifications of species were assigned using the naïve Bayesian algorithm CLASSIFIER of Ribosomal Database Project (RDP) (21). Species were considered unassigned when confidence value score was lower than 0.8, and were annotated using upper taxonomic ranks. EzBioCloud Identify (22) and RDP Sequence match tools (23) were used to identify the closest cultivable strains to interesting species.

Protein extraction, separation, identification and data processing

Protein extraction was performed from faecal bacteria as previously described (24). Briefly, faecal bacteria were disrupted by mechanical lysis in BugBuster Protein Extraction Reagent (Novagen) and separated on a 12% acrylamide separating gel. After electrophoresis, peptide lysates were generated from protein bands by trypsin digestion, and analysed by nano-HPLC system Advion NanoMate and Orbitrap Fusion mass spectrometer (Thermo Fisher Scientific). 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 was used to obtain only rank 1 peptides with a threshold of FDR <1%. Mass spectrometry proteomics data were deposited to the ProteomeXchange Consortium via the PRIDE (25) partner repository with the dataset identifier PXD009056. Protein abundances were calculated based on normalized spectral

abundances that allow relative comparison of protein abundances over different samples (24, 26, 27). "PROteomics results Pruning & Homology group ANotation Engine" (PROPHANE) (28) was used to assign proteins to their taxonomic and functional groups using the functional annotation of Clusters of Orthologous Genes (COGs) (29). Metaboanalyst version 5.0 was used to analyse and visualize metaproteomic data (30). To compare experimental groups, the corrected metaproteome matrix was used for sparse Partial least squares discriminant analysis (sPLS-DA), a supervised model to reveal variation between experimental groups and determine features that enable discrimination with the lowest possible error rate.

Measurement of histidine metabolome and histidine ammonia-lyase activity

Determination of histidine metabolome and histidine ammonia-lyase activity was performed as described by Acuña et al (31). Briefly, faecal, microbial, plasma, urine and hippocampal matrices were extracted, mixed with of UHPLC-MS-grade acetonitrile, filtered, separated by chromatography with a Waters Acquity UPLC™ System I-Class (Waters, UK), and analysed with a Waters triple quadrupole mass spectrometer (XevoTQ-XS, Waters, UK). Histidine ammonia-lyase activity was determined at 37ºC in protein extracts using 1 mg mL^{-1} histidine as substrate. For urine samples, creatinine levels were simultaneously analysed to adjust for quantitation of urine analyte.

Transplantation of microbiota

Faecal microbiota transplantation (FMT) of sixteen stool samples was performed under anaerobic conditions in an anoxic glove chamber. Approximately 0.3 g of stool was resuspended in 5 mL of anaerobic PBS that contained 2 mM DTT as a reducing agent. Each stool sample was vortexed for 1 min and centrifuged at 500xg for 1.5 min. The suspension was removed from the anaerobic chamber, and then immediately used. 200 μL of each fecal

suspension were inoculated via oral gavage into two randomly selected to 9 weeks-old C57BL/6 male mice. Mice from the same infant donor transplantation were housed together in ISOCAGE N isolator cages, kept under a 12 hours light/dark cycle, and fed autoclaved water and 7017 NIH-31 mouse diet produced by Harlan Teklad (Madison, WI) ad libitum. Freshly mouse stool and urine samples were collected and immediately placed at -80ºC. After behavioural tests, blood was collected from mouse tail. Mice underwent cervical dislocation, the brain was removed, and the hippocampus was sliced and stored at -80ºC.

Novel object recognition

Memory functions were assessed with the novel object recognition test (NORT) two weeks after transplantation. NORT is a widely-used relatively low-stress and efficient method to test neuropsychological changes on learning and memory in mice upon treatment (32). NORT is based on a natural curiosity towards novelty, i.e. an inclination to explore novel objects, in mice with no need for any positive or negative reinforcement that could confound the data. NORT was completed over 2 days, which included a habituation day and a training / testing day. The habituation consisted of an open field test (OFT) where mice were placed individually in the centre of an open field box of 50 x 50 x 30 cm (length x width x height). Mice behaviour was recorded for 5 min with a video-computerized tracking system. Time spent in the central and peripheral area of the field, the number of central area entries, distance travelled and speed were calculated. Twenty-four hours after habituation, mice were placed in the centre of the same arena, equidistant from the two identical objects located in the central symmetrical positions of the arena, and given 5 minutes to explore the objects. Two hours later, mice were exposed to the familiar object together with a new object for 5 min. After each test session, the objects were cleaned with 70% ethanol. All mice explored for more than 5 s. Discrimination index was calculated as d_1/e_2 where d_1 is the time

of exploration of new object minus familiar object, and e_2 is the total exploration time. *t*-tests were used to compare the discrimination value of a fictive group (with random exploration, no discrimination ability and standard deviation observed in our experimental groups) with each mice group independently, and the discrimination measure between experimental mice groups (33). Full effect of the condition was considered when significant differences were observed in both comparisons for an experimental mice group.

Statistical analysis

Statistical analyses were carried out using SPSS version v22.0 (IBM, IL) and R with multiple analytical packages. For all determinations, the significance cut-off was set at $p \leq 0.05$ or False Discovery Rate (FDR) ≤ 0.05 when multiple test correction was applied. No power calculations were conducted to pre-determine appropriate mouse sample sizes. All date were tested for normal distribution and equality of variances before statistical testing. The Benjamini–Hochberg procedure was used for multiple testing corrections. The design of the mice experiment and the number of animal replicates was performed according to recommendations for causality experiments with gut microbiota (34) (35). The effect size and significance of study variables on gut microbiota composition was determined using the *envfit* function in *vegan* package. Ordination plots of β-diversity for compositional data were calculated with Unifrac (Gunifrac) and Aitchison distances (36) (this distance has scale invariance, perturbation invariance, permutation invariance and sub-compositional dominance), and Bray–Curtis dissimilarity metrics (Phyloseq), followed by a PERMANOVA test (Vegan) to detect differences between the experimental groups. Alpha-diversity was analysed at the species level. In the human study, significant differential phylotype abundance at several different taxonomy levels was constructed from non-normalized raw count tables using a two-sided Wald's test adjusted for covariates and with

multiple comparisons correction using DESeq2 package. Associations between taxa and CC after partialling out the effect of microbiota covariates were assessed by fitting a generalized linear model (GLM) with the glm R function. In the mouse study, the random forest regression model (randomForest) was carried out to identify the key discriminatory features which assigns an importance score to each determination by estimating the increase in error caused by removing that determination from the set of predictors. Training (80% of samples) and test (20% of samples) datasets were randomly created to build a predictive model of discriminating features. The model was further refined using Boruta package that performs a top-down search for relevant features by comparing original attributes' importance with importance achievable at random, estimated using their permuted copies, and progressively eliminating irrelevant features to stabilise that test. All the features confirmed as important were treated as relevant and used to examine the predictive power of the model. Correlations using metabolome and taxa data were determined with Spearman's rank correlations, followed by the *corr.test* (Psych) for multiple comparison corrections.

RESULTS

Study population characteristics and BSID-III scores

Our analysis focused on BSID-III scales of infant development collected from full-term healthy infants of 6-months of age (n = 225). Infants met the threshold for typical healthy neurodevelopment according to their scores in three individual BSID-III domains: composite cognition, language (receptive language, expressive language and composite language) and motor (gross motor and fine motor). The medians [ranges] for BSID-III scores were as follows: composite cognition (125[85-125]), receptive language (12[5-15]), expressive language (10[5-15]), composite language (109[83-129]), gross motor (9[2-17]) and fine motor (12[5-17]) (Supplementary Table S1). Infants were dichotomized into two groups, above and below the median (50th percentile) for each BSID-III scale.

Gut microbial community diversity, structure and composition differ between infant cognition groups

We sought to determine whether the gut microbiota associated with language, motor and cognition scores as early as 6-months of age. We collected faecal samples from healthy infants at month 6 to characterize the gut microbial composition by high-throughput amplicon sequencing of 16S rRNA gene. After quality filtering, 4,435,206 read sequences (Good's coverage > 98.5% per sample) rendered a gut microbial profile consisting of 645 species-level species that narrowed to 102 distinct genera belonging to 46 families, after high confidence phylogenetic annotation (Supplementary Figure S1 and Table S2). In agreement with previous reports (37, 38), taxonomic classification against RDP database showed a community membership dominated by taxa within *Firmicutes* (479), followed by *Bacteroidetes* (101), *Proteobacteria* (30), *Actinobacteria* (23), an unassigned phylum (8) and *Fusobacteria* (4). The most abundant genera were an unassigned genus within

Lachnospiraceae (*unclass_Lachnospiraceae)*, an unassigned genus within *Enterobacteriaceae* (*unclass_Enterobacteriaceae*), *Bacteroides*, *Lachnospiraceae incertae sedis*, and *Enterococcus*, accounting for 61.4% of total reads. Phylotype occurrence showed high inter-sample variation in infants' gut microbiota. Only one species, assigned to *unclass_Enterobacteriaceae*, was present in all samples and accounted for 4.84% of total reads. Twenty-five species were highly abundant (>1% of all sequence reads) but only six of them were highly prevalent (>90% of samples). These frequent and highly abundant species belonged to *Firmicutes*, *Bacteroidetes* and *Proteobacteria*.

We first tested whether measures of gut microbial community diversity and structure differed between infants categorized as above or below median scorers in each of the seven individual BSID-III scales (Supplementary Table S3). Considering all species, the unique BSID-III scale with a strong association with gut microbial community structure was composite cognition (CC). Microbial α-diversity (intra-sample diversity) metrics showed that higher evenness, Shannon's and Simpson's diversities and reduced dominance characterized the gut microbiota of infants with above median CC scores (Inf-aboveCC) compared to infants with below median CC scores (Inf-belowCC) (Figure 1A). No differences in the number of taxa (richness) and Faith's phylogenetic diversity were observed between CC groups. At species level, redundancy analysis using Aitchison distance, that corrects for compositionality, to assess β-diversity showed significant differences in microbial community structure between infant CC groups (Figure 1B). At genus level, differences between microbial profiles of infant CC groups were significant using Bray-Curtis distance that considers dissimilarity in species abundance (Supplementary Figure 2A). Finally, we determined the proportion of inter-individual variation in overall

microbiota composition that could be explained by anthropometric, perinatal, maternal and nutritional variables using distance-based PERMANOVA test. At species level, no significant association with neonate weight, gender, maternal age, maternal pregestational BMI, maternal IQ, gestational diabetes (Yes/No), type of delivery (C-section, vaginal), drinking alcohol during pregnancy (Yes/No), smoking during pregnancy (Yes/No), days of breastfeeding up to the third or sixth month, or type of breastmilk feeding up to the third or sixth month (formula, mixed or exclusive breastfeeding) was observed. At genera level, a significant explaining effect of gut microbiota variation was observed with maternal age and gestational diabetes accounting for 8.53% and 9.41% of total variance, respectively (Supplementary Figures 2A-B). The significant effect of these variables on microbiota variance was considered in downstream statistical analyses when required.

Signature taxa of composite cognition performance

Since gut microbial community diversity and structure was different between infant CC groups, we used DESeq2 to identify taxa with differential abundances between Inf-aboveCC and Inf-belowCC groups. The gut microbiota of Inf-aboveCC infants was enriched in *Bacteroidetes* and depleted in *Firmicutes* (Figure 1C). As a result, the *Firmicutes*:*Bacteroidetes* ratio was significantly lower in Inf-aboveCC compared to Inf-belowCC infants (Figure 1C). The relative abundances at genus level the were different between infant groups (Figure 1D). The gut microbiota of Inf-aboveCC was characterized by an enrichment in *Bacteroides, Streptococcus, Parabacteroides, Clostridium sensu* stricto, *Holdemanella* and *Ruminococcus2*, and by a significant decrease in *Lachnospiracea incertae sedis*, *Haemophilus* and *Lactococcus* compared to Inf-belowCC infants (Figure 1E and Supplementary Figure S2C). Fitting GLM models to partial out maternal covariate contributions confirmed significant associations between the relative abundances of

Bacteroides and *Lachnospiracea incertae sedis* with infant cognition (Figure 1F). At species level, twelve species were differentially abundant between infant CC groups. Ten species were enriched in Inf-aboveCC infants that assigned to *Bacteroidia* and *Clostridia* classes, with single representations from *Erysipelotrichia* and *Negativicutes* classes. One representative of *Bacilli* class and one of *Clostridia* class were enriched in the gut microbiota of Inf-belowCC infants (Figure 1G and Supplementary Figure S2D). Assignment of rDNA sequences in taxonomy databases (Supplementary Table S4) showed that these species shared sequence identity (similarity higher than 96%) with culturable strains of *Phocaeicola dorei*, *Phocaeicola vulgatus*, *Bacteroides caccae*, *Bacteroides stercoris*, *Parabacteroides bouchesdurhonensis*, *Streptococcus lutetiensis*, *Streptococcus gallolyticus*, *Holdemanella porci*, *Ruminococcus bromii* and *Veillonella ratti* for Inf-aboveCC microbiota, and to strains of *Lactococcus lactis* and *Sellimonas intestinalis* for Inf-belowCC microbiota. Except for *Lactococcus*, members of these strains have been linked to memory and cognitive performances in humans and animal models (39) (40) (41) (42) (43) (44).

Phylogenetic assignment of metaproteomes

The above results suggested differences in the composition of gut microbiota of infant CC groups. To better understand the relationship between microbiota and health outcomes in humans, functional-based studies are required to identify molecular mechanisms (45). For this purpose, we performed a metaproteomic analysis on infant´s gut microbiota. Metaproteomics offers large-scale functional profiling of expressed proteins within microbial ecosystems and, thus, a feasible approximation to associate biological activities with bacterial taxa (46). We first investigated the microbial community taxa that had potentially produced the identified proteins in infant metaproteomes. Peptides were assigned to 6,063 bacterial protein groups, and unambiguously matched (>99%) to fully

sequenced genomes from closely related genera to sample strains and other documented gut genera (Supplementary Table S2). These protein groups were assigned to 111 genera belonging to 50 families (Supplementary Table S2). Community compositional contributions to gut metaproteomes did not show a strong association with infant CC groups (Supplementary Figure S3A), suggesting high inter-individual similarity in the phylogenetic origin of bacterial protein groups. Zooming on the phylogenetic assignment of metaproteomes, the contribution of *Eggerthella* within *Actinobacteria* to gut metaproteomes was negatively associated with CC while that of un unassigned genus within *Erysipelotrichaceae* (*unclass_Erysipelotrichaceae*) in *Firmicutes* was positively associated with CC (Supplementary Figure S3B).

Metaproteomic enrichment analysis reveals cognition-specific functional signatures in infant microbiota

In comparative proteomics, the global analysis of function based on peptide annotation of proteins provides insights into community-wide metabolic relationships and molecular mechanisms behind health and disease states (24). 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, 6,063 bacterial protein groups narrowed to 656 non-redundant COG functions (Supplementary Table S2). The mean number of protein groups and COG functions per sample was 176[73,279]. Overall distribution of main and secondary COG tiers across samples exhibited a rather similar pattern where the most abundant secondary COGs belonged to Metabolism main category (Supplementary Figure S4A). Twenty-four COG functions were found across 90% infant metaproteomes (core), comprising twelve

transporters and membrane proteins and twelve enzymes involved in carbohydrate, amino acid and energy metabolisms (Supplementary Table S2). Five of these core enzymes were also found to be highly prevalent in adult metaproteomes: phosphoketolase, glutamate dehydrogenase, enolase, glycealdehyde-3-phosphate dehydrogenase and fucose isomerase. These results are consistent with previous reports on the functional profile of protein groups expressed by gut microbiota (24) (38) (47).

We next determined whether the functional capacity of the gut microbiota differed between infant CC groups. We searched for over- and under-represented COG functions between infant CC metaproteomes. A high proportion of 19.8% and 25.1% COG functions occurred only in Inf-aboveCC and Inf-belowCC metaproteomes, respectively (Supplementary Figure S4B). The gut microbiota of Inf-belowCC infants was enriched in proteins involved in Intracellular Trafficking Secretion and Vesicular Transport ($p<0.027$) while there was a trend for proteins involved in Carbohydrate Transport and Metabolism to be higher in Inf-aboveCC metaproteome ($p<0.065$) (Supplementary Figure S4C). We performed an sPLS-DA model to explore which protein groups could discriminate infant metaproteomes, and determined variable importance score of discriminant features. sPLS-DA plot showed a clear discrimination between infant metaproteomes (Figure 2A) and identified protein groups contributing to discrimination (Figure 2B). Significant differential abundances were observed for *arylsulfatase A or related enzyme AlkP superfamily* (AslA) involved in sulphur modification of organic metabolites, *aspartate carbamoyltransferase catalytic subunit* (PyrB) involved in the first steps of pyrimidine biosynthesis, *guanylate kinase* (Gmk) involved in the recycling of GMP, and *histidine ammonia lyase* (HutH), the gate enzyme that catalyses the non-oxidative deamination of histidine. HutH, also known as histidase, was the only protein to be significantly enriched in Inf-aboveCC metaproteome.

These observations were further tested using DESEq2 analysis (Figure 2C) and Wilcoxon rank sum test with multiple test correction (Supplementary Figure S5), confirming significant differential abundances of these proteins between infant metaproteomes. When enzymes involved in pathways related to these proteins were investigated in metaproteomes, we observed higher abundances of two downstream enzymes of histidine catabolism, urocanate dehydratase (HutU) and imidazolone propionase (HutI), in Inf-aboveCC metaproteome, though differences between infant groups were not statistically significant. Our findings uncovered a cognition-associated metaproteomic signature in infants.

Higher gut microbial histidine ammonia lyase activity in Inf-aboveCC microbiota

The fact that HutH, HutI and HutU proteins are involved in histidine metabolism stood out because histidine is the precursor of the neurotransmitter histamine, both reported to modulate human cognition (48) (49) (50) (51). When assessment of protein (enzyme) function in complex mixtures is feasible, determination of enzymatic activity escalates from prediction to actual biochemical pathways, validating proteomic data and mapping reactions into operating metabolic networks (52). For this purpose, we developed a test to assess the transformation of histidine into urocanate by HutH on protein extracts from faecal bacteria (31). Our results confirmed that HutH activity was 1.3-fold higher in the gut microbiota of Inf-aboveCC compared to Inf-belowCC group though differences were not statistically significant (Figure 2D).

Histidine metabolome associate with composite cognition in infants

The metaproteomic and biochemical results on HutH prompted us to investigate the extent to which CC was associated to an altered histidine metabolome in infants. We quantified the levels of histidine, histamine, urocanate, imidazole acetate, imidazole propionate, N-acetylhistamine and glutamate extracted in gut microbes and faecal content of

infants by targeted metabolomics (31). We first tested the correlation between intracellular and fecal levels of histidine metabolites because it would provide an insightful view of intracellular microbial physiological states within stool context. Histamine and N-acetylhistamine were very poorly detected in intracellular content. Intracellular levels of histidine, imidazole propionate, glutamate and urocanate significantly correlated with fecal content levels (Supplementary Figure S6A). The levels of urocanate in faeces were significantly lower in Inf-aboveCC compared to Inf-belowCC infants (Figure 3A) whereas no differences were observed for the rest of histidine metabolites (Supplementary Figure S6B). We also calculated the ratios between two single metabolites because alterations may point at perturbations in pathways relevant for a biological system or a neurodevelopmental disorder (53) (54) (55) (56). The fecal urocanate/histidine ratio (Figure 3B) and urine the histamine/urocanate ratio (Figure 3C) were significantly lower in Inf-aboveCC infants compared to Inf-belowCC infants. Furthermore, considering the CC discriminating taxa, we observed that fecal urocanate positively correlated with unclass_Lachnospiraceae_41 while fecal histidine correlated positively with Ruminococcus_53 and negatively with *Lachnospiracea incertae sedis* genus. The urocanate/histidine ratio positively correlated with unclass_Lachnospiraceae_41 and *Lachnospiracea incertae sedis* genus, and positively with Bacteroides_3 (Figure 3D). In urine, the levels of histidine positively correlated with Streptococcus_42 and *Bacteroides* genus while the levels of urocanate negatively correlated with Holdemanella_16 and positively with *Streptococcus* genus. Finally, the urine histamine/urocanate ratio negatively correlated with *Streptococcus* genus (Figure 3E).

Fecal transplants of Inf-aboveCC donors into germ-free mice promote better memory functions than those from Inf-belowCC donors

While studies on microbiota associations with Human Health are powerful, causative

experiments are required to further stablish the involvement of gut microbiota in infant neurodevelopment (34). To investigate whether gut microbiota contribute to cognition, a FMT experiment was performed to (i) determine the transmissibility of memory functions, (ii) comparatively analyse the gut microbial profile and metabolism of mice, (iii) narrow down to the species that associate with memory functions, and (iv) link memory responses to alterations in histidine metabolites. Sixteen fecal samples derived from Inf-aboveCC (n = 8) and Inf-belowCC (n = 8) donors were transplanted into GF mice (n = 32). Each donor fecal sample was transplanted into two independent "humanized" mice, subsequently bred in the same ISO-cage. Donor faecal samples were rationally selected by ranking each infant donor based on infant CC records and features discriminating CC (α-diversity values, signature taxa and proteomic abundances, and histidine metabolite levels).

To test whether colonization of GF mice with infant gut microbiota resulted in behavioural differences, two well-established behavioural tests, OFT and NORT tests, were performed on week 2 post FMT. Habituation to explore the arena in OFT can assess exploratory behaviours and locomotor abilities because mice will typically spend a significantly greater amount of time exploring the periphery of the arena, usually in contact with the walls (thigmotaxis), than the unprotected centre area. After OFT, NORT assessed memory learning by comparing visual exploration of a novel object in the test session that replaced one of the two identical objects explored in the training session. Concerning OFT, mice recipient of Inf-belowCC microbiota displayed increased duration of immobility in centre compared to those with Inf-aboveCC microbiota (Figure 4A). These differences were not the result of locomotion deficits since mice spent the same time in centre and periphery of the arena, travelled similar distances with no differences in mean speed (Supplementary

Table S5). The NORT test showed that mice recipient of Inf-aboveCC microbiota had significantly better discrimination index than mice recipient of Inf-belowCC microbiota (Figure 4B). No association between discrimination index in mice with anthropometric, perinatal, maternal and nutritional variables of infant donors was observed. In this humanized behavioural model, our results demonstrate that memory functions were transmissible via infant gut microbiota suggesting a causal relationship.

Mice colonized with infant gut microbiota harbor different bacterial taxa that associate with memory functions

To assess the validity of microbiota transplantation and its association with CC, we collected fresh fecal samples from each individual mouse of FMT experiment. Donor infant and mice samples rendered 1,071,999 read sequences (Good's coverage > 99.5% per sample) containing 398 species-level bacterial OTUs that narrowed to 196 distinct genera belonging to 93 families after high confidence phylogenetic annotation (Supplementary Table S2). As expected, a shift in α- and β-diversity was observed between donor and recipient microbiota. We observed a loss of bacterial species when transferred to mice, possibly consequence of sample processing and/or species/host incompatibility (Supplementary Figure S7A). On average, sixty nine percent of bacterial species in donor microbiota were present in their corresponding pair of humanized mice. Taxa within *Bacteroidetes* and *Verrumicrobia* thrived whereas others within *Actinobacteria* and *Proteobacteria* failed to grow in mice gut. When binned by donor CC scores, the microbiota of Inf-aboveCC mice had higher evenness, Shannon and Simpson diversity indexes values compared to those harbouring Inf-belowCC microbiota though differences did not reach statistical significance as was observed in donor infant gut microbiota (Supplementary Figure S7B). β-diversity analysis using Aitchison

distance showed that donor infant microbiota and recipient mice samples had different community structures (Supplementary Figure S7C) that significantly clustered according to cognitive performances (Figure 5A). Structural differences in microbial community structure between mice groups were also accounted with Bray-Curtis distance at genus level (Supplementary Figure S7D). Phylum-level composition of Inf-aboveCC mice was significantly enriched in *Actinobacteria* and depleted in *Firmicutes* compared to Inf-belowCC mice (Figure 5B). We used a robust machine learning non-parametric classification and regression tool (random forest classifier algorithm, RF) that builds prediction rules from study variables without making any prior assumption on the form of their association with the response variable. We searched for genera that distinguished mice groups. Using RF, a model was trained with 80% of the samples produced an accuracy of 76.2% with a Matthews correlation coefficient (MCC) of 83% and an area under the curve (AUC) of 84.6% when tested on the remaining 20% of samples (Supplementary Figure S8A). We then used a feature selection process to identify the relevant genera. Nine genera belonging to phyla *Actinobacteria*, *Bacteroides* and *Firmicutes* increased predictive accuracy to 96.9% and AUC to 100%. The genera discriminating memory functions were *unclass_Erysipelotrichaceae*, *Parabacteroides*, *unclass_Lachnospiraceae*, *Anaerotipes*, *Lacrimispora*, *Bifidobacterium*, *Hungatella*, *Eubacterium* and *Lachnospiracea incertae sedis*, in order of their contribution to model accuracy (Figure 5C). Differential abundance analysis using DESEq2 confirmed significant differences in *unclass_Erysipelotrichaceae*, *Parabacteroides*, *Bifidobacterium*, *Anaerotipes*, *Hungatella*, *Lacrimispora*, and *Lachnospiracea incertae sedis* between inf-aboveCC and Inf-belowCC mice (Figure 5D). When RF was used to discriminate mice groups with species data, the prediction performed on the 20% of the samples reserved as a testing dataset produced an accuracy of 88.5% with

MCC of 93.3% and an AUC of 91.5% (Supplementary Figure S8B). The top-down cross-validation test reduced the model to twelve relevant discriminating species with a predictive accuracy of 96.1%, a MCC of 100% and an AUC of 99.4%. Discriminating microbes mostly belonged to *Bacteroidia* and *Clostridia* classes with two representatives of *Actinobacteria* class and a single representative of *Erysipelotrichia* class (Figure 5E). These observations were further corroborated by DESEq2 analysis (Figure 5F). Except for Lachnospiracea incertae sedis_7 and Anaerostipes_42, discriminant microbes were enriched in Inf-aboveCC mice compared to Inf-belowCC mice. The closest described culturable relatives (similarity higher than 96%) are strains of *Clostridium innocuum*, *Ruminococcus gnavus*, *Lacrimispora xylanolytica* and *Anaerostipes caccae* in *Firmicutes*, *Phocaeicola dorei*, *Phocaeicola vulgatus*, *Bacteroides xylanisolvens*, *Bacteroides luhongzhouii*, *Bacteroides faecichinchillae* and *Parabacteroides distasonis* in *Bacteroidetes*, and *Bifidobacterium pseudocatenulatum* and *Bifidobacterium longum* in *Actinobacteria* (Supplementary Table S5). Together, GF mice recipient of infant gut microbiota maintained differences between Inf-aboveCC and Inf-belowCC microbial profiles that discriminated memory functions in mice.

The microbiota modulates histidine metabolite levels

Based on the association of histidine metabolome with CC outcomes in infants, we hypothesized that microbial metabolism may modulate histidine metabolites. We utilized targeted metabolomic profiling of histidine metabolites in fecal, plasma, urine and hippocampal samples of humanized mice from FMT experiment. We detected all metabolites in mice matrices. Except for N-acetylhistamine, significant correlations were observed between intracellular and fecal levels of histidine metabolites in mice (Supplementary Figure S9A). We next used RF to assess whether histidine metabolome was predictor of memory functions in mice. Metabolomic profiles of histidine metabolites in

mice discriminated memory functions with a predictive accuracy of 52%, a MCC of 50% and an AUC of 62.3% (Supplementary Figure S9B). After performing a feature selection process, two metabolites, histidine levels in fecal content and urocanate levels in hippocampus, remained relevant and contributed highly to discrimination of mice groups, increasing the predictive accuracy of the model to 68%, a MCC of 66.7% and an AUC of 69% (Figure 6A). Fecal histidine level was significantly higher (Figure 6B) while hippocampal urocanate level was not different between Inf-aboveCC compared to in Inf-belowCC mice. In the case of histidine metabolome ratios, the predictive accuracy was 50.0%, a MCC of 50% and an AUC of 51.8% (Supplementary Figure S9C). The feature selection process identified two ratios as relevant that increased model accuracy to AUC of 81.1%, namely, hippocampal urocanate:glutamate and urocanate:histidine ratios that were significantly lower in Inf-aboveCC compared to in Inf-belowCC mice (Figure 6C). We finally tested whether the levels of histidine metabolites discriminating memory performances correlated with bacterial taxa. Spearman's correlation showed that fecal histidine positively correlated with Phocaeicola_13, unclass_Erysipelotrichaceae_22, unclass_Bacteroidaceae_150, and *unclass_Erysipelotrichaceae* genus, and negatively with Anaerostipes_42 and *Anaerotipes* genus (Figure 6D). Negative correlations were observed between hippocampal urocanate and urocanate ratios with species assigned to *Bacteroides*. These results revealed that the gut microbiota may modulate histidine metabolites in mice.

DISCUSSION

In this study we demonstrate that variation in the composition of the gut microbiota is significantly associated with infant cognitive performance as soon as 6-months of age. Moreover, comparisons of gut metaproteomes identified a cognition-associated signature including histidine ammonia-lyase, the gate-keeper enzyme of histidine catabolism. Differences in this enzymatic activity were biochemically confirmed that associated with distinct fecal and urinary histidine metabolomes between infant cognition groups. Most importantly, memory functions via fecal transplant of gut microbiota from infant donors were transmissible to GF mice, which, to the best of our knowledge, has not been reported before. Mice with microbiota of infants with better cognitive performances exhibited higher memory functions, an enrichment in taxa including *Bacteroides* and *Bifidobacterium* species and different histidine metabolomic profile compared to those with microbiota of infants with poorer cognitive performances. These results suggest that the gut microbiota may influence in infant cognition, possibly through the modulation of histidine metabolism.

There have been several reports on the association between gut microbiota and cognition in infants. Carlson et al (57) and Tamana et al (58) reported that one and two years-old infants clustered in a *Bacteroides*-dominant enterotype showed better learning composite scores than those clustered in enterotypes dominated by *Faecalibacterium* or by *Ruminococcaceae*. In contrast, Rothenberg et al (59) and Sordillo et al (60) showed positive association with the abundances of *Faecalibacterium*, *Sutterella*, and *Clostridium* cluster XIVa genera with infant cognition and personal and social skills while *Lachnospiraceae* abundances were inversely associated. In these studies, alpha-diversity measures were either inversely correlated or were not associated with cognition. The characteristic gut microbiota associated to infant cognition identified here partially replicates these previous observations.

While our data showed a more diverse and even gut microbial ecosystem associated to cognition, the gut microbiota of infants with better cognition was enriched in *Bacteroides* and depleted in *Lachnospiracea incertae sedis* and *Firmicutes*:*Bacteroidetes* ratio. The disparity between studies may rely on differences in ethnicity, infant's age, microbiota sampling to infant neurodevelopmental assessment, psychological test, sample sizes as well as on statistical approaches to identify discriminating taxa. Still, our findings and those previously reported consistently demonstrate an association between infant cognitive performance and gut microbiota during the critical period of early growth. The association between gut microbial profiles and cognition was unrelated to infant or maternal covariates.

Causative proof of the influence of commensal gut microbiota on neurological development has been established with gnotobiotic animal models (6). Mice without microbiota or with antibiotic-induced dysbiosis showed multiple signals of neurological and behavioural abnormalities (61). Colonization of gnotobiotic mice with gut microbiota rescues most neurological alterations that sometimes depends on a specific temporal window (4) (62) (63). The perinatal period is a critical developmental window characterized by the co-evolution of gut microbial colonization alongside with neuronal organization. To scale up from association to causation, we transferred gut microbiota from 6-months old full-term healthy infants to GF mice. In studies using FMT during early development, there is often a mismatch between age of the GF animal and donor contributing the sample, altering microbial developmental trajectories (64). Our FMT experiment matched the age of GF mice and donor samples. Mice receiving the microbiota from infants with better cognitive responses led to higher memory outcomes, exhibiting that the gut microbiota can serve as a memory-promoting entity. Additionally, these mice showed reduced immobility in arena centre during habituation task, that can be interpreted as a sign of better exploratory

behaviour (44). We expected that discriminant taxa of cognition in infants would be poorly captured in mice microbial communities due to habitat filtering, as in other FMT experiments (35). Despite the loss of many species, memory functions were transmissible from infants to mice, further supporting the hypothesis of high functional stability and resilience of the gut microbiota, due to metabolic plasticity and redundancy, within very diverse compositional contexts (65). Still, we found that *Lachnospiracea incertae sedis* and *Bacteroides* associations with cognition in infants were replicated in humanized mice. *Lachnospiraceae incertae sedis* is a phylogenetically heterogeneous genus of yet uncultured Candidatus species that has been associated with depression-like behaviours in mice after fecal transfer from human subjects with major depressive disorder (66) (67). Within *Lachnospiraceae*, we also observed that species assigned to *Ruminococcus gnavus* associated negatively with memory functions in mice. This finding is supported by Coletto et al reporting that GF mice monocolonized with *R.gnavus* ATCC 29149 did not improve memory functions (68). On the other hand, *Bacteroides* genus and species assigned to *Bacteroides* and *Phaeicola* were associated with better cognitive performances. The unique species discriminating cognition in both infants and recipient mice were those assigned to *Phaeicola dorei* and *Phaeicola vulgatus* (formerly *Bacteroides dorei* and *Bacteroides vulgatus*), none of which has been previously associated with cognition. In mice models of Alzheimer's disease and autism, both characterized by cognitive impairment, supplementation with *Bacteroides ovatus* or *Bacteroides fragilis* have been shown to rescue cognitive deficits (64, 69). Notably, members of the probiotic *Bifidobacterium* genus were enriched in mice with better memory functions. Beneficial effects of *Bifidobacterium* strains on functional memory in GF mice and in mice with Alzheimer's disease were reported by Luk et al (70) and Abdelhamid et al (71), respectively. Since our experiment establishes a

causal role for microbial effects, it is thus tempting the isolation of *Phaeicola* and *Bifidobacterium* strains identified in our study to test their efficacy on memory functions. Cognition is not the unique neurodevelopmental response that may be influenced by the gut microbiota (72). On the basis of our FMT results and of studies associating gut microbial profiles with early behavioural outcomes (73) (74) (75) (76, 77), we propose further research to identify specific gut microbiota species or phenotypically-selected consortia that contribute to the development on the four psychological domains (social interactions, stress and anxiety, learning and memory and, motor control), as such microorganisms may serve as treatments to improve neuropsychological deficits in infants.

One possible explanation of how microbiota may influence cognition is by the modulation of neurotransmitter levels (78). The catabolism of histidine produces the neurotransmitter histamine and ends in glutamate, all of which have been reported to be associated to memory and cognitive performance (48-51, 79). In our study, the metaproteomic signature of infant cognition rendered a number of discriminating proteins, mostly transporters and enzymes, of which histidine ammonia-lyase stood out as it controls the catabolism of histidine to glutamate through urocanate (His-Uro-Glu). Proof on the relationship between histidine ammonia-lyase and cognition was established with clinical evidence since histidine ammonia-lyase deficiency causes language and cognitive retardation (80). In addition, subjects with Alzheimer's, autism or depression disorders experienced cognitive dysfunction that was accompanied by altered output of histidine metabolism (81) (82) (83) (84). Using metabolomic analyses, we observed differential levels of histidine, histamine and urocanate between infants with better or poorer cognitive performances. Previous study by Matsumoto et al showed that restoration of GF gut microbiota with a SPF gut microbiota significantly reduced the levels of histidine and

rescued the levels of urocanate in faeces, suggesting their microbial origin (85). Consistent with this report, we found that colonization of GF mice with infant microbiota modified histidine metabolite levels in faeces, and urocanate and His-Uro-Glu ratios in hippocampus that associated with memory functions. It was recently reported that activation of His-Uro-Glu in the motor cortex and hippocampus promoted behaviours such as motor learning and recognition memory (86). Taken together, our results introduce the gut microbiota as a new regulatory factor of histidine metabolome with physiological impact on host neurodevelopment. However, the precise mechanism by which gut microbiota modulate cognitive performances through these compounds remains to be determined.

Our study is not without limitations. Using powerful DNA sequencing technologies, cross-sectional studies constitute a starting point to check hypothesis linking microbial compositional signatures to host phenotypes. GF animals provide an excellent tool to investigate function of the gut microbiota in a highly controlled environment though translation into applied medicine, for instance, to modulate cognition-deficits in human disorders, is daring but exciting. While our study is limited to 16 donor samples from our pediatric cohort, we are aware that the results herein demonstrate that the gut microbiota influences memory functions, though not exclusively, since most probably other factors such as host genetics, educational events and nutritional issues converge in the modulation of infant neurodevelopment during early life. In addition to histidine metabolites, we cannot exclude other potential immunological and metabolic mechanisms through which microbiota may impact cognitive performances. Indeed, our study identified several microbial proteins that call for investigation on their potential role on cognition. We believe that the association between histidine metabolites or specific bacterial strains derived from this study with cognition should be further tested with their administration to mice from

conception where behavioural assessment should be coupled to brain functionality tests. Given the mounting evidence on the co-evolution of gut microbiota and brain functioning throughout life, studies should also address long-term effects in animal models or other species including humans. Though infant donors were male and females, recipient mice in FMT were male so that gender-effects were not adequately assessed and require further investigation. In addition, donor infants belong to Spanish caucasian population residing in Andalucía, implying the need to rule out potential site-specific and ethnic biases.

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