Gut microbial genes are associated with neurocognition and brain development in healthy children

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Abstract

Both the brain and microbiome of humans develop rapidly in the first years of life, enabling extensive signaling between the gut and central nervous system (dubbed the "microbiome-gut-brain axis"). While emerging evidence implicates gut microorganisms and microbiota composition in cognitive outcomes and neurodevelopmental disorders (e.g., autism), the role of the gut microbial metabolism on typical neurodevelopment has not been explored in detail. We investigated the relationship of the microbiome with the neuroanatomy and cognitive function of 310 healthy children and demonstrated that differences in gut microbial taxa and gene functions, including genes for the metabolism and synthesis of glutamate and GABA, are associated with the size of brain regions important during development such as the cerebellum, and with overall cognitive function.

Introduction

The first years of life are a unique and dynamic period of neurological and cognitive development. Throughout childhood, a child's brain undergoes remarkable anatomical, microstructural, organizational, and functional change. By age 5, a child's brain has reached >85% of its adult size, has achieved near-adult levels of myelination, and the pattern of axonal connections has been established (Silbereis et al., 2016). This development is profoundly affected by the child's environment and early life exposures (Fox et al., 2010). The first years of life also witness dramatic changes in the gut microbiome. The gut microbial community is seeded at birth, and develops over the course of the first year from a low-diversity community dominated by Firmicutes and Proteobacteria, to a more diverse, adult-like microbiome upon the introduction of solid food. This microbial community development is also shaped by the environment, with factors such as mode of delivery and diet (breast milk vs formula) known to

50 have lasting effects on community composition (Bäckhed et al., 2015; Dominguez-Bello et al.,
51 2010).

52 The gut, the gut microbiome, and central nervous system are intricately linked through a
53 system known as the gut-microbiome-brain axis (Clarke et al., 2013), and the differences in
54 microbial communities have been shown to associate with, and in some cases cause, changes
55 in neurocognitive development and the outcome of neurological disorders such as autism
56 (Flannery et al., 2020; Gao et al., 2019; Sharon et al., 2019). However, the study of the
57 relationships between environmental exposures, neurocognitive development, and the gut
58 microbiome during neurotypical development remains in its infancy. This is the first study from
59 the RESONANCE consortium, part of the NIH initiative Environmental influences on Child
60 Health Outcomes (ECHO; Gillman & Blaisdell, 2018), a longitudinal observational study of
61 healthy and neurotypical brain development that spans the fetal and infant to adolescent life
62 stages, combining neuroimaging (magnetic resonance imaging, MRI), neurocognitive
63 assessments, bio-specimen analyses, subject genetics, environmental exposures such as lead,
64 and rich demographic, socioeconomic, family and medical history information (Table 1, Figure
65 1a).

66 Here, we focused on the relationship of microbial metabolic potential in the structural
67 development of the brain and in neurocognition. In particular, we show that microbial taxa and
68 genes with neuroactive potential, specifically genes encoding enzymes for the metabolism of
69 glutamate and GABA, are associated with the size of important brain regions and in cognitive
70 function. Understanding the relationship of intestinal GABA and glutamate metabolism may be
71 particularly important to understanding the role of the gut microbiome in early childhood
72 cognitive development, as together, GABA and glutamate responses make up the main
73 cerebellar output neurons (Carletti & Rossi, 2008; Hoshino, 2006). The cerebellum is one of the
74 earliest brain regions to develop (Leto et al., 2016; Silbereis et al., 2016), making it especially
75 vulnerable for disorder and disease (S. S.-H. Wang et al., 2014). Understanding how the gut

76 microbiome of healthy children interacts with the complex metabolism of these and other
77 neuroactive molecules will be critical in understanding the etiology of cognitive disorders and
78 how to promote healthy neurological development.

79 Results and Discussion

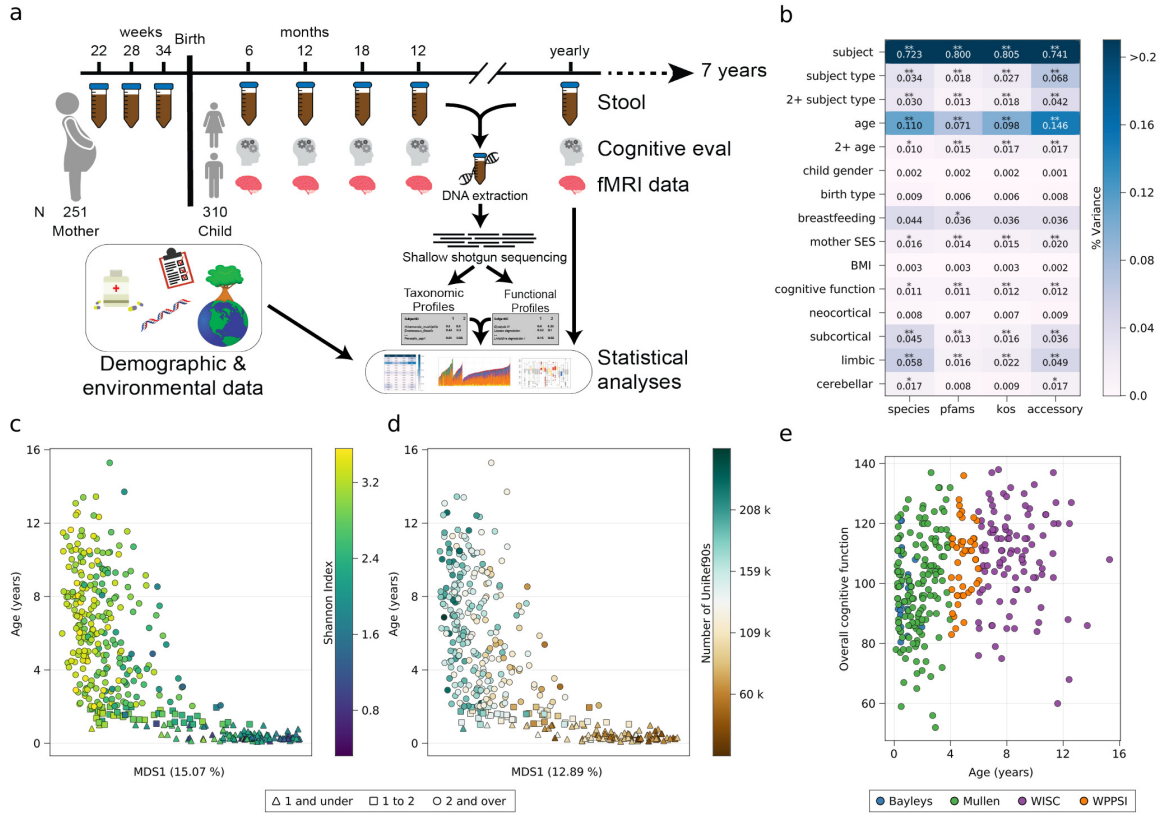
80 To examine the relationships between early childhood gut microbiome and
81 neurocognitive development, we collected stool samples from 251 pregnant women and 310
82 children enrolled in the RESONANCE study of child development. As an initial characterization
83 step, we used shotgun metagenomic sequencing to generate taxonomic and functional profiles
84 for each of our maternal and child fecal samples. Participant age was the greatest driver of both
85 taxonomic and functional diversity, as expected (Figure 1b-d). Children under one year of age
86 formed a distinct cluster from older children, characterized by high aerobe load and low alpha
87 (within-sample) diversity (Figure 1c, Supplementary Figure 2), and samples from children over
88 two years old were similar to maternal samples (Supplementary Figure 1).
89

90 Table 1

Table 1 - Baseline characteristics of ECHO RESONANCE participants	
All samples (n)	802
Total subjects (n)	561
Pregnant women (n)	251
Kids (n)	310
Kids under 1yo (n)	81
Kids over 2yo (n)	200
Kids with high-resolution scan (n)	141
Kids with cognitive function score (n)	263
Kids with both (n)	133
Non-white kids (%)	40.19
Mixed race kids (%)	23.49
Age in years (mean, SD)	4.27, 3.58
BMI (mean, SD)	16.66, 2.68
Maternal SES (mean, SD)	51.68, 10.86

91

Figure 1



The gut microbiome of health children changes dramatically over the first 2 years of life, and is associated with neurocognitive measures

a, Overview of ECHO RESONANCE cohort: Study design involves a longitudinal study of pregnant women and their children, combining stool samples with cognitive evaluation, structural and functional brain imaging, and rich demographic and environmental exposure information. **b**, PERMANOVA analysis for selected subject metadata vs. pairwise Bray-Curtis dissimilarity for species-level taxonomic or functional profiles. Functional profiles include Kegg-Orthology (KO), Pfams or accessory UniRef90s; subject and subject type include all subjects, 2+ includes only children over 2 years old, others include all children for which the measure was available; stars indicate significance after Benjamini-Hochberg FDR correction. (* <0.1, ** < 0.01, *** < 0.001). **c**, First principle coordinate (PCoA) based on Bray-Curtis dissimilarity in taxonomic (species) profiles vs age; younger children cluster away from older children, and are lower in diversity. **d,** same as (c) using first PCo axis for functional profiles (UniRef90 accessory genes) after removing gene families that were present in >90% of subjects in a given age group; variation is driven by similar effects as for taxonomic profiles. **e,** Child age vs. normalized cognitive function score; age-appropriate assessments achieve similar score distributions for their age groups.

111 As in previous adult and infant cohorts, functional beta diversity was generally lower than
112 taxonomic diversity (Supplementary Figure 2a), suggesting that healthy guts select for similar
113 gene functions even when different species contribute those functions. However, this
114 interpretation may be complicated by the fact that as many as 50% of sequencing reads in
115 some samples are not mapped to any of the reference genes used, and are thus unclassified
116 (Supplementary Figure 2b). Interestingly, although children under 1 tended to have substantially
117 fewer species and, therefore, fewer total genes (Figure 1c, right), those genes tended to be
118 better characterized (Supplementary Figure 2b, Vatanen et al., 2018). Consistent with previous
119 studies of adult cohorts from industrialized countries (Tett et al., 2019), another major driver of
120 variation visible from principal coordinates analysis was the presence of *Prevotella copri*
121 (Supplementary Figure 3). Like samples from very young children, samples with *P. copri* had
122 reduced diversity compared with samples from older children and pregnant mothers without *P.*
123 *copri* (Supplementary Figure 2, 3). Overall, these results are consistent with prior studies of
124 adult and childhood gut microbiomes.
125 To assess the potential role of the microbiome in neuro structural and cognitive
126 development, child stool samples were collected alongside MRI and age-appropriate
127 neurocognitive evaluations (Figure 2e). Measures of overall cognitive ability (e.g., intelligence
128 quotient, IQ, Mullen & others, 1995; Wechsler, 1949), MRI measures of cortical volume and
129 morphometry, as well as other potentially relevant clinical metadata, were compared to
130 taxonomic and functional profile dissimilarity by PERMANOVA (Anderson, 2017; McArdle &
131 Anderson, 2001). Consistent with previous studies, inter-individual variation accounted for the
132 majority of variation in microbial taxonomic and functional profiles ([79%, 77%], $q < 0.001$)
133 (Figure 1b, Supplementary Table 1). Subject type (child or mother) accounted for a large
134 amount of variation (4-6% $q < 0.001$), but this effect dropped to 2% when children under 2 years
135 of age were excluded, suggesting that age, rather than subject type, is actually responsible for
136 driving the taxonomic and functional variation. Among children's samples, age accounted for

137 over 20% of variation in both taxonomic and functional profiles, but this effect also largely
138 disappeared when children under 2 were excluded (Figure 1b, Supplementary Table 1),
139 suggesting that the age effect is primarily driven by the enormous changes in the microbiome
140 over the first year.

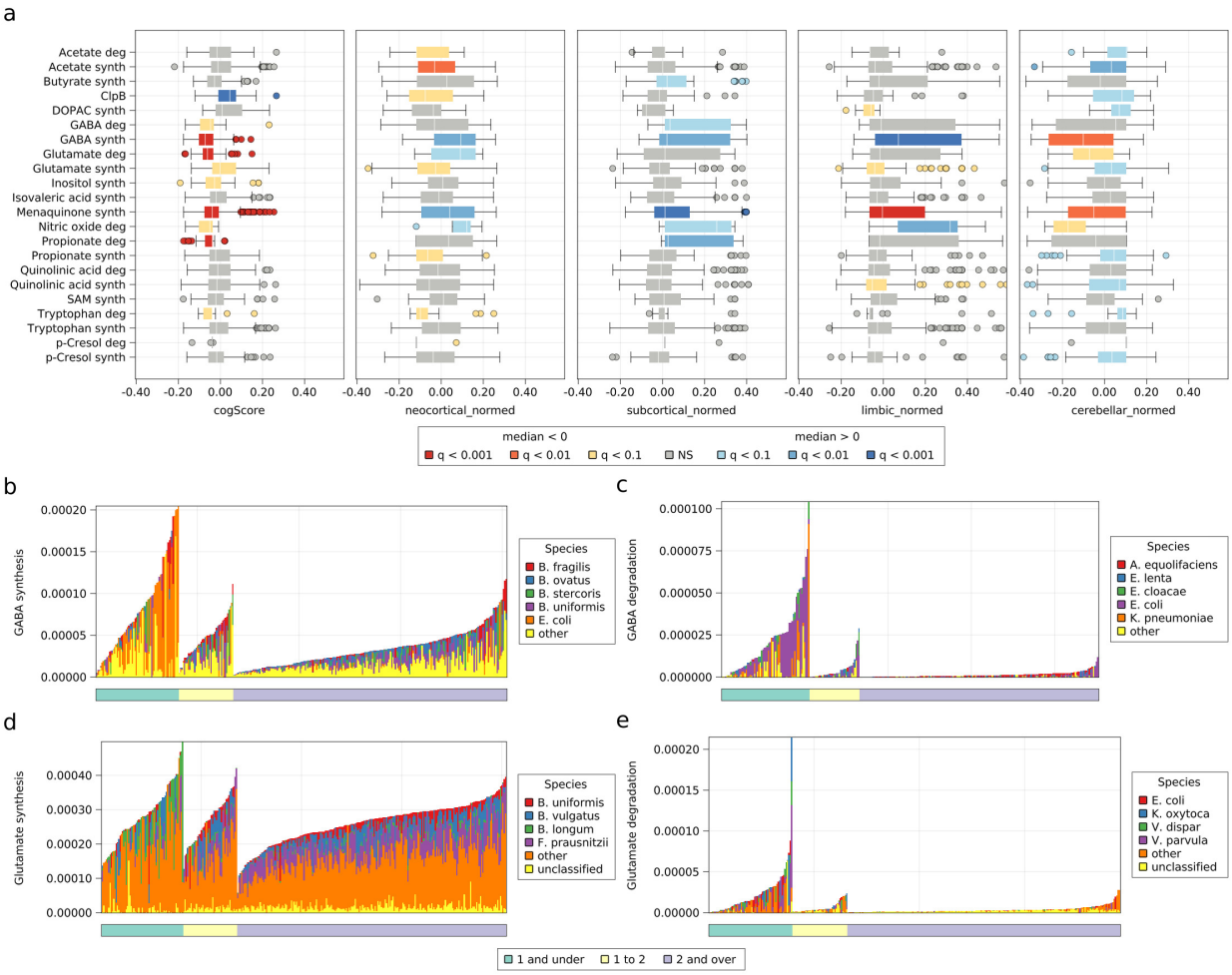
141 Microbiome taxonomic and functional variation was also associated with moderate but
142 significant differences in several neurocognitive measures including age-appropriate measures
143 of cognitive ability ([2.04%, 2.08%], $q < 0.01$, N=263). We also found significant associations
144 between regional brain volumes and microbial taxonomic and functional variation, including the
145 sizes of the cerebellum ([3.63%, 2.82%], $q < 0.01$), the subcortex ([3.64% 5.42%], $q <$ [0.01,
146 0.001]), neocortex ([1.95% 1.1%], $q <$ [0.01, NS]), and limbic regions ([4.31% 5.9%], $q <$ [0.01,
147 0.001]) (N = 141 for all high resolution scans) even after controlling for the effect of age on brain
148 volume. These results suggest that there is a strong relationship between the gut microbiome
149 and neurocognitive development (Figure 1b). Though the direction of causality cannot be
150 determined, experimental models of brain development neurological disorders have
151 demonstrated that microbes in the intestine may have causal effects on the functioning of the
152 central nervous system (Blacher et al., 2019; Clarke et al., 2013; Gao et al., 2019; Hsiao et al.,
153 2013).

154 To investigate potential mechanisms through which the gut microbiome might affect
155 neurostructural and neurocognitive development in infants and young children, we focused on a
156 group of microbial genes that code for enzymes that metabolize neuroactive compounds
157 (Valles-Colomer et al., 2019). We analyzed the association of each of these gene sets with our
158 neurocognitive measures using feature set enrichment analysis (FSEA; de Leeuw et al., 2016;
159 Metwally et al., 2018; Figure 2a, Supplementary Table 2). Briefly, we calculated the Pearson
160 correlation between the relative abundance of all identified UniRef90 gene families with each
161 neurocognitive measure, then calculated the Mann-Whitney U statistic for each potentially
162 neuroactive subset. Using this analysis, we observed that catabolic and anabolic pathways for

163 several molecules known to be important in the developing brain were significantly associated

164 with overall cognitive function scores and the size of brain subregions.

Figure 2



Microbial genes involved in the metabolism of neuroactive compounds are associated with cognition and brain structure

a, FSEA analysis for gene sets with neuroactive potential (see Methods); many gene sets with neuroactive potential are associated with cognitive function and brain structure. **b-e,** species contributions of GABA and glutamate synthesis and degradation; the microbiomes of children over 1 year old have substantially lower capacity for degradation of glutamate and GABA. There is a clear shift in glutamate synthesis from *B. longum* and other *Bifidobacterium spp.* to *F. prausnitzii* and *Bacteroides spp* (common species in adult microbiomes) as children age.

175 In particular, microbial genes for GABA synthesis were positively associated with
176 neocortical ($q < 0.01$, Figure 2a, Supplementary Table 2), subcortical ($q < 0.01$), and limbic ($q <$
177 0.001) volume, and negatively associated with cerebellar volume ($q < 0.01$) and overall
178 cognitive function ($q < 1e-5$). Interestingly, GABA degradation genes were also positively
179 associated with the size of the subcortex and negatively associated with cognitive function ($q <$
180 0.05). This may be due to higher GABA synthesis selecting for the ability to catabolize this
181 molecule, making it difficult to assess how actual GABA concentrations in the gut are associated
182 with brain development. GABA synthesis genes that could be assigned to specific taxa were
183 found primarily in *E. coli* in children under 1 year old, and in several different *Bacteroides*
184 species in older children (Figure 2b). GABA degradation in younger children was also seen
185 extensively in *E. coli*, but declines dramatically in abundance in older kids (Figure 2c).
186 Unlike the metabolism of GABA, glutamate synthesis and degradation genes have an
187 inverse relationship with neurocognitive measures (Figure 2a, Supplementary Table 2). The
188 glutamate degradation gene set was negatively associated with cognitive function ($q < 1e-4$)
189 and cerebellar volume ($q < 0.05$) and positively associated with the size of the neocortex ($q <$
190 0.05), while glutamate synthesis was marginally negatively associated with overall cognitive
191 function and the size of the neocortex, while positively associated with the size of the
192 cerebellum ($q < 0.05$). However, it remains difficult to predict how gut concentrations of
193 glutamate might be related to microbial metabolism; while it might be intuitive to expect that
194 higher glutamate synthesis and lower glutamate degradation would lead to higher gut
195 concentrations of glutamate, it might also be the case that lower glutamate concentrations
196 select for microbes that can synthesize it and against those that break it down. Glutamate is
197 also far more prevalent in the diet and can be rapidly metabolized by gut epithelial cells, making
198 the relationship between gut concentrations and microbial metabolism even more complex
199 (Reeds et al., 2000). Unsurprisingly, as glutamate is an essential amino acid, Glu synthesis

genes were found in a variety of taxa, including the most common taxa for each age group (eg. *B. longum* for children under 1 year old and *F. prausnitzii* in older children; Figure 2d).

This is the first look at an ongoing study of child neurocognitive and microbiome development. Using cross-sectional data, we have shown that differences in gut microbial taxa and genes are associated with the structural development of the brain and with cognitive development. In addition, we have shown that particular microbial gene sets with neuroactive potential are associated with neurocognitive development, thus perhaps playing a direct role in the gut luminal exposure of children to neuroactive metabolites. Glutamate and GABA metabolism are of particular interest, since these are critical molecules for signaling from the cerebellum during early development and learning, and the cerebellum is on the first brain structures to develop in the fetal brain (Leto et al., 2016; Silbereis et al., 2016). Neurodevelopmental disorders, such as autism spectrum disorder, have been associated with an imbalance of the inhibitory/excitatory system regulated by glutamate and GABA, with recent evidence suggesting an impaired conversion of glutamate to GABA in the disorder (Fatemi et al., 2012), and understanding these pathological outcomes will depend on a deeper understanding of developmental exposures in neurotypically developing children.

This study is ongoing, and we are collecting additional clinical data such as resting state functional brain imaging, participant genetic profiles, lead exposure, air quality data and nutritional information to understand how the environment, microbiome, and biological development interact to shape neurocognition. Future studies assessing gut metabolite pools combined with MR spectroscopic methods to quantify concentrations of neurotransmitters such as GABA and glutamate-glutamine in the brain, as well humanized mouse models and longitudinal human data, will provide further insight into the interactions of microbial metabolism and neurocognitive development. As we continue to follow these subjects, we will be able to identify how early-life microbial exposures, including exposures of mothers *in utero*, might affect future neurocognitive outcomes.

Materials and methods

Cohort description

Data used in this study were drawn from the ongoing longitudinal RESONANCE study of healthy and neurotypical brain and cognitive development, based at Brown University in Providence, RI, USA. From the RESONANCE cohort, 310 typically-developing children and 251 healthy pregnant women were selected for analysis in this study. General participant demographics are provided in **Table 1**, with children being representative of the RI population. As a broad background, children in the BAMBAM cohort were born full-term (>37 weeks gestation) with height and weight average for gestational age, and from uncomplicated singleton pregnancies. Children with known major risk factors for developmental abnormalities at enrollment were excluded. In addition to screening at the time of enrollment, on-going screening for worrisome behaviors using validated tools were performed to identify at-risk children and remove them from subsequent analysis.

Additional data collection

Demographic and other non-biospecimen data such as race and ethnicity, parental education and occupation, feeding behavior (breast- and formula-feeding), child weight and height, were collected through questionnaires or direct examination as appropriate. All data were collected at every assessment visit, scheduled on the same day of the MRI scan or at least within 2 weeks of the scan date.

Approval for human subject research

All procedures for this study were approved by the local institutional review board at Rhode Island Hospital, and all experiments adhered to the regulation of the review board.

248 Written informed consent was obtained from all parents or legal guardians of enrolled
249 participants.

250 Stool sample collection and handling

251 Stool samples (n=802) were collected by parents in OMR-200 tubes (OMNIgene GUT,
252 DNA Genotek, Ottawa, Ontario, Canada), stored on ice, and brought within 24 hrs to the lab in
253 RI where they were immediately frozen at -80˚C. Stool samples were not collected if the infant
254 had taken antibiotics within the last two weeks.

255 DNA extraction and sequencing of metagenomes

256 All processing of the samples was done at Wellesley College (Wellesley, MA). Nucleic
257 acids were extracted from stool samples using the RNeasy PowerMicrobiome kit automated on
258 the QIAcube (Qiagen, Germantown, MD), excluding the DNA degradation steps. Extracted DNA
259 was sequenced at the Integrated Microbiome Resource (IMR, Dalhousie University, NS, Canada).

260 Shotgun metagenomic sequencing was performed on all samples. A pooled library (max
261 96 samples per run) was prepared using the Illumina Nextera Flex Kit for MiSeq and NextSeq
262 from 1 ng of each sample. Samples were then pooled onto a plate and sequenced on the
263 Illumina NextSeq 550 platform using 150+150 bp paired-end “high output” chemistry, generating
264 ~400 million raw reads and ~120 Gb of sequence.

265 Computational methods, statistical analyses and data availability

266 Raw and processed data (excluding PHI) is available through SRA and Zenodo.org
267 (Bonham et al., 2020). All code used for statistical and other analysis is available on github
268 (Kevin Bonham, 2020). Software packages included vegan (R package) for PERMANOVAs,

MultivariateStats.jl for MDS analysis, HypothesisTests.jl for Mann-Whitney U tests (used in FSEA analysis), MultipleTesting.jl for false discovery rate correction, and Makie.jl for plotting.

Metagenomic data were analyzed using the bioBakery (McIver et al., 2018) family of tools with default parameters. Briefly, KneadData (v0.7.1) was used to trim and filter raw sequence reads and to separate human reads from bacterial sequences. Samples that passed quality control were taxonomically profiled to the species level using MetaPhlAn2 (v2.7.7). Stratified functional profiles were generated by HUMAnN2 (v0.11.1).

MRI Acquisition and data processing

Structural T_1-weighted MRI scans were acquired on a 3T Siemens Trio scanner with a 12-channel head RF array, preprocessed using a multistep registration procedure. Cortical reconstruction and volumetric segmentation was performed with the Freesurfer image analysis suite, which is documented and freely available for download online (http://surfer.nmr.mgh.harvard.edu/). Brain regions were divided into neocortex, cerebellum, limbic and subcortical regions (for more details on acquisition and processing, see extended methods).

Neurocognitive assessments

Overall cognitive function was defined by the Early Learning Composite as assessed via the Mullen Scales of Early Learning (MSEL; Mullen & others, 1995), a standardized and population normed tool for assessing fine and gross motor, expressive and receptive language, and visual reception functioning in children from birth through 68 months of age.

The third edition of the Bayley Scales of Infant and Toddler Development (Bayley's III) is a standard series of measures primarily to assess the development of infants and toddlers, ranging from 1 to 42 months of age (Bayley, 2006).

The Wechsler Intelligence Quotient for Children (WISC; Wechsler, 2012) is an individually administered standard intelligence test for children aged 6 to 16 years. It derives a full scale intelligence quotient (IQ) score, which we used to assess overall cognitive functioning.

The fourth edition of the Wechsler Preschool and Primary Scale of Intelligence (WPPSI-IV; Wechsler, 2012) is an individually administered standard intelligence test for children aged 2 years 6 months to 7 years 7 months, trying to meet the increasing need for the assessment of preschoolers. Just as the WISC, it derives a full scale IQ score, which we used to assess overall cognitive functioning.

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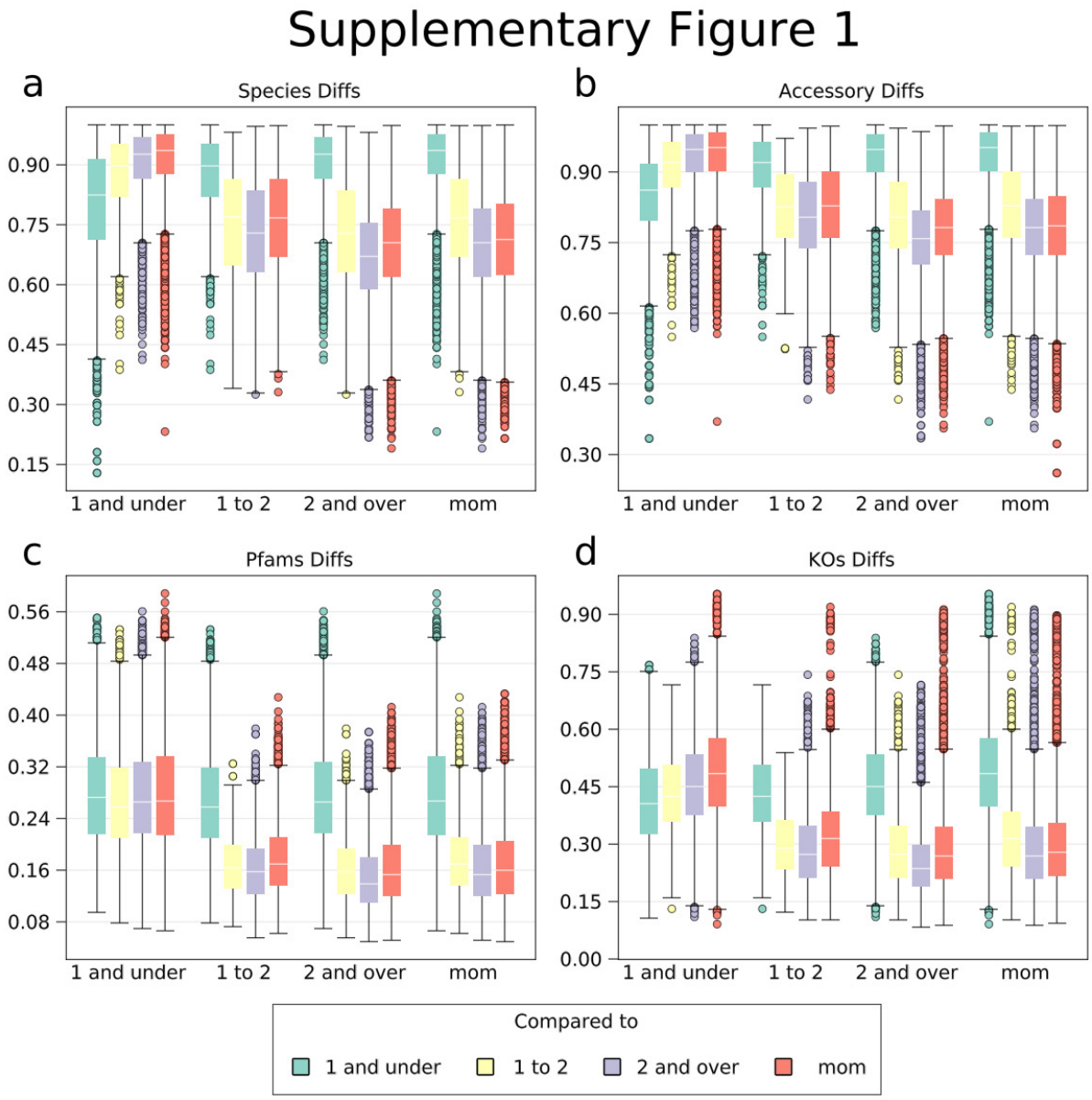
Supplemental Figures

Supplementary Figure 1



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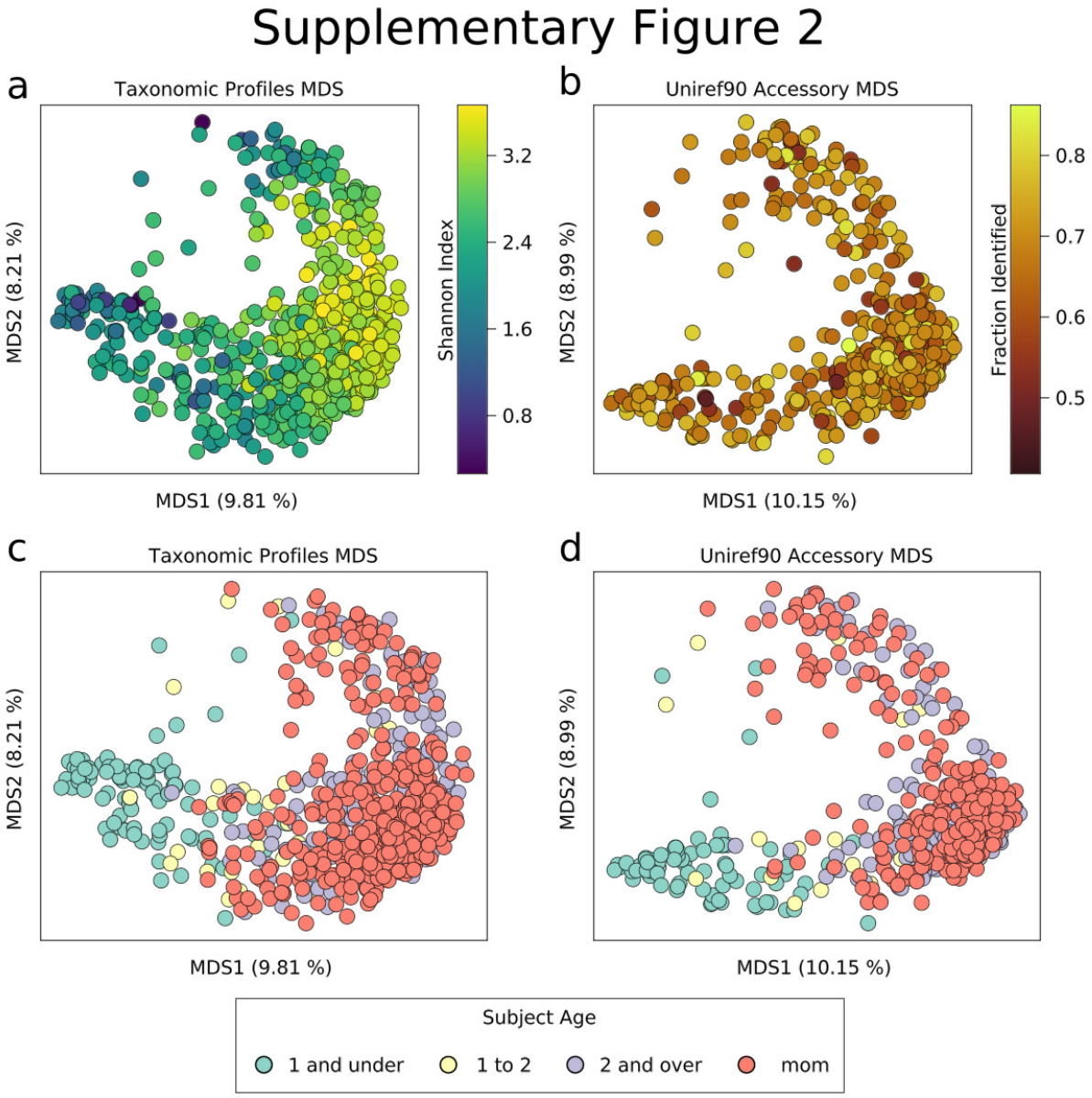
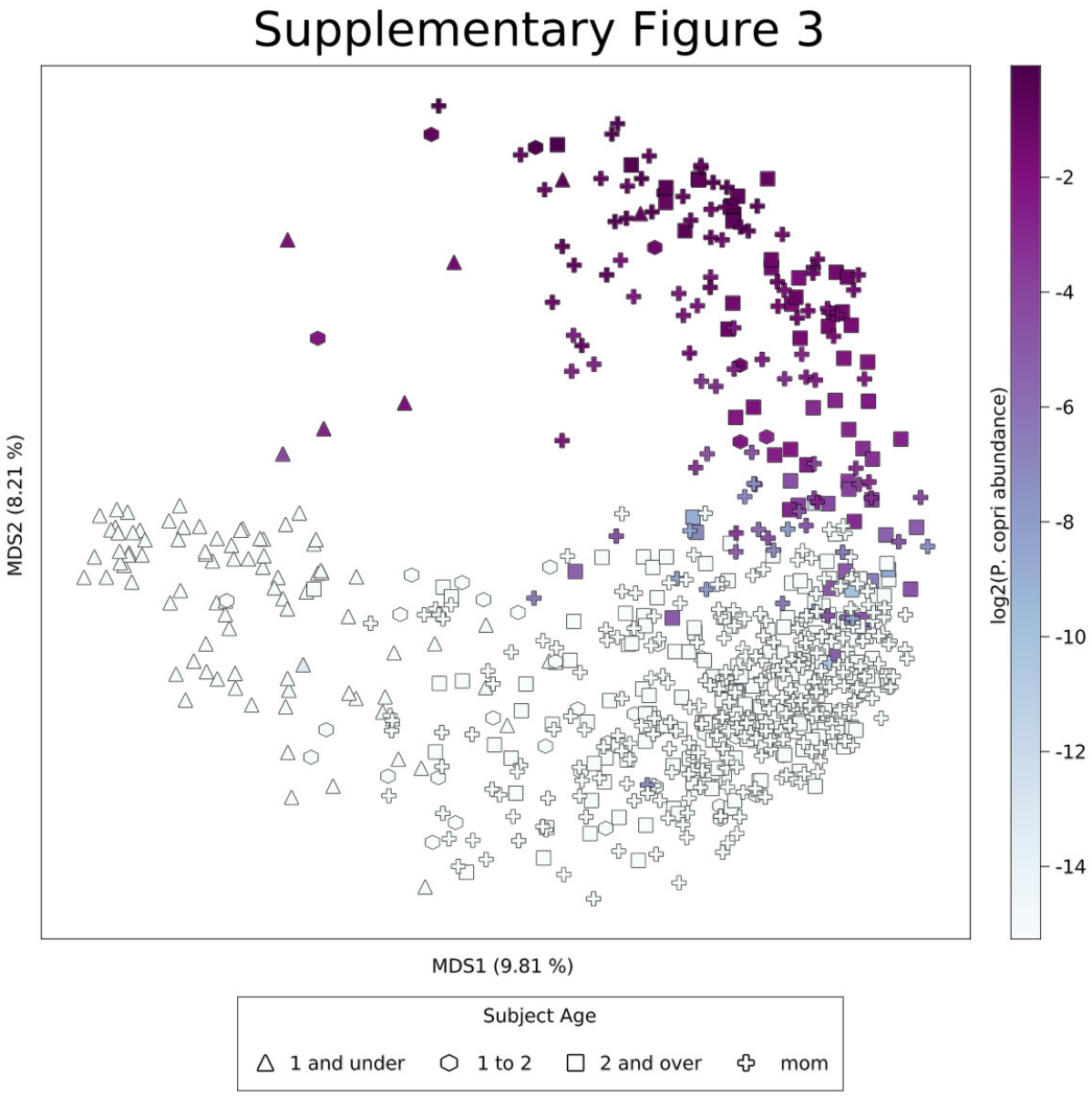


Supplementary Figure 3



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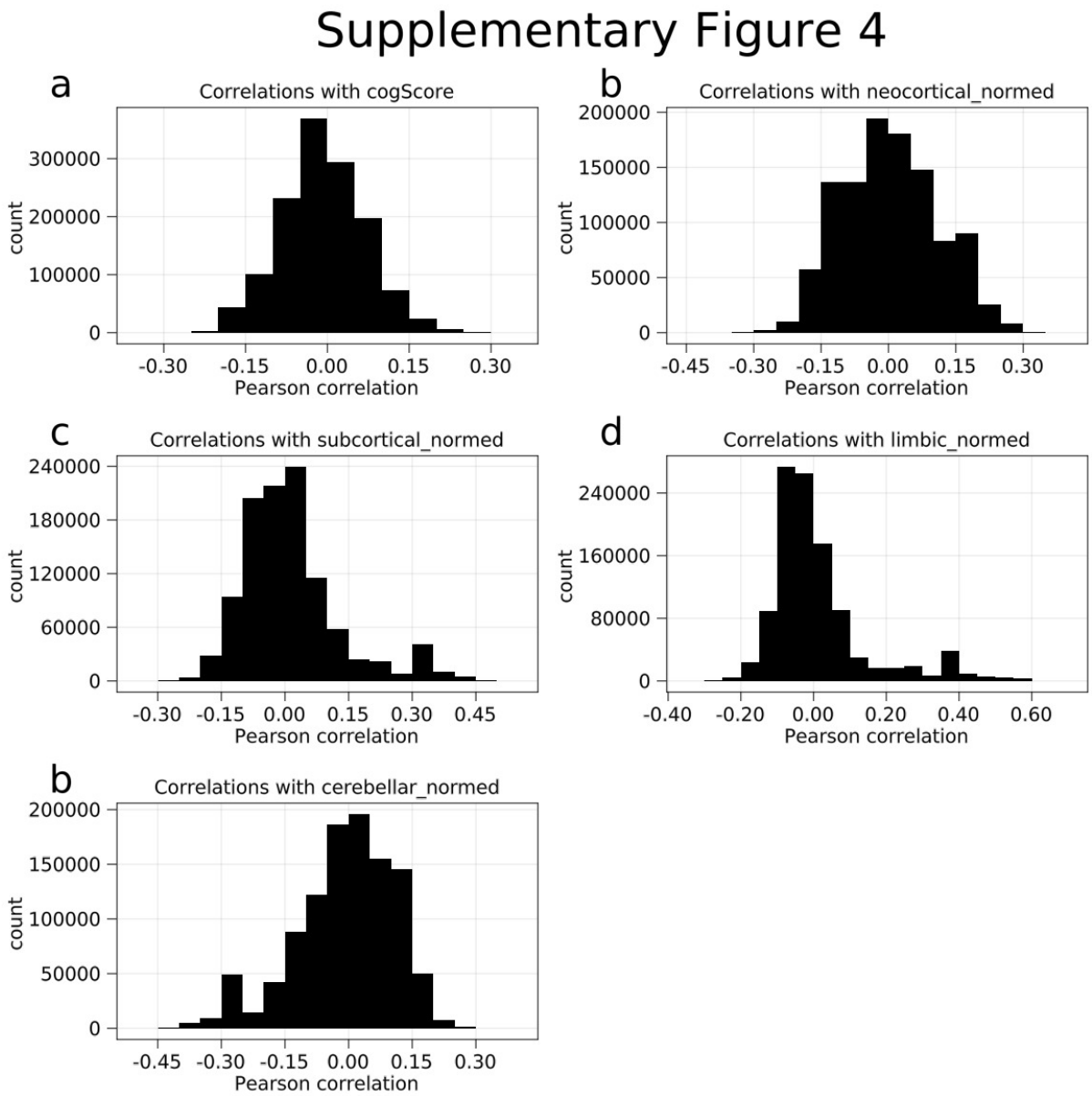
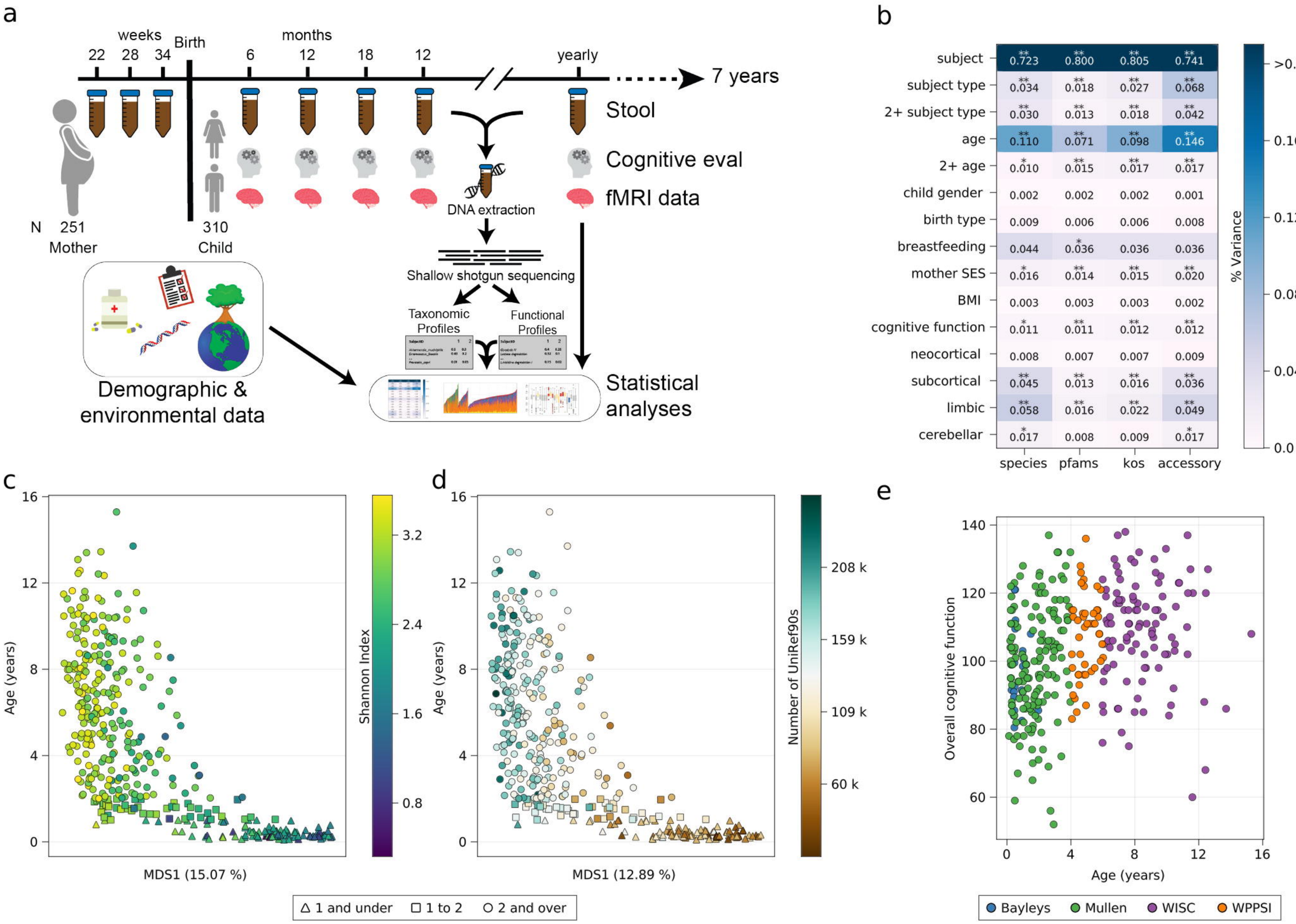
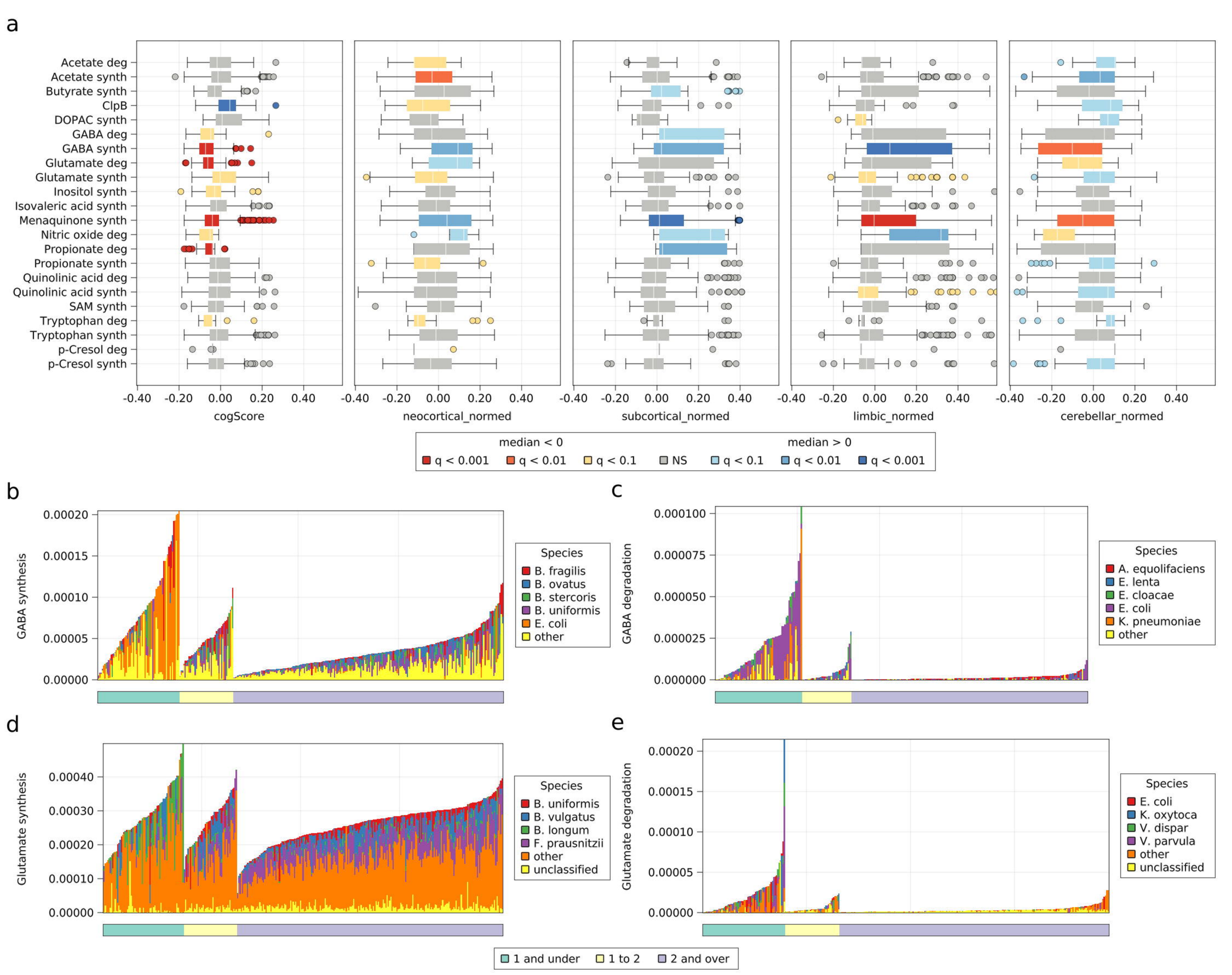



a
weeks
Birth
months
22
28
34
6
12
18
12
yearly
7 years
Stool
Cognitive eval
fMRI data
DNA extraction
Shallow shotgun sequencing
Taxonomic Profiles
Functional Profiles
N 251
Mother
310
Child
Demographic & environmental data
Statistical analyses
b
subject
subject type
2+ subject type
age
2+ age
child gender
birth type
breastfeeding
mother SES
BMI
cognitive function
neocortical
subcortical
limbic
cerebellar
species
pfams
kos
accessory
% Variance
>0.2
0.16
0.12
0.08
0.04
0.0
c
Age (years)
MDS1 (15.07 %)
Shannon Index
3.2
2.4
1.6
0.8
d
Age (years)
MDS1 (12.89 %)
Number of UniRef90s
208 k
159 k
109 k
60 k
△ 1 and under
□ 1 to 2
○ 2 and over
e
Overall cognitive function
Age (years)
Bayleys
Mullen
WISC
WPPSI


a
Acetate deg
Acetate synth
Butyrate synth
ClpB
DOPAC synth
GABA deg
GABA synth
Glutamate deg
Glutamate synth
Inositol synth
Isovaleric acid synth
Menaquinone synth
Nitric oxide deg
Propionate deg
Propionate synth
Quinolinic acid deg
Quinolinic acid synth
SAM synth
Tryptophan deg
Tryptophan synth
p-Cresol deg
p-Cresol synth
-0.40 -0.20 0.00 0.20 0.40
cogScore
neocortical_normed
subcortical_normed
limbic_normed
cerebellar_normed
median < 0
q < 0.001
q < 0.01
q < 0.1
NS
median > 0
q < 0.1
q < 0.01
q < 0.001
b
GABA synthesis
0.00020
0.00015
0.00010
0.00005
0.00000
Species
B. fragilis
B. ovatus
B. stercoris
B. uniformis
E. coli
other
c
GABA degradation
0.000100
0.000075
0.000050
0.000025
0.000000
Species
A. equolifaciens
E. lenta
E. cloacae
E. coli
K. pneumoniae
other
d
Glutamate synthesis
0.00040
0.00030
0.00020
0.00010
0.00000
Species
B. uniformis
B. vulgatus
B. longum
F. prausnitzii
other
unclassified
e
Glutamate degradation
0.00020
0.00015
0.00010
0.00005
0.00000
Species
E. coli
K. oxytoca
V. dispar
V. parvula
other
unclassified
1 and under
1 to 2
2 and over