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Deviated and early unsustainable stunted development of gut microbiota in children with autism spectrum disorder

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ABSTRACT

Objective Recent studies have provided insights into the gut microbiota in autism spectrum disorder (ASD); however, these studies were restricted owing to limited sampling at the unitary stage of childhood. Herein, we aimed to reveal developmental characteristics of gut microbiota in a large cohort of subjects with ASD combined with interindividual factors impacting gut microbiota.

Design A large cohort of 773 subjects with ASD (aged 16 months to 19 years), 429 neurotypical (NT) development subjects (aged 11 months to 15 years) were employed to determine the dynamics change of gut microbiota across different ages using 16S rRNA sequencing.

Result In subjects with ASD, we observed a distinct but progressive deviation in the development of gut microbiota characterised by persistently decreased alpha diversity, early unsustainable immature microbiota, altered abundance of 20 operational taxonomic units (OTUs), decreased taxon detection rate and 325 deregulated microbial metabolic functions with age-dependent patterns. We further revealed microbial relationships that have changed extensively in ASD before 3 years of age, which were associated with the severity of behaviour, sleep and GI symptoms in the ASD group. This analysis demonstrated that a signature of the combination of 2 OTUs, *Veillonella* and *Enterobacteriaceae*, and 17 microbial metabolic functions efficiently discriminated ASD from NT subjects in both the discovery (area under the curve (AUC)=0.86), and validation 1 (AUC=0.78), 2 (AUC=0.82) and 3 (AUC=0.67) sets.

Conclusion Our large cohort combined with clinical symptom analysis highlights the key regulator of gut microbiota in the pathogenesis of ASD and emphasises the importance of monitoring and targeting the gut microbiome in future clinical applications of ASD.

INTRODUCTION

Autism spectrum disorder (ASD) is a group of neurodevelopmental disorders characterised by repetitive behaviours and impairments in social communication and interaction.¹ Since Leo Kanner first described early infantile autism clinically,² the

Significance of this study

What is already known on this subject?

- ⇒ Increasing evidences have provided insights into the gut microbiota in autism spectrum disorder (ASD); however, these studies were restricted owing to the small sample size or limited sampling at the unitary stage of childhood.
- ⇒ Dynamic characteristics of gut microbiome development in children with ASD associated with clinical symptoms remained unknown.

What are the new findings?

- ⇒ We first reported that children with ASD displayed a progressive deviation in development of gut microbiota when compared with that of the neurotypical group based on a large cohort of stool samples.
- ⇒ In subjects with ASD, deviated development in ASD was manifested as persistently decreased alpha diversity, early unsustainable and immature microbiota, difficulty or obstruction in the colonisation of common foundational bacterial groups in the early life stage and altered microbial relationships.
- ⇒ We concluded that several bacterial taxa, bacterial metabolic function and alteration of microbial relationship that were associated with the severity of behaviour, sleep and GI symptoms in children with ASD.
- ⇒ Microbiota-based disease diagnostic models showed admired efficiency across age and region.

How might it impact on clinical practice in the foreseeable future?

- ⇒ Our findings provide admired visible and interpretable microbiota-based disease diagnostic models for the prevention and treatment of ASD.

worldwide morbidity of ASD has increased, ranging between 0.75%³ and 1.85%,⁴ and continues growing. Accumulating evidences have revealed that both genetic (eg, rare inherited and de novo

variants)⁵ and environmental factors (eg, perinatal events)⁶ are potential triggers of ASD.⁷ An encouraging hypothesis recently proposed that gut microbiota may be an important factor in a broad range of neurological and psychiatric disorders and diseases.⁸ Although recent studies have provided insights into the gut microbiota in ASD, most of these studies have been restricted owing to the small sample size or limited sampling at the unitary stage of childhood.^{9–10} Accordingly, whether there is a real difference in the gut microbiota between healthy individuals and those with ASD has been questioned.¹¹

The symbiotic microbiota affects the host nervous system development through multiple forms across different host life stages, such as via the maternal gut-immune axis in the sterile fetal period^{12–13} or the gut microbiota-brain axis in the post-partum microbiota involved in host development.¹⁴ Recently, Roswall *et al* reported that gut microbiota of healthy children matured along similar trajectories at different speeds, and individual dynamics of gut microbiota may indicate sensitive points for gut microbiota development in early life.¹⁵ To further explore the gut microbiota profile in children with ASD, we used a large cohort of 1222 subjects to determine the dynamics change of gut microbiota across different age. We first identified the effects of multiple factors, including age, region, sex, clinical comorbidity, perinatal events and other factors on the gut microbiota of the present cohort. Then, using our multiregional large cohort and clinical metadata, we examined the impact of both population-wide and interindividual factors on the gut microbiota and determined whether alterations in gut microbiota impact the pathophysiological state of autism.

METHODS

Cohort description and study subjects

In total, 1222 participants from 25 provinces of China (mainly from Hunan, Shandong, Zhejiang and Guangdong) including 773 participants with clinical definition as ASD (aged between 16 months and 19 years) and 429 neurotypical (NT) children (aged between 11 months and 15 years) and 20 unrelated healthy adults (aged 16–24 years) were recruited (online supplemental table S1, S2). Informed consent was obtained from all the guardians of the participants for the collection of stool samples and trial information. Table 1 lists a detailed demographic and age distribution for all samples in both ASD and NT groups. Other detailed information about two cohorts and validation cohort 1–3^{10–16} are shown in online supplemental methods 1. The usage of antibiotics in the past 3 months before sampling was recorded in detail (online supplemental table S1). The detailed clinical evaluation standard is shown in the online supplemental table S3. The summaries of age, demographic, clinical and district characteristics are provided in online supplemental table S2 and S4.

16S rRNA gene sequencing

PCR amplification for V4 region of bacterial 16S rRNA gene was performed. Sample-specific paired-end 6 bp barcodes were incorporated into the TrueSeq adaptors for multiplex sequencing; 2×150bp pair-end sequencing was performed using the Illumina NovaSeq6000 platform at GUHE Info Technology (Hangzhou, China).

Bioinformatics and statistical analysis

The criteria for sequences filter are detailed in online supplemental methods 1. The resultant clean reads were blasted, dereplicated, clustered and chimera detected using VSEARCH (V2.4.4) against the SILVA138 database.¹⁷ Sequences with similarity ≥97% were

assembled into operational taxonomic unit (OTU) using Quantitative Insights Into Microbial Ecology (QIIME2, V2020.6) pipeline. Microbial functions were predicted by PICRUSt (Phylogenetic investigation of communities by reconstruction of unobserved states). The output file was further analysed using Statistical Analysis of Metagenomic Profiles (STAMP) software package V2.1.3. Host multifactorial effects on gut microbiota was assessed by EnvFit based on NMDS with Bray-Curtis dissimilarity. MaAslin2¹⁸ was used to determine multivariable associations via generalised linear regression between the relative abundance of microbial signatures and metadata.

Random forest analysis was performed to discriminate the samples from different groups using the R package ‘randomForest’ with 1000 trees and all default settings off.^{19–20} The generalisation error was estimated using 10-fold cross-validation. SHapley Additive exPlanations (SHAP) value was evaluated according to the unified framework proposed by Scott M. Lundberg and Su-In Lee²¹ to interpret the kind of host factor that affected the selected feature. The decision tree was visualised using *treeheatr* R package.²²

Definition of the 30 age-discriminatory bacterial taxa

Age-discriminatory bacterial taxa list containing feature importance was obtained using the random forests machine learning algorithm proposed by Subramanian *et al*.²³ The relative abundance of OTUs was then regressed against their physiological age using random forest regression (default parameters), and the most 30 taxa were extracted to map the developmental spectrum of gut microbiota in both ASD and NT.

Deep neural network for microbiota age quantification

Microbiota age was quantified using a neural network approach similar to that described by Galkin *et al*.²⁴ All deep neural networks (DNNs) were implemented using the Python V3.6 Keras library with Tensorflow backend. The detail process of model construction is described in online supplemental methods 1.

Taxa detection rate analysis

Taxa with at least 10 samples were piped into the detection rate analysis. The detection rate for each taxon is defined as:

$$D = \frac{\text{number of samples in which the taxon was detected}}{\text{total samples}}$$

The detection rate in the NT and ASD cohorts was calculated and compared using Fisher’s exact test.

Absolute microbial abundance change analysis

The absolute microbial abundance change was analysed following the previous method.²⁵ False discovery rate (FDR) q value <0.05 were used to filter significantly changed taxa.

Microbial relationship alteration analysis

Alteration in the paired microbial relationship between the NT and ASD groups, and alteration of microbial relationship with increasing ASD score were derived using PM2RA (profile monitoring for microbial relationship alteration).²⁶ The detailed analysis method is shown in online supplemental methods 1.

RESULTS

General characteristics of the gut microbiota and clinical information of the cohort

To characterise the gut microbiota profile in ASD across age, we enrolled 773 subjects clinically diagnosed with ASD (aged 16 months to 19 years), 429 NT subjects (aged 11 months to 15 years) (figure 1A and B and online supplemental table S1–S4).

Table 1 Information regarding detailed demographic and age distribution for all samples in both ASD and NT group

Age	11–23 months				24–35 months				36–47 months				4–5 years				5–6 years				6–7 years				7–8 years				8–9 years				>9 years			
	No.	ASD	NT		No.	ASD	NT		No.	ASD	NT		No.	ASD	NT		No.	ASD	NT		No.	ASD	NT		No.	ASD	NT		No.	ASD	NT		No.	ASD	NT	
Hunan	18	18	15	71	26	63	35	45	16	37	32	14	28	15	17	9	17	13	11																	
Guangdong	0	0	0	12	2	17	0	18	0	26	0	6	0	9	0	4	0	12	0																	
Shandong	1	0	0	10	1	19	21	10	35	17	34	4	25	0	3	4	1	0	0																	
Beijing	0	0	0	9	0	14	0	11	0	13	0	2	0	2	0	0	0	5	0																	
Sichuan	0	0	0	4	0	7	0	6	0	8	0	1	0	3	0	1	0	6	1																	
Hubei	0	0	0	3	2	10	5	4	2	5	3	1	0	2	4	2	4	1	8																	
Shanghai	0	2	1	1	0	5	2	4	2	8	4	2	4	2	3	2	2	1	3																	
Zhejiang	0	2	0	0	9	7	6	7	6	4	2	1	7	1	4	1	3	2	11																	
Jiangsu	1	0	0	2	0	10	0	1	0	3	0	0	0	1	1	1	2	1	0																	
Jiangxi	2	0	0	0	0	2	0	3	0	9	0	0	0	0	0	2	0	0	0																	
Shanxi	0	0	0	1	0	5	0	4	0	2	0	0	0	1	0	0	0	2	0																	
Hebei	1	0	0	1	0	6	0	1	0	4	0	0	0	0	0	0	0	0	0																	
Chongqing	0	0	0	3	0	1	0	6	0	0	0	1	0	0	0	0	0	0	0																	
Tianjin	0	0	0	1	0	3	0	2	0	4	0	1	0	0	0	0	0	0	0																	
Xinjiang	0	0	0	0	0	2	0	1	0	2	0	2	0	3	0	1	0	0	0																	
Anhui	0	0	0	5	0	2	0	0	0	0	0	0	0	0	0	0	1	0	0																	
Henan	0	0	0	2	0	1	0	3	0	0	0	0	0	0	0	3	0	0	0																	
Gansu	0	0	0	0	0	1	0	5	0	0	0	0	0	0	0	0	0	0	0																	
Guangxi	2	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0																	
Fujian	0	0	0	1	0	0	1	0	2	0	0	0	0	0	0	0	0	0	0																	
Hainan	0	0	0	1	0	1	0	1	0	0	0	0	0	0	1	0	0	1	0																	
Liaoning	0	0	0	0	0	3	0	1	0	0	0	0	0	0	0	0	0	0	0																	
Neimeng	0	0	0	0	0	1	0	1	0	2	0	0	0	0	0	0	0	0	0																	
Guizhou	0	0	0	0	0	0	1	1	0	1	0	0	0	0	0	0	0	1	0																	
Jilin	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0																	
No significant differences in age distribution can be observed across the regions. A multifactor analysis of variance (provided by bruceR 0.7.2 package at R V.3.6.3) was performed to test age divergence among regions. As most of provinces collected samples in the 36–47 months age bracket and the age bracket has the highest number of samples in all provinces. The age divergence is represented by the ratio of the sample number of a certain age bracket to that of the age 36–47 months bracket. Provinces with sample numbers larger than 20 were included in this analysis.																																				
ASD, autism spectrum disorder; NT, neurotypical.																																				

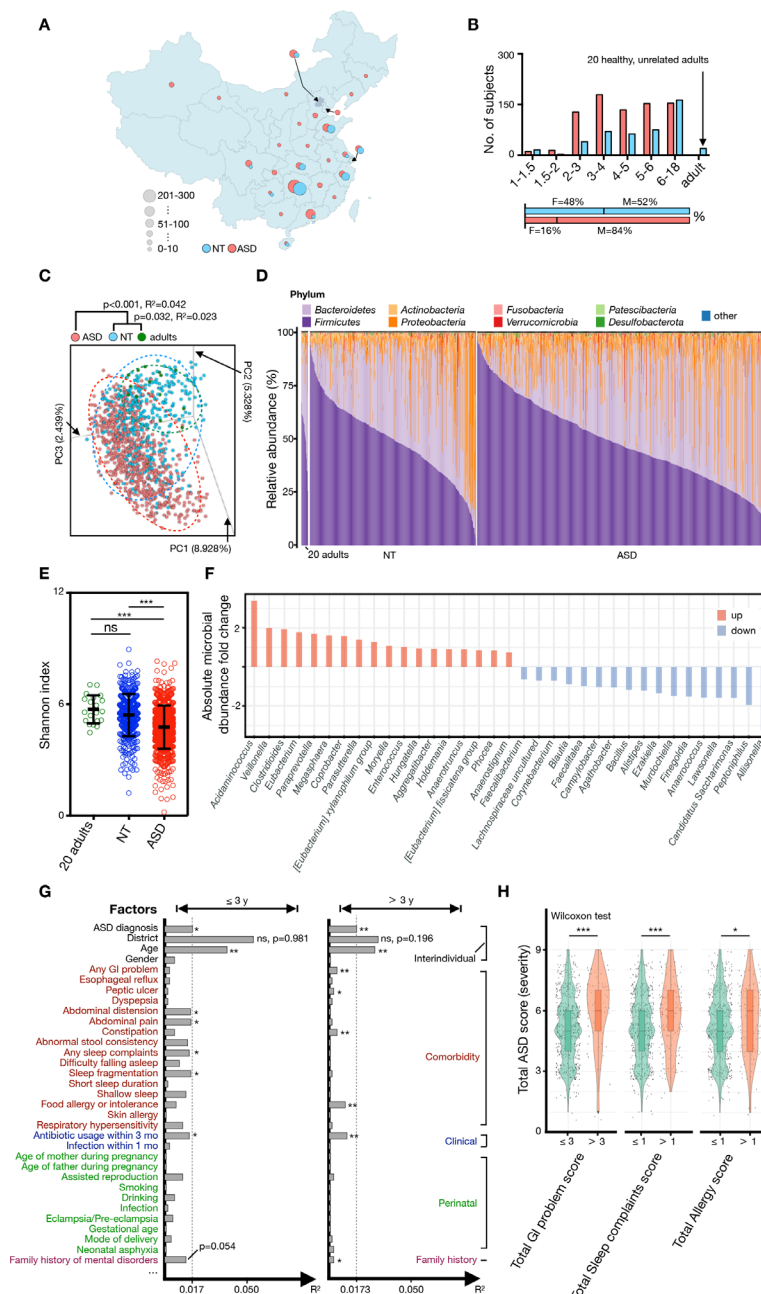


Figure 1 General characteristics of gut microbiota and clinical information of cohorts. (A) Geographical features of residence of the studied cohort. Subjects with ASD (n=773) were from 25 provinces of China, while NT subjects (n=449, 20 adults included) were from 14 provinces of China. (B) Histogram showing the summative distributions of grouped subjects according to age and gender. (C) Unweighted PCA at OTU level (for PC1, PC2 and PC3) showed that the gut microbial composition of subjects with ASD was separated from that of NT and healthy adults. The p values between each group were tested using mutational multivariate analysis of variance (Adonis). (D) Phylum-level distribution of gut microbiota in ASD, NT and healthy adults. (E) The Shannon diversity index of each group or age category. The mean values \pm SEM are plotted. One-way analysis of variance, *** $p < 0.0001$. (F) Diverging bar chart of absolute microbial abundance changes by Analysis of Compositions of Microbiomes with Bias Correction (ANCOM-BC) between NT and ASD. (G) Horizontal bars indicate the impact (R^2) of each host factor on gut microbiota variations. Subjects were subdivided into two groups (group 1: age ≤ 3 years or group 2: age > 3 years), and the effect of each host factor was determined by EnvFit (vegan). Factors were roughly classified according to metadata categories, and the factors with significant effects are indicated with an asterisk (FDR adjusted p value, * $p < 0.05$ and ** $p < 0.01$). (H) The severity of ASD showed a significant correlation with severity of GI (Wilcoxon signed-rank, $p = 8.274 \times 10^{-6}$), sleep disorder (Wilcoxon signed-rank test, $p = 0.0001537$) and allergy (Wilcoxon signed-rank test, $p = 0.03008$). ASD, autism spectrum disorder; NT, neurotypical; ns, not significant; OTU, operational taxonomic unit; PC, principal component; PCA, principal component analysis.

The 20 adults were observational cohort, mainly for monitoring the development of both alpha diversity and gut microbiota age.

Consistent with previous studies,^{9,10,16} although subjects with ASD were separated from NT and healthy adults (figure 1C), obvious variations in microbial composition were still detected

among individuals in the same group (figure 1C and D). At the phylum level, *Firmicutes*, *Bacteroidetes*, *Proteobacteria* and *Actinobacteria* were dominant in the different groupings (figure 1D). The alpha diversity of gut microbiota in the ASD group showed a significant decrease when compared with those of the NT and

adult groups (figure 1E and online supplemental table S5); 18 and 17 genera showed elevated or decreased absolute abundance in the ASD group relative to the NT group, respectively (figure 1F). The gut microbiome has been reported to be affected by multiple factors, such as age, region, food, gender, clinical comorbidity and perinatal factors.^{27,28} Thus, we further analysed the effects of these factors on the gut microbiota of the present cohort. A total of 12 host factors were detected to significantly affect the gut microbiota of children under 3 years of age (≤ 3 years) or/and more than 3 years of age (> 3 years) (figure 1G and online supplemental table S6). Unsurprisingly, regional differences provided the greatest contribution (≤ 3 years, $R^2=0.0544$; > 3 years, $R^2=0.0295$) of gut microbiota variations across all factors but with no significance difference (figure 1G). Consistent with a recent analysis of gut microbiota in children with ASD,²⁹ age afforded the second highest variance in gut microbiota in ASD, although the effect decreased after 3 years of age (figure 1G). The reduced covariance of age from 0.0384 (≤ 3 years) to 0.0283 (> 3 years) could be attributed to the development of the gut microbiota from a highly chaotic and changeable state to a relatively mature state; additionally, we noted that the clinical conditions of food allergy or intolerance of individual significantly affected gut microbiota only in subjects over 3 years of age (figure 1G). At this age bracket, their diet, usually transformed from a diet based on dairy products to concentrate on fewer food types and became diverse, can be largely affected by the food susceptibility. In the present cohort, the incidence rates of comorbidities were associated with ASD (online supplemental table S1 and S7) especially the GI problems, approximately sixfold higher in the ASD group (63.9%) than in the NT group (10.7%) (online supplemental table S7). Impressively, children with ASD who presents serious GI (scores > 3), sleep (scores > 1) and allergy problems (scores > 1) showed more severe ASD symptoms (figure 1H). To investigate which bacteria are associated with GI problems in ASD, we further compared differential bacteria between ASD patients with/without GI problems. We identified that 12 genera showed significant differential relative abundance between ASD with/without GI problems, and the most common comorbidity, that is, GI problems presenting a significant positive association with the differential bacteria, such as *Clostridia Vadin BB60 group*, *UBA1819* and *Erysopelotoclostridium* (online supplemental figure S1A). Moreover, a small number of differential genera were associated with social retardation, language retardation and total ASD score (online supplemental figure S1B). The analysis highlighted the interaction between gut microbiota and other host factors in the pathological process of ASD.

Deviated development in diversity and microbial relationship of gut microbiota in ASD group

To explore the effect of age on gut microbiota, we further tracked the principal component spectrum with age and described two simultaneously evolving temporal organisations of gut microbiota with different origins (figure 2). Age-mediated changes in gut microbiota mainly contributed to the first axis of taxonomy-based principal components, and the diagnosis of ASD contributed to the second and third axes (figure 2A). Other tracking methods according to the significant gut microbiota affecting factors showed no potential rules (online supplemental figure S2A–R). The development of gut microbiota is the replacement of dominant bacteria,^{30,31} and the spatiotemporal dislocation of functional bacterial groups indicates immaturity.^{23,32} Using the random forests machine learning algorithm,²³ we observed that

the relative abundance of 27 taxa from among the top 30 age-discriminatory bacterial taxa, were relatively consistent in the ASD and NT groups (figure 2B). Unlike those in children with immature or stunted gut microbiota,^{23,33} only taxa *Veillonella ratti* (OTU 359954), *Clostridium* (OTU 3203801) and *Enterobacter* (OTU 2119418) were significantly disturbed in children with ASD (especially in subjects aged > 3 years) (figure 2B and online supplemental figure S3A–C). It is noteworthy that subjects with a higher abundance of age-discriminatory taxa were more likely to be distributed close to the ends of axis PC1 (online supplemental figure S4A–C), which was consistent with the age-related subject distribution in PCA (figure 2A).

To further evaluate the relationship between gut microbiota and age, we conducted a DNN to quantify the physiological age based on the gut microbiota (see ‘Methods’ section). The predicted microbiota age linearly fitted the physiological age, with $R^2=0.04373$ in the NT group and $R^2=0.08405$ in the ASD group (figure 2C, top panel). According to the method developed by Subramanian *et al.*,²³ developmental disorders of gut microbiota were investigated in two dimensions: (1) deviations between the predicted microbiota age and their physiological age and (2) microbiota-for-age Z score (MAZ) of each subject with ASD. Regardless of whether the predicted microbiota age was compared among themselves or with NT (ASD cohort only and for MAZ calculation), only early unsustainable immaturity (18–20, 20–22, 22–24, 26–28 and 28–30 months) of gut microbiota in the subjects with ASD were found (figure 2C, bottom panel and online supplemental table S8). The validation cohort 1 showed similar encounter in the development of gut microbiota (24–35 and 36–47 months) (online supplemental figure S4D). Due to the inconsistent age distribution between the ASD and NT groups, especially the lack of samples at younger ages, validation cohort 2 did not show similar changes when compared with the current cohort and validation cohort 1 (online supplemental figure S4E).

Alpha diversity in the NT group increased rapidly from newborn to 2–3 years of age and entered a relatively stable stage (figure 2D), consistent with findings in a recent longitudinal birth cohort.¹⁵ However, the ASD group showed a mostly persistent decrease in bacterial alpha diversity (especially the Shannon diversity index) (figure 2D, online supplemental table S5). In line with the alpha diversity analysis across age, the genera detection rate with age was always lower in the ASD group than that in the NT group, and the detection rates of the NT group-enriched genus *Blautia* and *Faecalibacterium* fluctuated (figure 2E, online supplemental table S9 and S10). The genera detection rate in the ASD and NT groups after 3 years of age was associated with the rate before 3 years of age (online supplemental figure S5A). Accordingly, the difference in the genera detection rate between the NT and ASD groups remained constant along with age (figure 2E, online supplemental figure S5B) and online supplemental table S10). The histogram presenting changes in absolute microbial abundance changes indicated that the gut microbiome of subjects with ASD showed partial recovery after 3 years of age (online supplemental figure S5C, online supplemental table S11). The results suggested the challenges or hindrances in the colonisation of common foundational bacterial groups in subjects with ASD during early life stages.

Some studies have indicated that gut microbiota evolves towards an adult-like composition 2–3 years after birth.^{34,35} Revealing the relationship alteration (RA) of gut microbiota between groups of subjects provides additional ecological perspective.^{36,37} To reveal the microbial RA between two groups with age, using our newly developed analysis tooling called PM2RA,²⁶ we quantified the

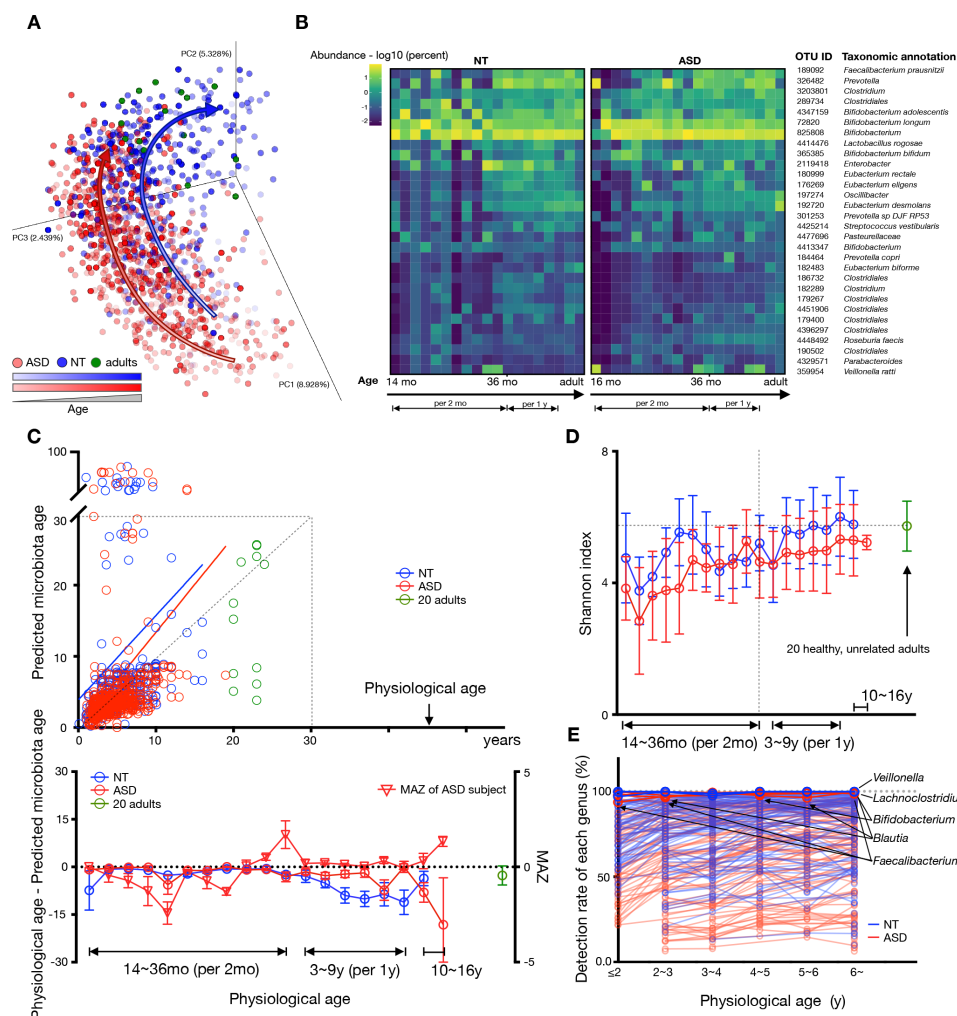


Figure 2 Deviated developmental spectrum of gut microbiota in children with ASD. (A) Three-dimensional diagram of unweighted PCA based on OTU-level Bray-Curtis dissimilarity. Plots of each sample were dyed gradients according to their physiological age. Arrows with gradient colours showed the developmental trends of the gut microbial community in ASD (red) and NT (blue) from young to old. (B) Heat map showed the mean relative abundance changes (10-based logarithm) of 30 age-discriminatory bacterial taxa across the physiological ages of subjects. (C) Predictions of microbiota age in both ASD, NT and adult subjects (above). Each circle represents an individual faecal sample, and the curves are a smoothed linear fit between the microbiota age and physiological age. The values of physiological age minus (–) predicted microbiota age of each group and the microbiota-for-age Z score (MAZ) of the subjects with ASD are shown in the Figure 2C chart below. Mean values±SEM are shown. (D) Shannon diversity index with age. (E) The taxon detection rate difference between NT and ASD remained constant with age. The detection rate curves of *Bifidobacterium*, *Veillonella*, *Faecalibacterium*, *Lachnospira* and *Blautia* are highlighted. Arrows indicated the time points of a specific bacteria with an abnormally fluctuating detection rate. ASD, autism spectrum disorder; MAZ, microbiota-for-age Z score; NT, neurotypical; OTU, operational taxonomic unit; PC, principal component; PCA, principal component analysis.

RA between ASD and NT groups before and after 3 years of age, respectively. As shown in figure 3A, RAs between ASD and NT groups showed complex alterations before 3 years of age; however, after 3 years of age, RAs between the two groups were significantly reduced, and only a few RAs occurred (figure 3B). Moreover, the microbial co-occurrence network showed a similar alteration in the microbial network to that observed in the PM2RA method (online supplemental figure S6A-B). Consistent with these results, we observed that the PM score which quantified the total RAs between NT and ASD after 3 years of age was also significantly reduced when compared with that before 3 years of age (figure 3C, online supplemental table S12). For example, the RA of *Chloroplast* and *Fenollaria* between the NT subjects and subjects with ASD under 3 years of age was considerably higher than after 3 years of age (figure 3D and E). RAs between the NT group before and after 3 years of age were

less complex than those between the ASD group before and after 3 years of age (online supplemental figure S6C-D), suggesting that the relationship between microbes in the ASD group were greatly altered with age. For example, the RA between *Desulfovibrio* and *Ezakiella* remained mostly unchanged in the NT group before and after 3 years of age (online supplemental figure S6E), however, the RA between the two microbes were considerably altered, and the PM score was substantially higher than that of NT (online supplemental figure S6F). Given that *Veillonella* showed different dynamic changes with age in ASD and NT (online supplemental figure S7A), we further compared the relationship between *Veillonella* and other bacteria in ASD and NT groups. Consistent with the whole relationship change with age, correlations between *Veillonella* and other bacteria in the NT group showed a simpler microbial network with age (online supplemental figure S7B-C), however, ASD showed a

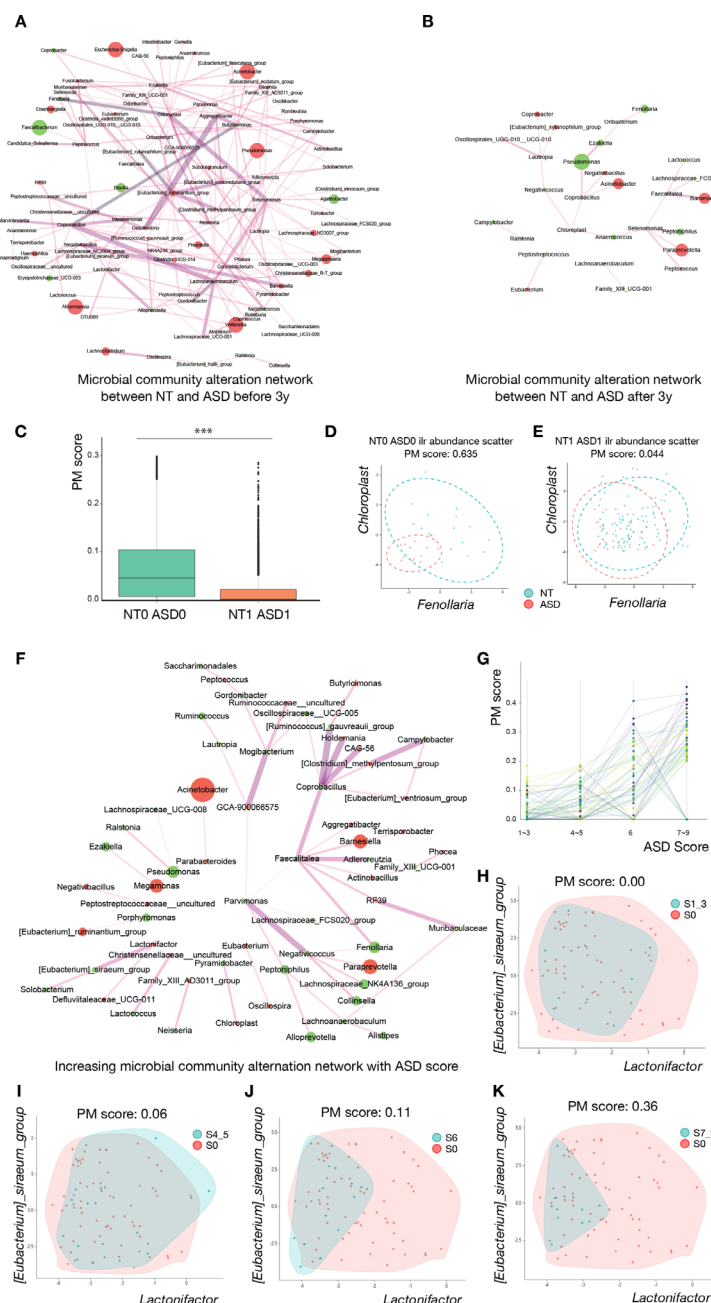


Figure 3 Deviated development in the microbial relationship in children with ASD. (A) Altered microbial community network between NT and ASD before 3 years of age. (B) Altered microbial community network between NT and ASD after 3 years of age. (C) Box plot of profile monitoring (PM) scores between NT and ASD at different ages. The PM score, that is, the microbial relationship alteration, is significantly reduced in NT subjects and children with ASD after 3 years of age when compared with that for children under 3 years of age (Wilcoxon signed-rank test, $p < 2.2 \times 10^{-16}$). (D, E) The isometric log-ratio transformed abundance scatter plot of *Fenollaria* and *Chloroplast* before and after 3 years of age, respectively. The relationship between these two taxa is significantly altered between NT and ASD groups under 3 years of age. This difference disappeared in NT subjects and children with ASD after 3 years of age. (F) Expanded alteration in the microbial community relationship with increasing ASD score. The edge width is proportional to the linear slope in the regression of the PM score to ASD symptom severity. (G) The identified PM score for 54 altered genera relationships is shown in figure 3F, which increased with ASD severity. (H–K) The isometric log-ratio transformed abundance scatter plot of (*Ruminococcus*) *gavvreauii* group and *Coprobacillus* in different ASD symptom severity groups. The microbial community alteration networks in A, B and F were derived using PM2RA. The edge width represented the interaction of the PM score. The node size represents the relative abundance change, as well as the label of the nodes specified taxonomic affiliation. The red node represents the increase of taxon abundance in ASD, and the green nodes represent the decrease. $p < 0.05$ *, $p < 0.01$ **, $p < 0.001$ *** and $p < 0.0001$ ****. ASD, autism spectrum disorder; NT, neurotypical.

substantially more complicated microbial network with age (online supplemental figure S7D–E), which implies that *Veillonella* partially contributed to microbiota immaturity in the ASD group and played an important role in the microbiota development.

To further identify the correlation of clinical symptoms of ASD with RA, we compared RA in ASD groups with different clinical symptoms, and observed that the alteration in the microbial relationship increased with the severity of ASD (figure 3F). Notably, the total PM score of 54 paired microbial

relationships gradually increased with the aggravation of clinical ASD symptoms (figure 3G and online supplemental table S12). For example, the PM score for RA of (*Eubacterium*)_siraenum and *Lactonifactor* increased from 0.00 to 0.36, along with an increase in the ASD score (figure 3H–K).

In summary, the above analysis suggested that ASD and NT groups were not synchronised in gut microbiota development. Furthermore, we noted that the development of gut microbiota in subjects with ASD deviated from NT development in terms of bacterial diversity, colonisation and microbial relationships.

Significant changes in microbial taxa and metabolic features across ages

Next, we deconstructed whether the signatures of gut microbiota between the two groups in an age-based dependent manner. In result, we observed that 20 microbial taxa showed significant different abundance across age between ASD and NT groups (figure 4). The total abundances of these 20 microbial taxa in different age brackets ranged between 33.41% and 65.90% in the NT group and 35.69% and 62.41% in the ASD group, representing the main proportion of the gut microbiota (online supplemental table S13). The *Enterobacteriaceae* family, *Bifidobacterium* genus and *Lachnospiraceae* NK4A136 group fluctuated substantially across different age brackets (figure 4A). Although *Veillonella* only showed a non-statistical increase in abundance in age brackets 7 and 8 years (figure 4A), it was positively correlated with the severity of ASD in subjects with >4 years of age (figure 4C, online supplemental figure S8A). Impressively, the abundance changes of *Veillonella* between ASD and NT groups was significantly negatively associated with the clinical diagnosis and age (online supplemental figure S9A). In agreement with its reported neuroactive potential,³⁸ *Faecalibacterium* was inversely correlated with ASD severity (mainly in subjects >3 years), while GI and sleep problems were significantly associated with age (online supplemental figure S9A). Unlike the previously reported loss of probiotics in children with ASD,³⁹ the relative abundance of *Bifidobacterium* flattened before the age of 3 years between the two groups, significantly increasing in the ASD group at the age of 4–5 years (figure 4A and online supplemental table S13).

To further investigate the potential microbial metabolic function in ASD across age brackets, we compared the differential microbial function of ASD and NT groups. Accordingly, we found 325 microbial-metabolic functions, 39 functions annotated as gut-brain modules (GBM) and 286 functions as members of the biocycle (METACYC), with a significant shift across age (figure 4B). Compared with dynamic changes in taxa, variations in microbial functions exhibited more obvious age dependence (figure 4B). The influence of early childhood on gut microbial function was mainly attributed to the conversion of the diet structure from breast milk or formula milk to complementary food.⁴⁰ Correspondingly, in the present cohort, the shift in cofactor biosynthesis and carbohydrate metabolic pathways were differentially enriched in the age brackets of 3–9 years (figure 4B and online supplemental table S14–S15). Furthermore, we revealed changes in the abundances of gut microbial taxa, such as *Veillonella*, *Faecalibacterium* and *Blautia*, as well as functions, such as MGB-004, MGB-027, PWY-7374, PWY-7254 and CODH-PWY, that were significantly related to the subjects' GI and sleep problems (online supplemental table S16–S18).

Although with moderate complexities, correlation networks between microbial functions and phenotypes were more closely interconnected with the clinical manifestations of ASD (online

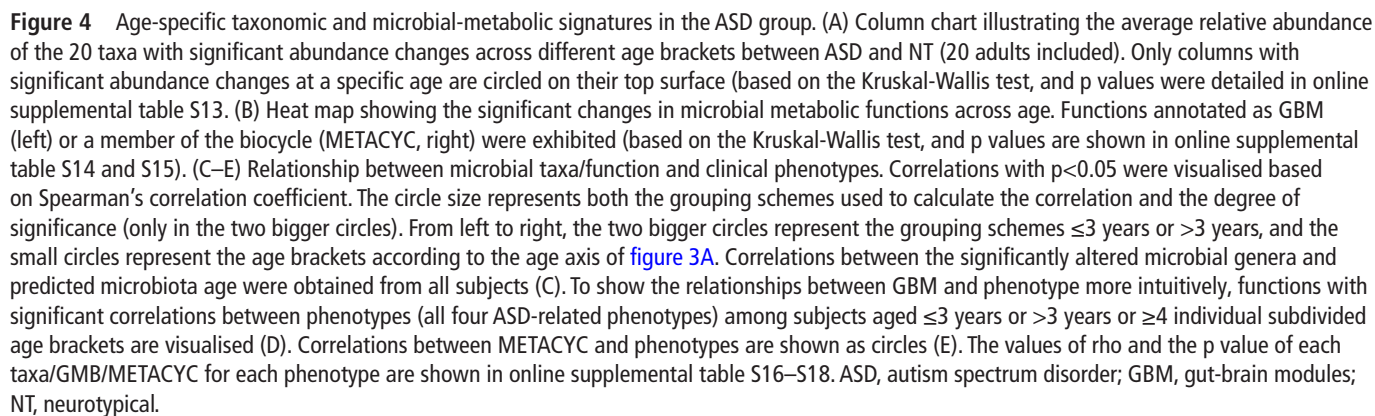
supplemental figure S8B). For example, glutamate degradation I (MGB-050) was positively correlated with the severity of both clinical manifestations before 3 years of age (figure 4D and online supplemental table S17). In contrast, glutamate degradation II (MGB-051) was inversely related to ASD severity in subjects after 3 years of age. From a higher level of functional annotation, functions (METACYC) correlated with the severity of ASD, with significance mainly in amino acid metabolism, aromatic compound metabolism and cofactor biosynthesis (figure 4E). Additionally, changes in the abundance of MGB-56, MGB-004, PWY-5188, PWY-5189 and X1CMET2-PWY-N10 between two groups showed a significant association with the clinical condition of ASD (online supplemental figure S9B and S9C). In brief, the analysis further suggested that gut microbes may involve in the pathological process of ASD via deregulation of various metabolic activities.

Gut microbiota as biomarkers for ASD and NT

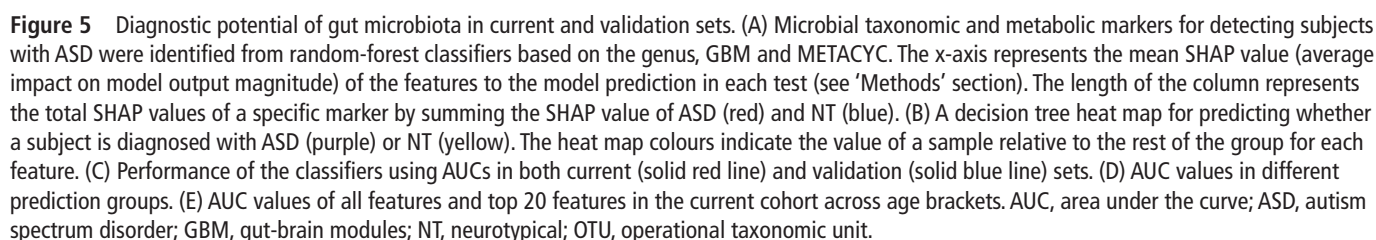
To define ASD-associated microbes or metabolic pathway markers, we devised a random forest model to correlate ASD and NT with gut microbiota data at OTUs, genus, GBM and METACYC levels in the current and validation cohorts. A unified framework for interpreting predictions, namely, SHAP, was conducted (see 'Method' section). Given that the microbial ecosystem differed dramatically in subjects before and after 3 years of age, we first defined two sets of markers for children ≤3 years of age and >3 years of age, respectively (online supplemental figure S10A–D). The predictive accuracy in the group ≤ or >3 years of age is 0.83 and 0.86 AUC, respectively. To provide a prediction tool for clinical applicability in all children, we selected the 20 top features with the highest model-building importance value and lowest inner subcategory bias to re-establish the prediction model. All 20 features showed no intergroup specificity, and in part, changes in abundance between the two groups remained consistent in the validation cohorts (figure 5A and online supplemental figure S11A–C). Each feature had an equal importance value to the ASD (red) or NT (blue), and the contribution of the subjects' ages was at the middle level (figure 5A).

To create an interpretable decision model with greater practical clinical value, we visualised a typical decision tree (figure 5B) using *treeheatr* (see 'Methods' section). The metabolic pathway of propionate synthesis III (MGB-055, no. 9), which showed significantly increased abundance changes in the ASD cohort (age brackets 3, 6 and 9) (figure 4B and online supplemental table S14), was placed at the top of the tree and tagged with a cut-off value (accounted abundance, 41119.31) (figure 5B). Other features were also distributed on the tree's leaf nodes and branched with specific cut-off values. The MGB-055 abundance value >41119.31 distinguished more subjects with ASD at the left bottom of the tree, consistent with the finding in an animal study indicating that propionic acid may cause autism-like behaviours in mice.⁴¹ Almost all subjects with an abnormal abundance of MGB-055, MGB-044 and PWY-5088 which were annotated to metabolic activities of intestinal microbes were addressed to the ASD group (figure 5B).

As most microbial features were age-dependent, we incorporated individual physiological ages into the performance verification. At the OTU level, our model showed 56%–79% accuracy in the current and validation sets, respectively (figure 5C–5D, online supplemental figure S10E–J). The model based on genus level was slightly inferior, with 62%–72% accuracy. The accuracy of the GBM and METACYC models in distinguishing



Most METACYC features in the ‘top 20’ belonged to amino acid metabolism, aromatic compound metabolism and carbohydrate metabolism (online supplemental table S15 and S18). Unexpectedly, the accuracies of the top 20 features was slightly improved (86%) when compared with that of all features (85%) in the present current cohort and was maintained in the validation set



In short, the results indicated that the predictive model based on these identified biomarkers showed a admired discriminant ability to predict the ASD status.

DISCUSSION

Based on our population-based multiregional gut microbiota results, we demonstrated that gut microbiota development significantly deviated and was unsustainably immature in children with ASD, considering microbial composition, function and relationship profiles compared with that in NT subjects. We further explored and confirmed the diagnostic potential of gut microbiota in large-scale human cohorts, suggesting that the gut microbiome can be considered a non-invasive method for the early warning of ASD. In addition to behaviour symptoms, comorbidities such as GI dysfunction, sleep disturbance and food allergies are frequently reported in children with ASD.^{42–44} Correspondingly, we illustrated that the abundances and functions of microbial taxa were significantly related to the mentioned comorbidities in subjects with ASD.

We identified significant changes in microbial relationships in individuals with ASD, especially before 3 years of age, and the degree of the altered relationship correlated with the severity of ASD, indicating that alteration in microbial relationships occurred in the early stages of microbiome development in children with ASD, which is consistent with the nodes when behavioural defects in children with ASD occur. Increasing evidence has suggested that the gut microbiota plays a key role in biological and physiological features underlying neurodevelopment.⁴⁵ The analysis further implicates that the establishment of early community relationships among microbes may potentially impact neurodevelopment in children.

Unlike growth faltering caused by severe paediatric pathological conditions, such as severe acute malnutrition^{23 32} and cystic fibrosis,⁴⁶ only transient dysplasia of gut microbiota was observed in the children with ASD in the present cohort; however, detachments of early predominant bacteria, such as *Veillonella*, were delayed. Roswall *et al* have recently reported that *Veillonella* and *Clostridium* showed dynamic changes during the early developmental stage of healthy children similar to that observed in the present cohorts.¹⁵ However, both OTUs were significantly disturbed in children with ASD, suggesting that both OTUs play an important role in establishing of the gut microecological system at the early stage of life.

Most disrupted microbial functions in ASD belong to GBM, amino acid metabolism and aromatic compound metabolism. Previous studies have indicated that these functions are involved in individual nervous system development, neurotransmitter biosynthesis and neuronal response regulations.⁴⁷ For instance, we observed that the bacterial pathways for tryptophan metabolism, including the production of neuroprotective kynurenic acid (kynurenine synthesis, MGB-004) and neurotoxic quinolinic acid (tryptophan synthesis, MGB-055), were significantly correlated with the severity of ASD. Similar correlations were also shown in propionate and dopamine metabolism, which is involved in the metabolic network of neurotransmitters. Our findings highlight that the gut microbiota may profoundly impact neural development by regulating neurotransmitter metabolism.

Most previous studies that constructed gut microbiota-based diagnostic models are typically described in a ‘parts list’, enumerating of component members and model’s efficiency,^{9 48 49} thus limiting the interpretation and practical application of gut microbiota features in human disease progression. In the current study, we visualised the decision-making process of our model and revealed the inner-group specificity of the factors in our model using *treeheat*²² and SHAP.²¹ Accordingly, the decision-making process was visualised by distributing each factor on a tree’s leaf nodes and branched with specific cut-off values. Moreover, our

model indicates the specific cut-off values and their final judgement results of a factor, which can provide practically available indexes for an independent individual in clinical warning or treatment as well as for the scientific exploration of potential pathogenic factors.

In summary, the progressive deviation in the development of gut microbiota of subjects with ASD highlighted the influence of age on the composition of gut microbiota, which suggested that individuals with ASD should be compared with healthy controls at the same physiological age to exclude ‘age-discriminatory’ features for both clinical application and scientific research. As to the construction of animal model based on faecal microbiota transplantation, researchers should consider the conversion and matching of age between human faeces donor and recipient mice. In the future, by constructing longitudinal cohorts of children with ASD and NT, and integrating metagenomics and metabolomics analyses, we can precisely identify potential developmental windows during which the gut microbiota may be particularly sensitive to ASD development, and further provide critical clues to reveal how gut microbiota participates in the pathogenesis of autism by regulating metabolic pathways.

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Contributors YW, XL, GJ, AC and ML conceived and devised the study. CJ, KM, ZZ, XP, ZL, JQ, QZ and XL performed data analysis and draw figures. XL, ZZ and CJ drafted the manuscript. ML, AC, YW, XX, SQ, HL, YL, LC and XQ conducted subject recruitment and collected clinical samples and data. YN, GJ, XS, ZH and YJ assisted with sample collection and data arrangement. XL, GJ, JL and YW supervised the study and revised the manuscript. All of authors interpret and confirm these data and manuscript.

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Competing interests None declared.

Patient consent for publication Consent obtained from parent(s)/guardian(s)

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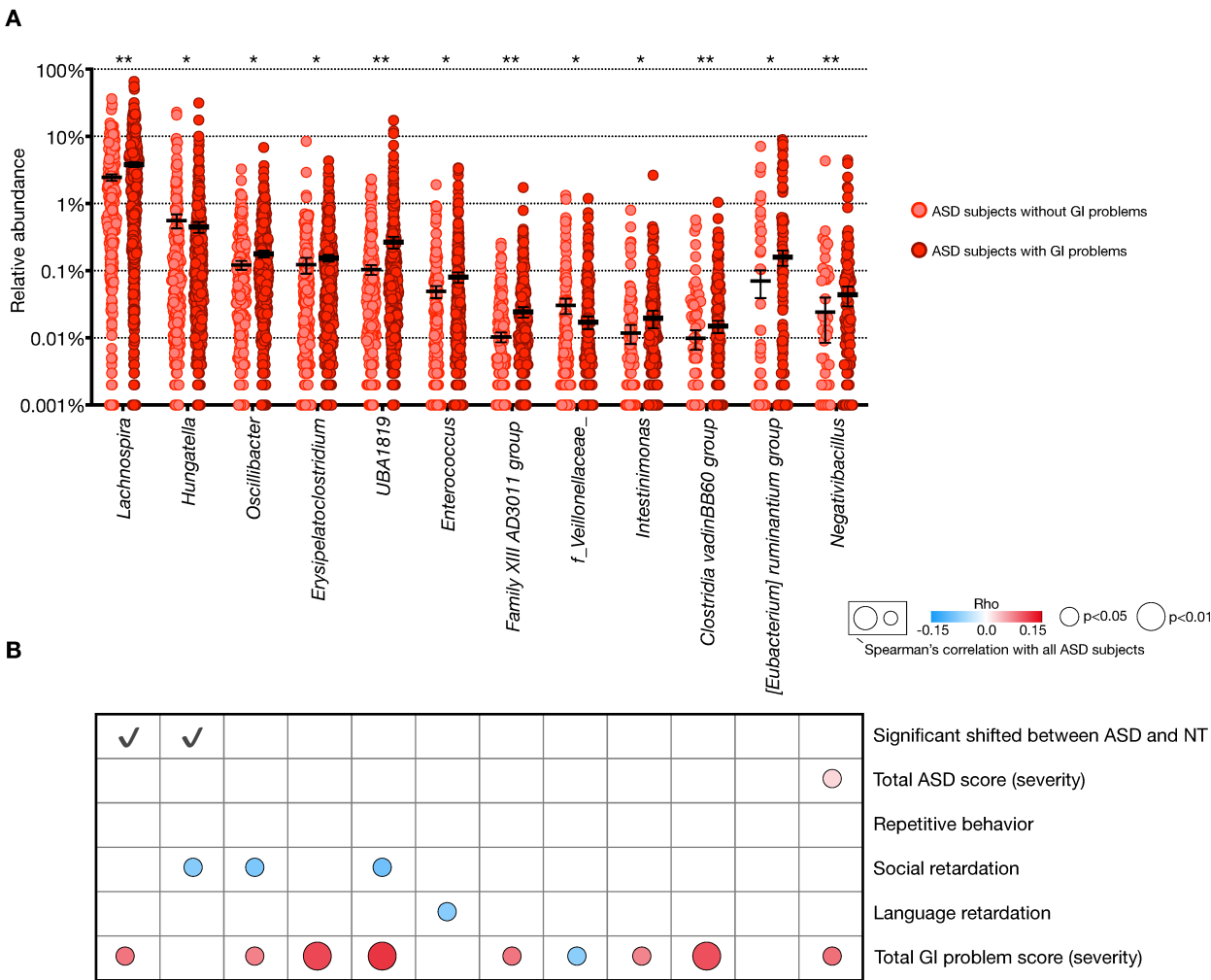
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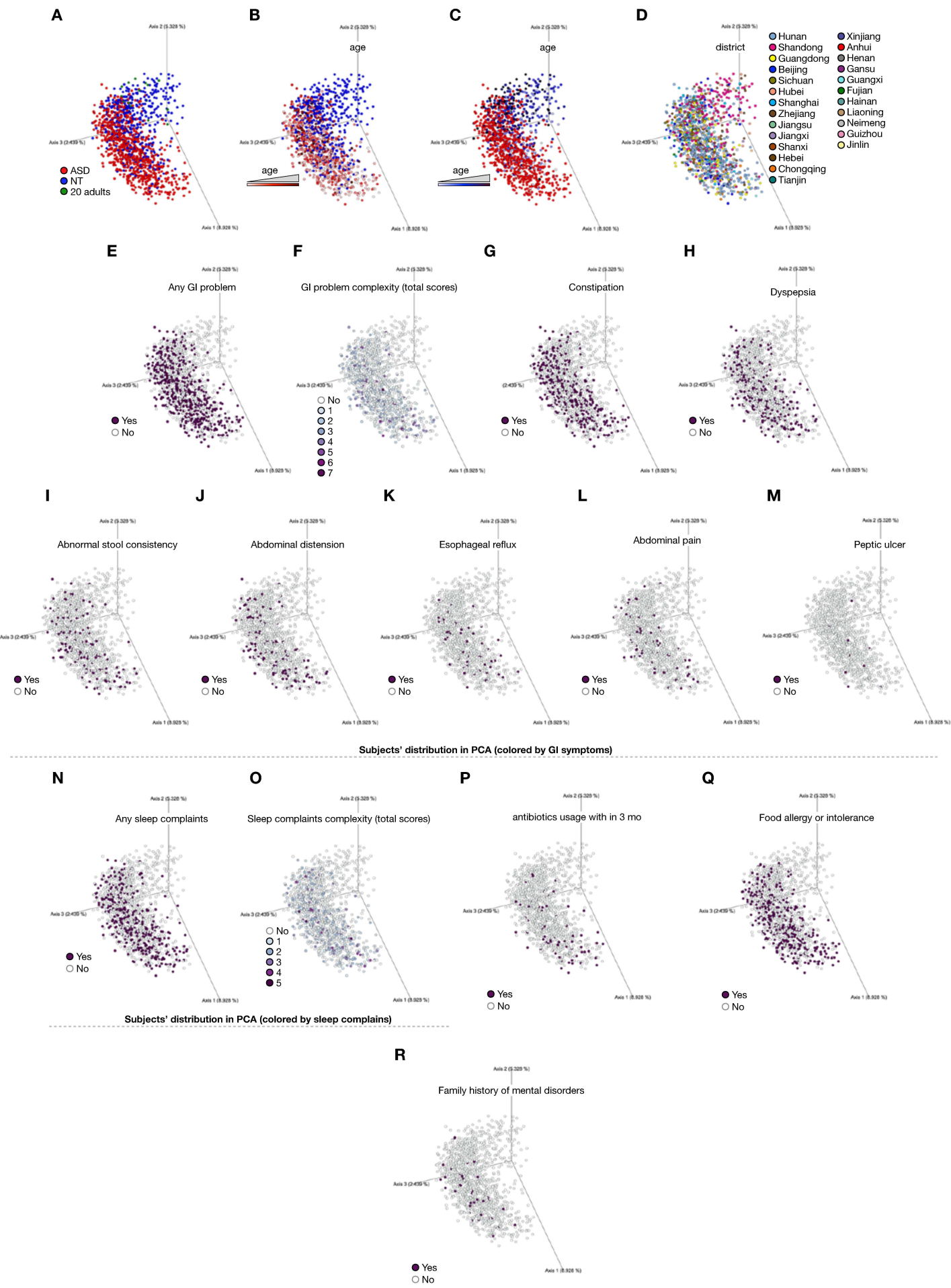
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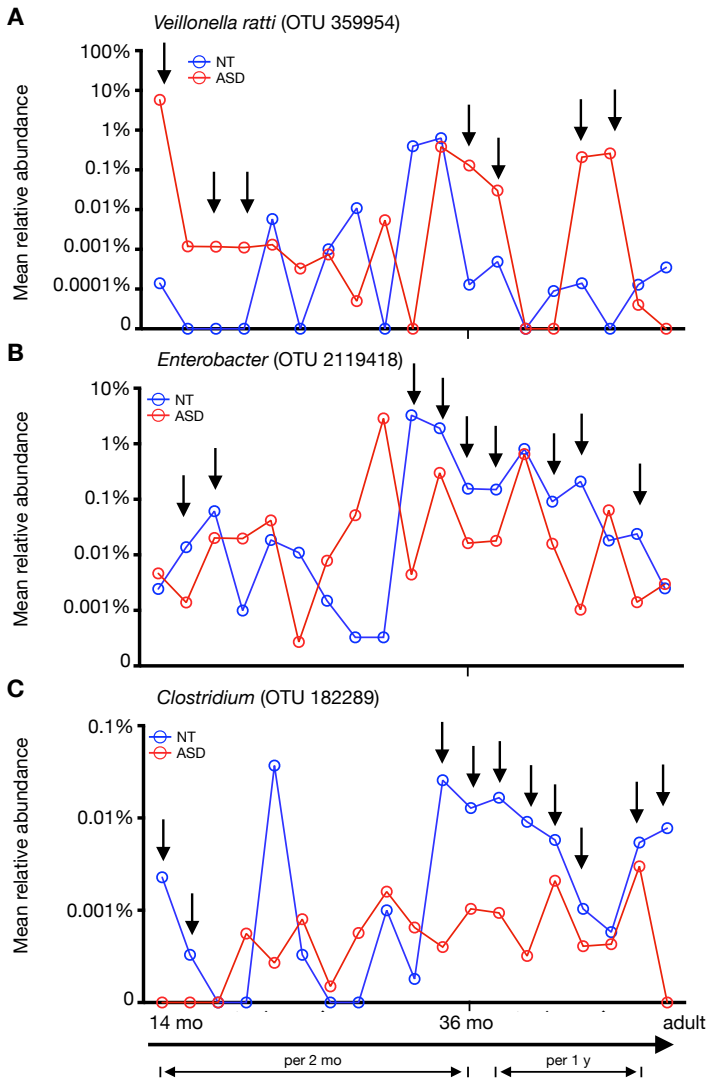
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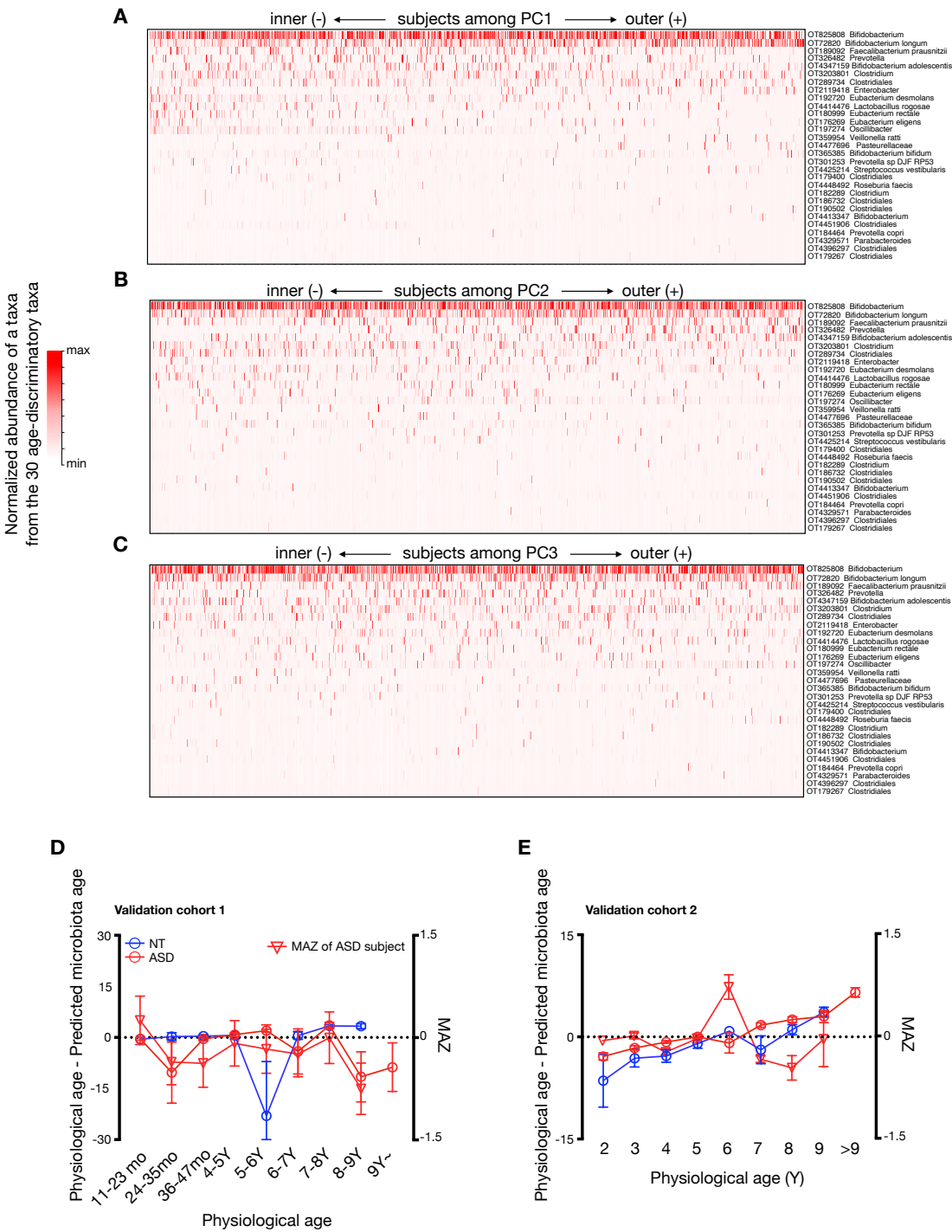
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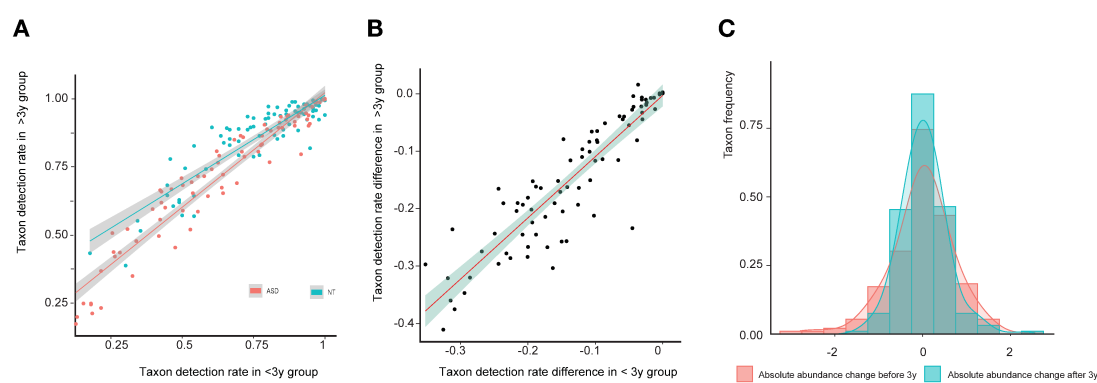
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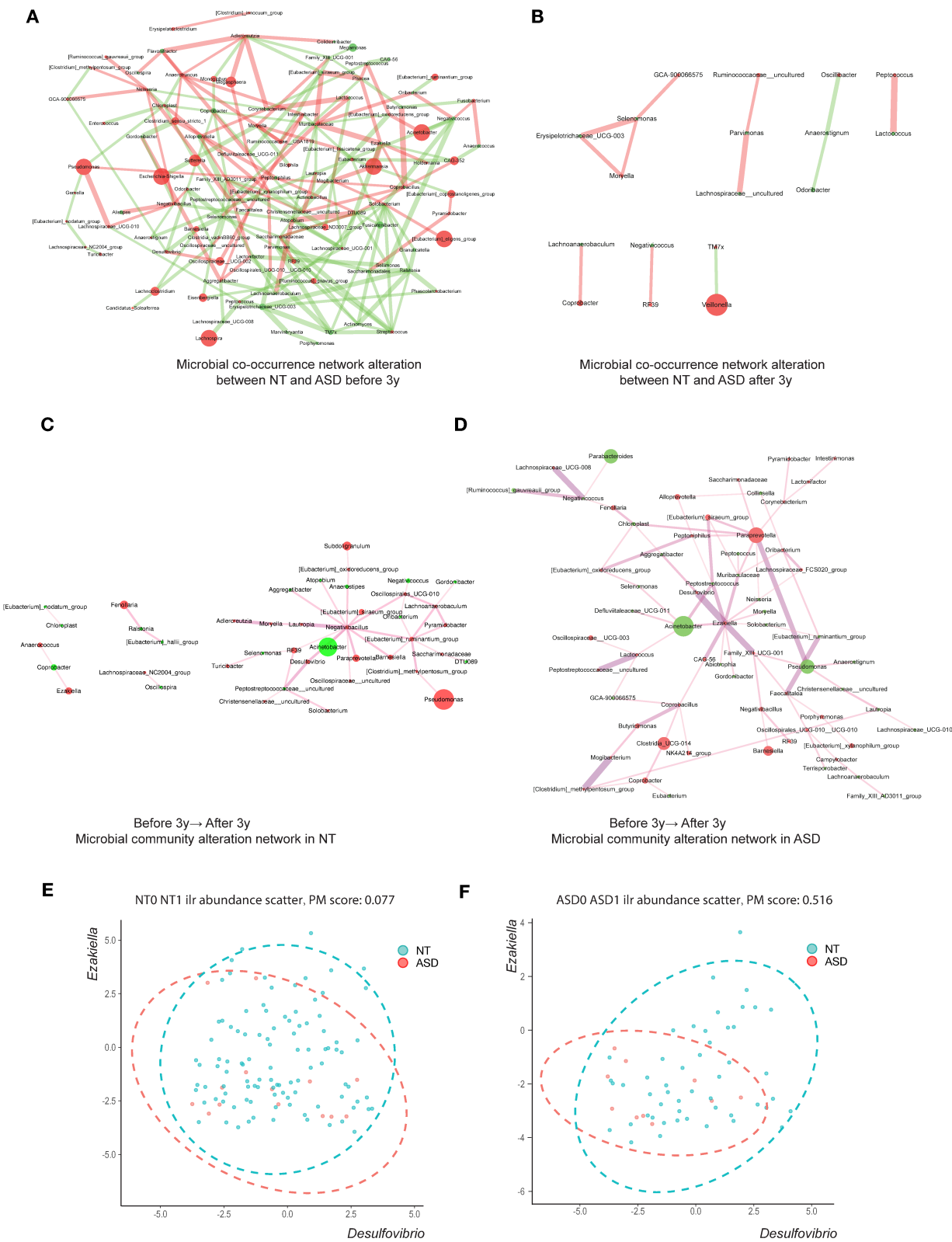
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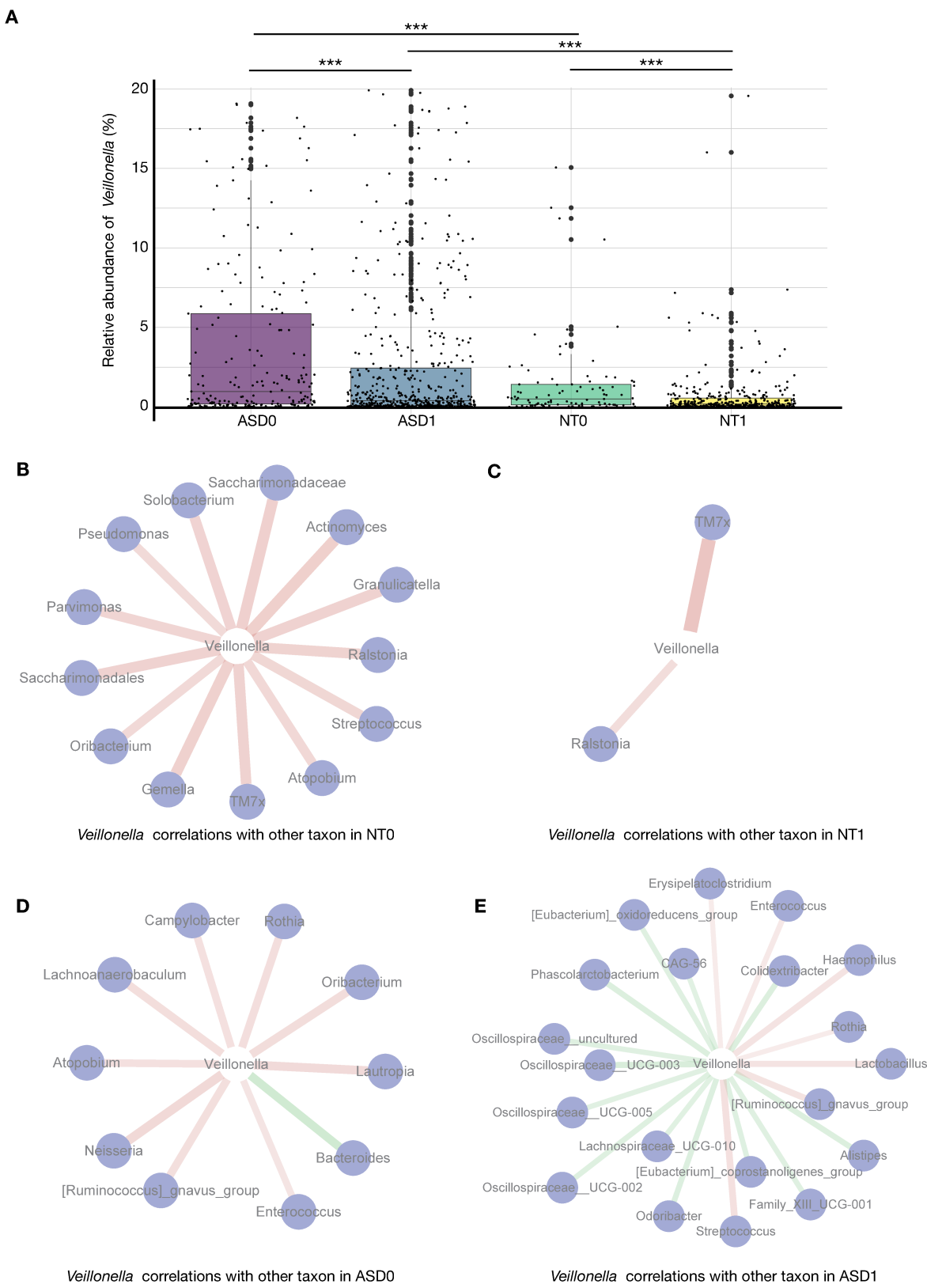
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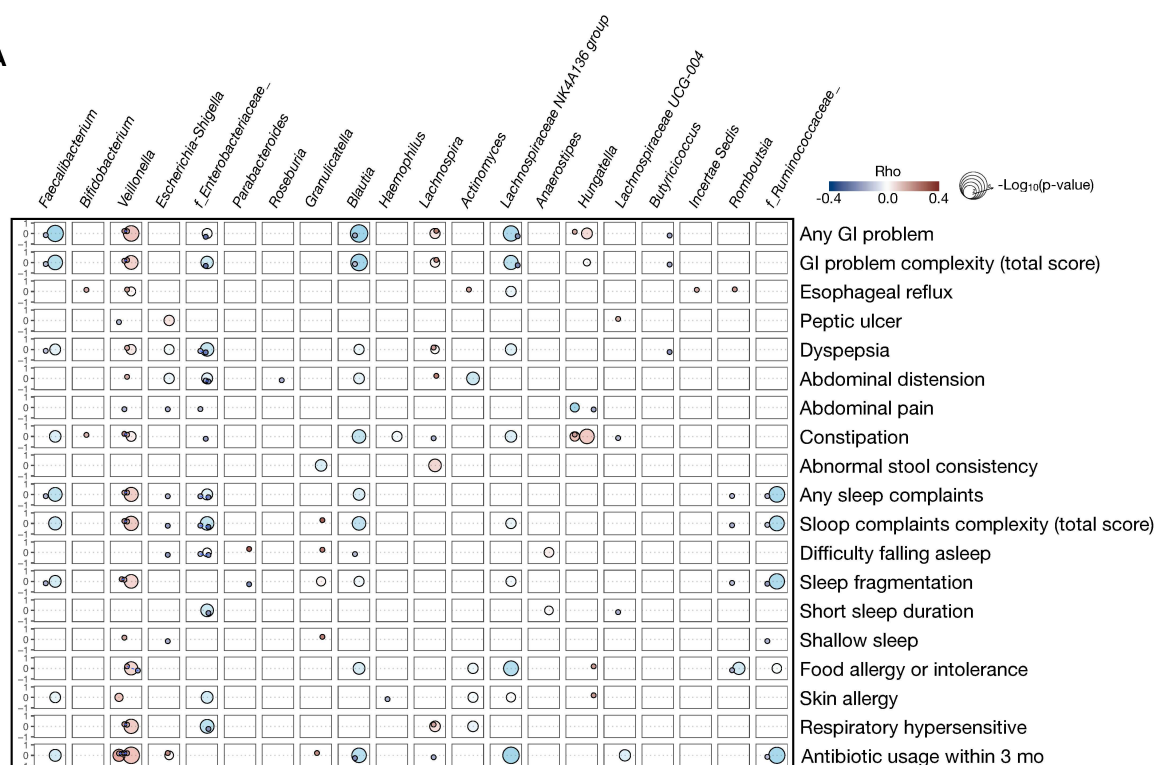
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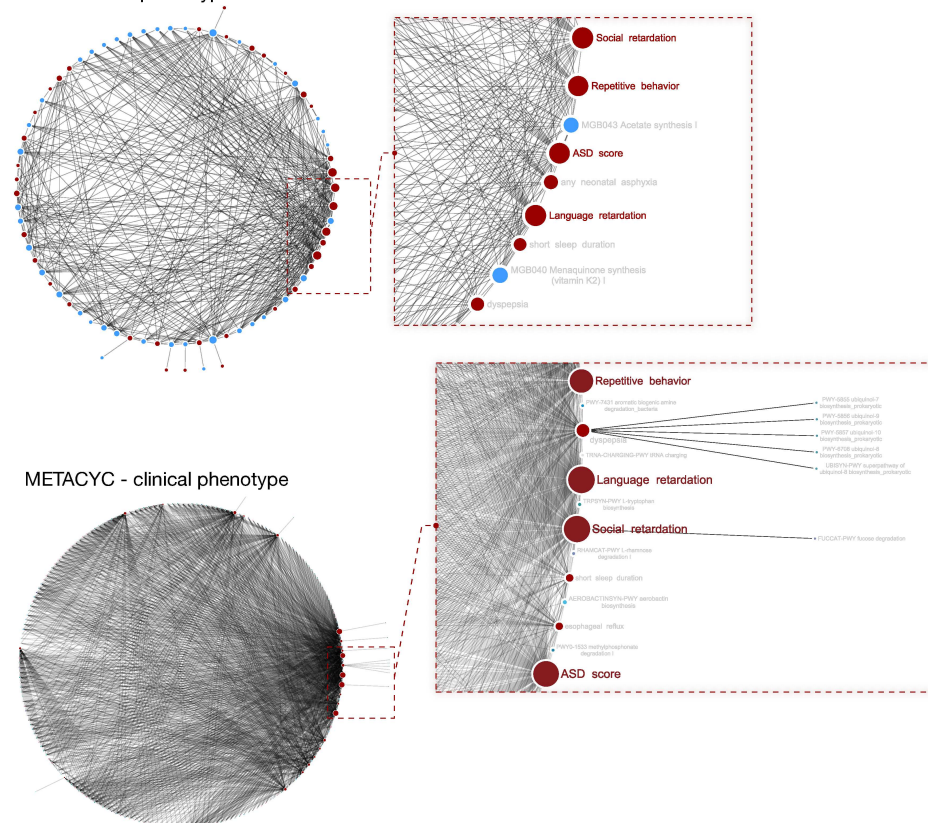
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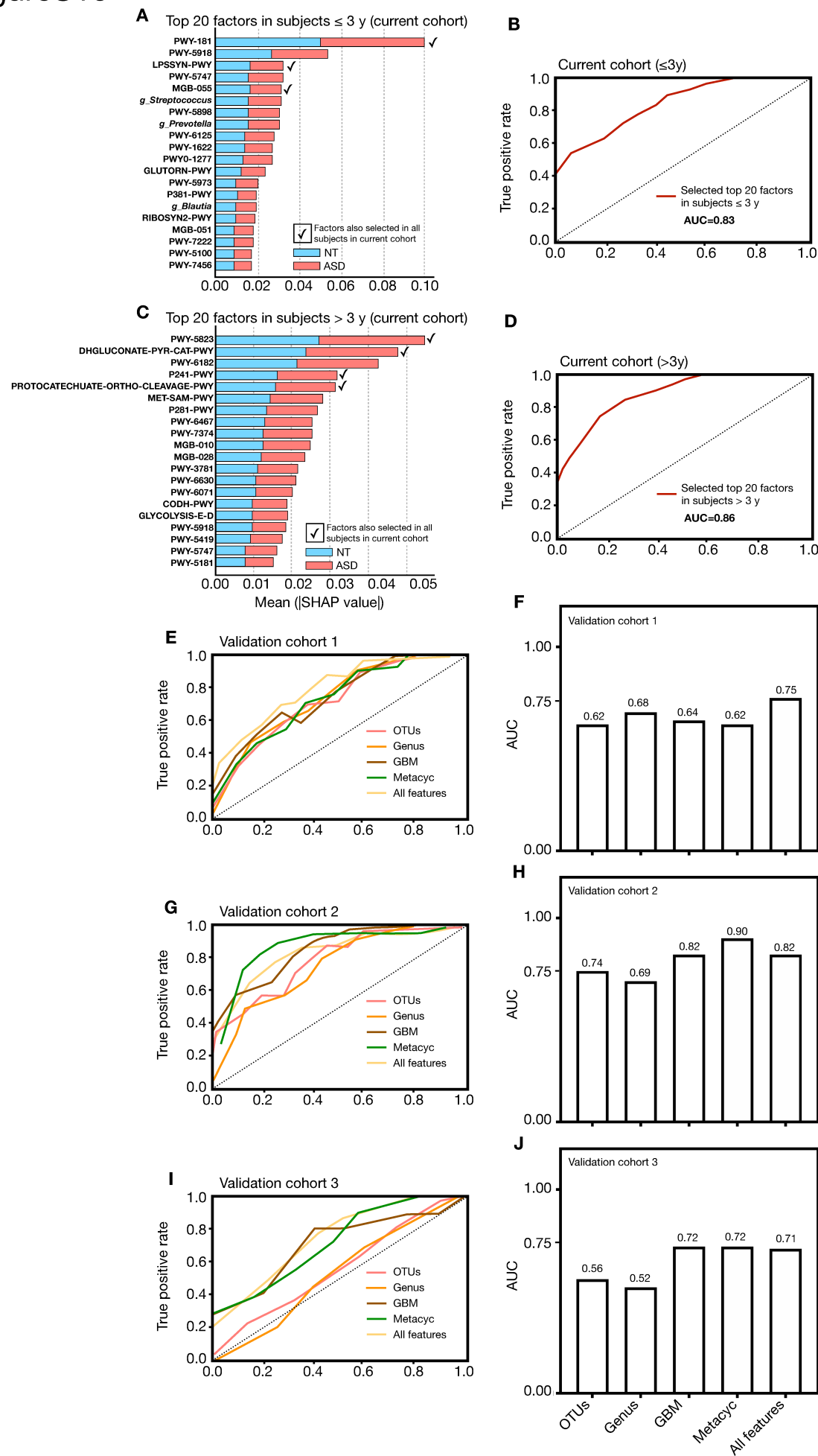


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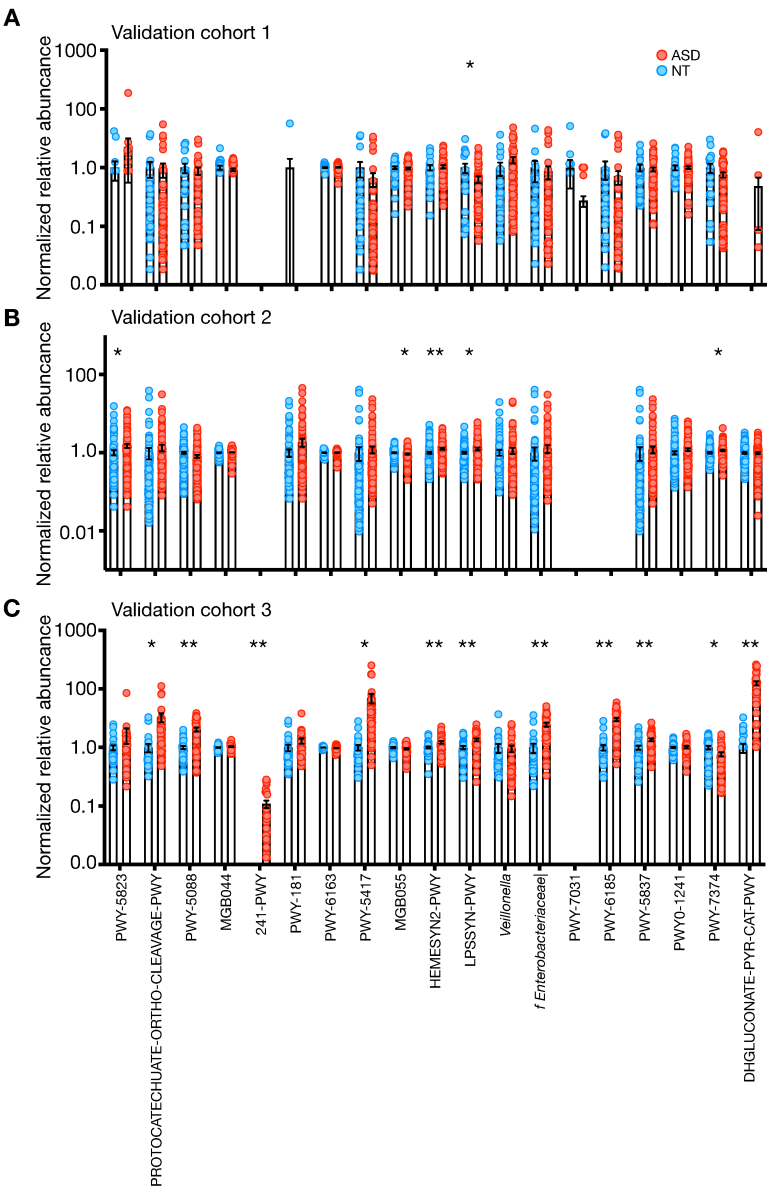
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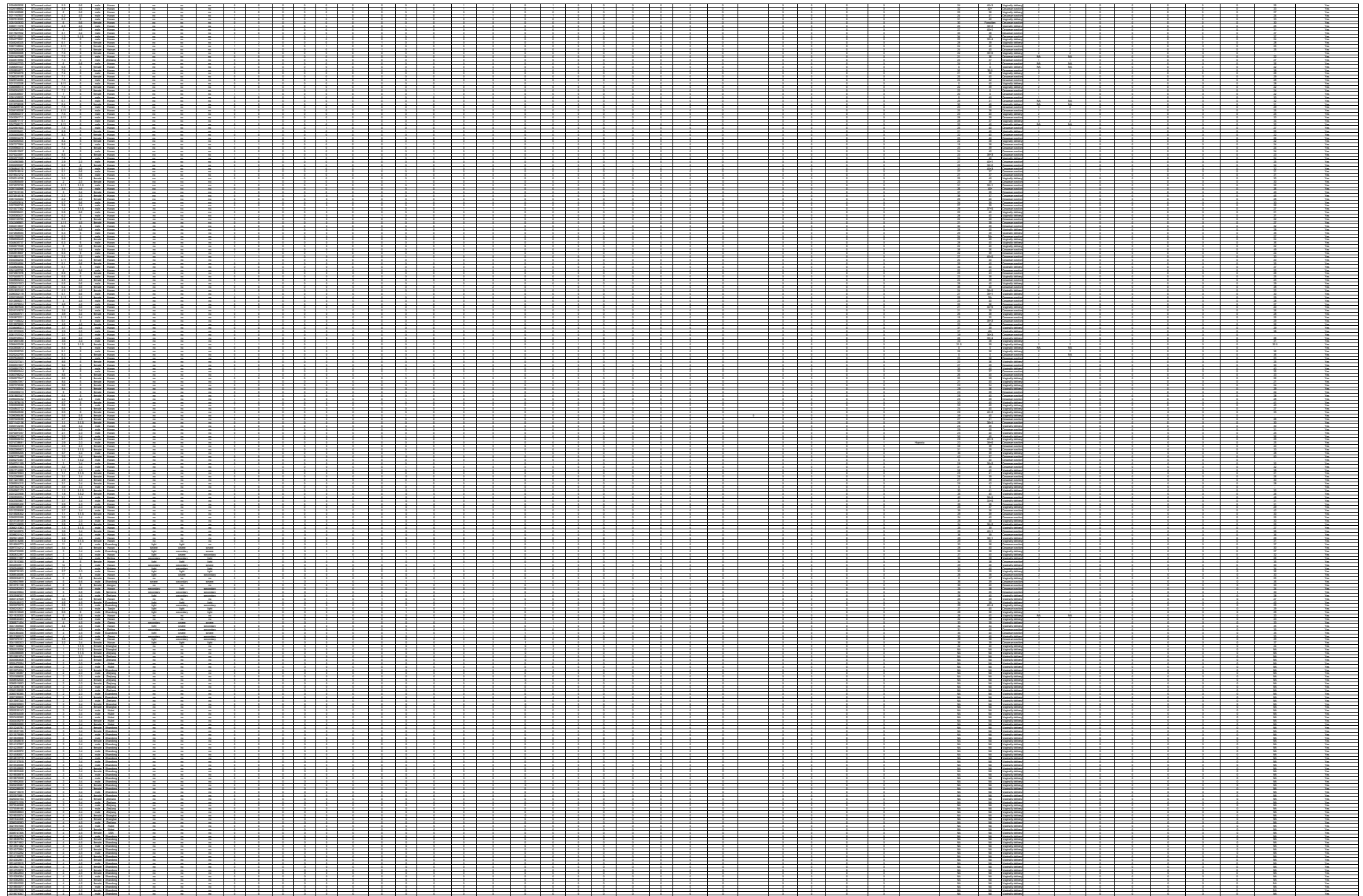
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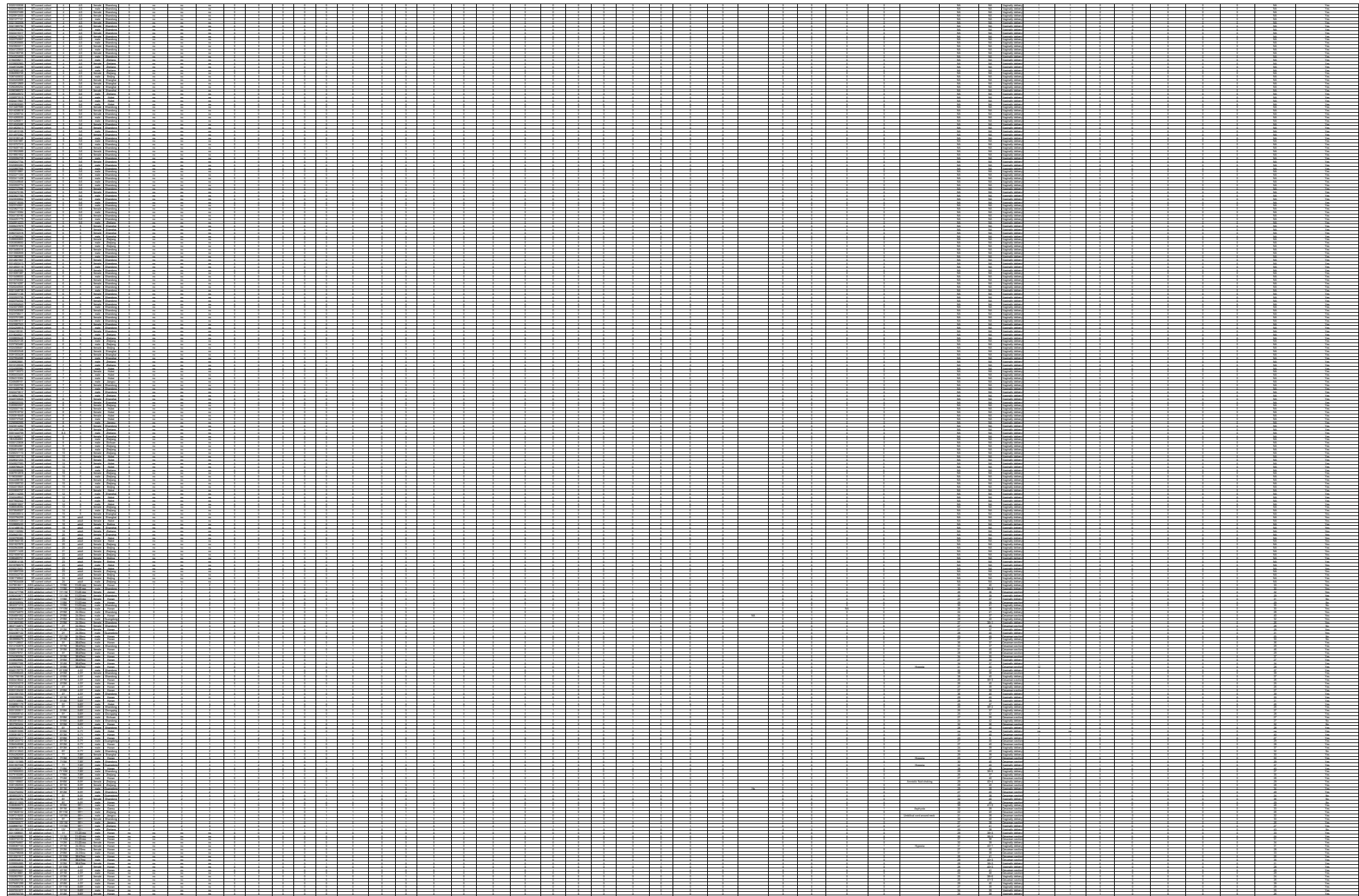


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Table 2 Summary of subject's distributions according to age categories

Current cohort	age brackets (y)	ASD	NT
	1-1.5	11	16
	1.5-2	14	3
	2-3	128	40
	3-4	179	70
	4-5	134	63
	5-6	152	75
	6-7Y	34	65
	7-8Y	40	33
	8-9Y	29	29
	9-12Y	39	28
	12-15Y	9	8
	adult	3	20
	total	772	450
Validation cohort 1	age brackets	ASD	NT
	11-23 mo	8	4
	24-35mo	9	3
	36-47mo	8	3
	4-5Y	9	6
	5-6Y	8	5
	6-7Y	8	4
	7-8Y	8	3
	8-9Y	7	4
	9Y~	8	none
	total	73	32

Table 3 Identification of severity levels for autism spectrum disorder based on DSM-IV and DSM-V

	Social communication	Restricted, repetitive behaviors	Language development delay
Mild	Requiring support. Without supports in place, deficits in social communication cause noticeable impairments. Difficulty initiating social interactions, and clear examples of atypical or unsuccessful response to social overtures of others. May appear to have decreased interest in social interactions.	Requiring support. Inflexibility of behavior causes significant interference with functioning in one or more contexts. Difficulty switching between activities. Problems of organization and planning hamper independence.	Difficulty in putting words together into a sentence or leaving words out of a sentence.
Moderate	Requiring substantial support. Marked deficits in verbal and nonverbal social communication skills; social impairments apparent even with supports in place; limited initiation of social interactions; and reduced or abnormal responses to social overtures from others.	Requiring substantial support. Inflexibility of behavior, difficulty coping with change, or other restricted/repetitive behaviors appear frequently enough to be obvious to the casual observer and interfere with functioning in a variety of contexts. Distress and/or difficulty changing focus or action.	Imitation, just babbling or inability to speak in short sentences.
Severe	Requiring very substantial support. Severe deficits in verbal and nonverbal social communication skills cause severe impairments in functioning, very limited initiation of social interactions, and minimal response to social overtures from others.	Requiring very substantial support. Inflexibility of behavior, extreme difficulty coping with change, or other restricted/repetitive behaviors markedly interfere with functioning in all spheres. Great distress/difficulty changing focus or action.	Language only for needs or not talking.

Table 4 Summary of **subject's distributions** according to district categories

	Table 4 Summary of subject's distributions according to district categories		
	Region	ASD	NT
Current cohort	Hunan	285	198
	Guandong	104	2
	Shandong	65	120
	Beijing	56	10
	Sichuan	36	1
	Hubei	28	33
	Shanghai	25	25
	Zhejiang	24	57
	Jiangsu	20	3
	Jiangxi	18	0
	Shanxi	15	0
	Hebei	13	0
	Chongqing	11	0
	Tianjin	11	0
	Xinjiang	11	0
	Anhui	9	0
	Henan	9	0
	Gansu	6	0
	Guanxi	5	0
	Fujian	4	0
	Hainan	4	1
	Liaoning	4	0
	Neimeng	4	0
	Guizhou	3	1
	Jilin	2	0
Validation cohort 1	Region	ASD	NT
	Hunan	31	32
	Shandong	20	0
	Guandong	6	0
	Zhejiang	6	0
	Chongqing	2	0
	Hubei	2	0
	Jiangsu	2	0
	Gansu	1	0
	Jiangxi	1	0
	Shanghai	1	0
	Sichuan	1	0

Table5 Alpha diversity under different under different parameters						
shannon	20 adults	5.73 ± 0.756	p value	Adults vs. NT	Adults vs. ASD	NT vs. ASD
	NT	5.4206 ± 1.14		0.4691	0.0007	<0.0001
	ASD	4.77 ± 1.16				
simpson	20 adults	0.94 ± 0.04	p value	Adults vs. NT	Adults vs. ASD	NT vs. ASD
	NT	0.91 ± 0.09		0.3992	0.0141	<0.0001
	ASD	0.87 ± 0.12				
chao1	20 adults	2355.07 ± 1274.39	p value	Adults vs. NT	Adults vs. ASD	NT vs. ASD
	NT	2977.13 ± 1583.84		0.1283	0.9587	<0.0001
	ASD	2267.15 ± 1294.37				
ace	20 adults	2394.17 ± 1329.29	p value	Adults vs. NT	Adults vs. ASD	NT vs. ASD
	NT	3052.70 ± 1630.33		0.1118	0.9717	<0.0001
	ASD	2319.87 ± 1319.99				
goods_coverage	20 adults	99.51% ± 0.33%	p value	NA		
	NT	99.35% ± 0.39%				
	ASD	99.55% ± 0.29%				

Shannon index				
	NT		ASD	
	mean	sd	mean	sd
0-16mo	4.759399451	1.359547967	3.836082927	0.964003457
16-18mo	3.764213312	1.025357825	2.849265085	1.619845272
18-20mo	4.196894667	0.606140885	3.616706239	1.347681161
20-22mo	4.926090339	0.752870905	3.775844587	1.573532974
22-24mo	5.542285549	1.01700189	3.841787444	1.387423655
24-26mo	5.463804669	1.194155942	4.701179309	0.926512271
26-28mo	5.022808685	1.136191124	4.458895719	0.733147925
28-30mo	4.353316031	0.753168643	4.586324527	1.110997305
30-32mo	4.745291597	0.912644623	4.570967246	1.179000381
32-34mo	4.642527458	0.76418573	5.26308442	0.960932
34-36mo	5.21101315	0.84406933	4.647607285	1.099560467
3-4y	4.543924885	1.135308193	4.568936906	1.000936654
4-5y	5.59501416	0.995748552	4.928618373	1.122853308
5-6y	5.47851421	1.041302464	4.858354881	1.109141125
6-7y	5.743279675	1.157214041	4.97313772	1.213777533
7-8y	5.60510922	1.060199335	4.984953987	1.299020924
8-9y	6.011443948	1.199575446	5.329017922	1.058225814
9-16y	5.781164334	1.030728251	5.299373648	1.081186082
adults	5.7284785	0.757131446	5.230236748	0.22090182

Table 6 Host multifactorial effects on composition of gut microbiota calculated by EnvFit

Factors	EnvFit			
	≤3y		>3y	
	r-square	p value	r-square	p value
ASD diagnosis	0.0170	0.038	0.0173	0.001
District	0.0544	0.981	0.0295	0.196
Age	0.0384	0.001	0.0283	0.001
Gender	0.0059	0.292	0.0010	0.379
Any GI problem	0.0033	0.49	0.0050	0.01
Esophageal reflux	0.0033	0.505	0.0019	0.165
Peptic ulcer	0.0019	0.687	0.0030	0.041
Dyspepsia	0.0019	0.69	0.0022	0.106
Abdominal distension	0.0166	0.033	0.0003	0.753
Abdominal pain	0.0168	0.021	0.0016	0.198
Constipation	0.0064	0.266	0.0049	0.009
Abnormal stool consistency	0.0137	0.055	0.0006	0.569
Any Sleep complaints	0.0153	0.045	0.0011	0.334
Difficulty falling asleep	0.0089	0.149	0.0001	0.92
Sleep fragmentation	0.0155	0.042	0.0024	0.089
Short sleep duration	0.0021	0.638	0.0006	0.565
Shallow sleep	0.0131	0.057	0.0001	0.914
Food allergy or intolerance	0.0070	0.233	0.0103	0.001
Skin allergy	0.0015	0.724	0.0004	0.707
Respiratory hypersensitivity	0.0113	0.084	0.0027	0.072
Antibiotic usage with in 3 mo	0.0149	0.047	0.0113	0.001
Infection within 1 mo	0.0025	0.605	0.0002	0.791
Age of mother during pregnancy	0.0000	1.000	0.0000	1.000
Age of father during pregnancy	0.0000	1.000	0.0000	1.000
Assisted reproduction	0.0111	0.09	0.0028	0.069
Smoking	0.0000	1.000	0.0001	0.965
Drinking	0.0056	0.329	0.0002	0.825
Infection during pregnancy	0.0023	0.603	0.0007	0.489
Eclampsia/Pre-eclampsia	0.0052	0.37	0.0011	0.355
Gestational age	0.0000	1.000	0.0000	1.000
Mode of delivery	0.0037	0.454	0.0024	0.081
Neonatal asphyxia	0.0001	0.971	0.0028	0.062
Family history of mental disorders	0.0129	0.054	0.0030	0.049
...				

Table 7 Summary of demographic and clinical characteristics of healthy children (NT) and kids with ASD					
Current cohort	Diagnosis	Total ASD score	mean ± SEM	ASD	NT (healthy adults included)
		Repetitive behavior	mean ± SEM	1.38 ± 0.05	/
		Social retardation	mean ± SEM	2.00 ± 0.07	/
		Language retardation	mean ± SEM	2.04 ± 0.07	/
	Demographic	Gender	Female (Male)	127 (645)	217 (233)
		Any GI problems	Yes/all	494/772 (63.9%)	48/450 (10.7%)
	Comorbidity	Mean value of total GI problem score of each subject	mean ± SEM	1.05 ± 1.33	0.18 ± 0.56
		Esophageal reflux	Yes/all	54/772 (6.9%)	2/450 (0.4%)
		Peptic ulcer	Yes/all	10/772 (1.3%)	1/450 (0.2%)
		Dyspepsia	Yes/all	238/772 (30.8%)	15/450 (3.3%)
		Abdominal distension	Yes/all	129/772 (16.7%)	1/450 (0.2%)
		Abdominal pain	Yes/all	61/772 (7.9%)	9/450 (2%)
		Constipation	Yes/all	346/772 (44.8%)	19/450 (4.2%)
		Abnormal stool consistency	Yes/all	127/772 (16.4%)	7/450 (1.6%)
		Any sleep complaints	Yes/all	354/772 (45.8%)	21/450 (4.7%)
		Mean value of sleep complaints score	mean ± SEM	0.71 ± 1.02	0.07 ± 0.25
		Difficulty falling asleep	Yes/all	193/772 (25.0%)	2/450 (0.4%)
		Sleep fragmentation	Yes/all	216/772 (27.9%)	9/450 (2.1%)
		Short sleep duration	Yes/all	84/772 (10.9%)	7/450 (1.6%)
		Shallow sleep	Yes/all	119/772 (15.4%)	7/450 (1.6%)
		Total Allergy score	mean ± SEM	0.67 ± 0.03	0.13 ± 0.02
		Food allergy or intolerance	Yes/all	353/772 (45.7%)	15/450 (3.3%)
		Skin allergy	Yes/all	157/772 (20.3%)	15/450 (3.3%)
		Respiratory hypersensitivity	Yes/all	109/772 (14.1%)	5/450 (1.1%)
	Clinical	Antibiotic usage within 3 mo	Yes/all	67/772 (8.7%)	3/450 (0.6%)
		Any infection within 1 mo	Yes/all	21/772 (2.7%)	2/450 (0.4%)
		Any neonatal asphyxia	Yes/all	51/772 (6.6%)	1/450 (0.02%)
	Perinatal	Age of mother during pregnancy	mean ± SEM	29.28 ± 0.20	26.58 ± 0.34
		Age of father during pregnancy	mean ± SEM	31.83 ± 0.21	29.88 ± 0.26
		Premature delivery	Yes/all/NA or Forgotten	83/772 (10.7%) /0	18/197 (9.1%) /253
		Mode of delivery	Cesarean section/Vaginally delivery	446/327	106/343
		Assisted reproduction	Yes/all	97/772 (12.5%)	7/450 (1.6%)
		Eclampsia/Pre-eclampsia	Yes/all	9/772 (1.2%)	2/450 (0.4%)
		Infection during pregnancy	Yes/all	55/772 (7.1%)	1/450 (0.2%)
	Family history	Family history of mental disorders	Yes/all	42/772 (5.4%)	0/450 (0%)
Validation cohort 1	Diagnosis	Total ASD score	mean ± SEM	ASD	NT
		Repetitive behavior	mean ± SEM	6.21 ± 0.18	/
		Social retardation	mean ± SEM	1.62 ± 0.08	/
		Language retardation	mean ± SEM	2.34 ± 0.07	/
	Demographic	Gender	Female (Male)	15 (73)	14 (32)
		Any GI problems	Yes/all	45/73 (61.6%)	5/32 (15.6%)
	Comorbidity	Mean value of total GI problem score of each subject	mean ± SEM	1.33 ± 0.17	0.31 ± 0.15
		Esophageal reflux	Yes/all	8/73 (10.9%)	0/32 (0%)
		Peptic ulcer	Yes/all	3/73 (4.1%)	0/32 (0%)
		Dyspepsia	Yes/all	32/73 (43.8%)	3/32 (9.4%)
		Abdominal distension	Yes/all	19/73 (26.0%)	0/32 (0%)
		Abdominal pain	Yes/all	8/73 (10.9%)	1/32 (3.1%)
		Constipation	Yes/all	23/73 (31.5%)	4/32 (12.5%)
		Abnormal stool consistency	Yes/all	1/73 (1.44%)	2/32 (6.3%)
		Any sleep complaints	Yes/all	37/73 (50.7%)	2/32 (6.3%)
		Mean value of sleep complaints score	mean ± SEM	0.78 ± 0.13	0.09 ± 0.07
		Difficulty falling asleep	Yes/all	24/73 (32.9%)	0/32 (0%)

Validation cohort 1		Sleep fragmentation	Yes/all	13/73 (17.8%)	2/32 (6.3%)
		Short sleep duration	Yes/all	11/73 (15.1%)	0/32 (0%)
		Shallow sleep	Yes/all	9/73 (12.3%)	1/32 (3.1%)
		Total Allergy score	mean ± SEM	0.64 ± 0.10	0.28 ± 0.09
		Food allergy or intolerance	Yes/all	26/73 (35.6%)	3/32 (9.4%)
		Skin allergy	Yes/all	14/73 (19.2%)	5/32 (15.6%)
		Respiratory hypersensitivity	Yes/all	7/73 (9.6%)	1/32 (3.1%)
		Antibiotic usage within 3 mo	Yes/all	7/73 (9.6%)	2/32 (6.3%)
		Any infection within 1 mo	Yes/all	2/73 (2.7%)	0/32 (0%)
		Any neonatal asphyxia	Yes/all	6/73 (8.2%)	1/32 (3.1%)
	Clinical	Age of mother during pregnancy	mean ± SEM	28.83 ± 0.41	28.67 ± 0.27
	Perinatal	Age of father during pregnancy	mean ± SEM	30.00 ± 0.43	28.33 ± 0.80
		Premature delivery	Yes/all/NA or Forgotten	4/72 (5.5%) /1	1/32 (3.1%) /0
		Mode of delivery	Cesarean section/Vaginally delivery	31/42	12/20
		Assisted reproduction	Yes/all	11/73 (15.1%)	1/32 (3.1%)
		Eclampsia/Pre-eclampsia	Yes/all	1/73 (1.44%)	0/32 (0%)
		Infection during pregnancy	Yes/all	8/73 (10.9%)	0/32 (0%)
		Family history	Family history of mental disorders	Yes/all	3/72 (4.2%)

Table 8 Predicted microbiota age and Physiological age.

Group	Physiological age	Predicted microbiota age	Mean value of Physiological age - Predicted microbiota age of each subject mean ± SEM, p value were calculated by one-way ANOVA respectively.				Microbiota-for-age Z score (MAZ) of each subject. The value of MAZ of each subject was calculated as: (microbiota age - median microbiota age of healthy children of same physiological age) / (standard deviation of microbiota age of healthy children of the same physiological age).	
			NT		ASD		ASD	
			mean	SEM	mean	SEM	Physiological age	MAZ
ASD	1.1	1.063991283	0-16mo	-7.388849941	6.164149533	-0.867568166	1.1	-0.039034064
ASD	1.1	1.781075827	16-18mo	-0.698748974	0.742024791	-0.385390627	1.1	-0.00024896
ASD	1.11	3.15698653	18-20mo	-0.547379962	0.228995236	-0.080775177	1.11	0.074170215
ASD	1.4	0.565391134	20-22mo	-0.980615501	0.265384868	-0.109104888	1.4	-0.066001956
ASD	1.4	0.70140548	22-24mo	-2.569078497	0.184055844	-5.626443526	1.4	-0.05864532
ASD	1.4	2.292544432	24-26mo	-2.180152462	0.793446388	-1.061452151	1.4	0.027414951
ASD	1.4	4.436245802	26-28mo	-1.294482887	0.658205823	-1.170565375	1.4	0.143361783
ASD	1.4	0.377813951	28-30mo	-0.634614481	0.416162351	0.017160573	1.4	-0.076147483
ASD	1.5	1.282486519	30-32mo	-0.661850718	0.369507484	-0.904633062	1.4	-0.720506371
ASD	1.6	0.36967383	32-34mo	-0.515342378	0.713771612	-0.802546909	1.5	-1.46481042
ASD	1.6	0.529338239	34-36mo	-2.395941997	0.411907781	-3.125436405	1.6	-1.334620658
ASD	1.6	2.217356637	3-4y	-2.89390495	1.938138562	-1.476573692	1.6	0.041783222
ASD	1.6	2.241657771	4-5y	-5.079561805	1.481656833	-2.768736079	1.6	0.061598276
ASD	1.6	3.203106806	5-6y	-8.958558559	2.389885103	-2.195826422	1.6	0.845560218
ASD	1.7	0.840901886	6-7y	-10.03102089	2.415255895	-1.895388965	1.6	-3.011405719
ASD	1.7	1.937369565	7-8y	-8.594485612	3.648938311	-7.303945452	1.7	-0.186517188
ASD	1.8	0.318060694	8-9y	-11.1081029	3.833408235	0.063331717	1.7	-1.506895578
ASD	1.8	0.803481103	9-16y	-3.629092634	2.295032983	-8.023251275	1.8	-1.111085594
ASD	1.8	0.882089074	adults	-2.663537246	3.010763383	-18.17773156	1.8	-1.046988949
ASD	1.8	1.754950471					1.8	-0.335261034
ASD	1.8	1.960165915					1.8	-0.167929129
ASD	1.8	2.237232407					1.8	0.05798985
ASD	1.8	3.204896043					1.8	0.847019155
ASD	1.9	2.18280643					1.9	-0.468395672
ASD	1.9	3.550967351					1.9	1.935357817
ASD	2	0.467091715					2	-3.48277444
ASD	2	1.176347592					2	-2.236666425
ASD	2	1.259873981					2	-2.089916985
ASD	2	1.741770705					2	-1.243261513
ASD	2	1.770958338					2	-4.796785208
ASD	2	1.91951554					2	-4.430704934
ASD	2	1.926712148					2	-4.412970781
ASD	2	1.93072657					2	-4.40307829
ASD	2	1.943004082					2	-4.372823582
ASD	2	1.945972593					2	-4.365508465
ASD	2	2.108131684					2	-3.965909901
ASD	2	2.187255621					2	-3.770929706
ASD	2	2.29659678					2	-3.501487087
ASD	2	2.520844315					2	-2.948887834
ASD	2	2.935516641					2	-1.927036615
ASD	2	3.050283375					2	-1.64422409
ASD	2	3.161383723					2	-1.370446412
ASD	2	3.516339334					2	-0.495751377
ASD	2	3.709160877					2	-0.020593243
ASD	2	4.515946781					2	1.96751908
ASD	2	5.023961999					2	3.219389384
ASD	2	29.64666025					2	excluded
ASD	2	44.64305183					2	excluded
ASD	2	54.19206455					2	excluded
ASD	2.1	1.245212627					2.1	-1.044695913
ASD	2.1	1.2649716					2.1	-1.03346807
ASD	2.1	1.559959012					2.1	-0.865844365
ASD	2.1	1.766049805					2.1	-0.748735291
ASD	2.1	1.77713922					2.1	-0.74243384
ASD	2.1	1.96702221					2.1	-0.634534692
ASD	2.1	2.124653667					2.1	-0.544962161
ASD	2.1	2.251197644					2.1	-0.473054787
ASD	2.1	2.252430715					2.1	-0.472354107
ASD	2.1	2.527564928					2.1	-0.316011787
ASD	2.1	2.900313087					2.1	-0.104201299
ASD	2.1	3.562744701					2.1	0.272218973
ASD	2.1	3.674741159					2.1	0.335859863
ASD	2.1	3.822704226					2.1	0.419938425
ASD	2.1	3.318712701					2.1	0.133550181
ASD	2.11	1.760552255						
ASD	2.11	1.932818675						
ASD	2.11	2.595862962						
ASD	2.11	3.365695231						

ASD	2.11	3.590261744
ASD	2.11	4.105028244
ASD	2.11	4.652653031
ASD	2.11	7.336691931
ASD	2.11	8.579041071
ASD	2.2	0.383731536
ASD	2.2	1.133225705
ASD	2.2	1.909490027
ASD	2.2	3.842345749
ASD	2.2	3.848045799
ASD	2.2	3.92455407
ASD	2.2	4.01154422
ASD	2.3	1.753805439
ASD	2.3	1.76626794
ASD	2.3	1.768342674
ASD	2.3	1.775403517
ASD	2.3	1.934150894
ASD	2.3	1.951543423
ASD	2.3	1.969422452
ASD	2.3	1.975056921
ASD	2.3	2.32311893
ASD	2.3	2.840479095
ASD	2.3	2.916988897
ASD	2.3	3.070233725
ASD	2.3	3.506979659
ASD	2.4	1.182039351
ASD	2.4	1.433945643
ASD	2.4	1.769037947
ASD	2.4	1.770056576
ASD	2.4	1.777827079
ASD	2.4	1.799120598
ASD	2.4	2.436815482
ASD	2.4	2.49150978
ASD	2.4	3.041442528
ASD	2.4	1.589790419
ASD	2.5	0.888687275
ASD	2.5	1.696395082
ASD	2.5	1.924532332
ASD	2.5	1.933812387
ASD	2.5	1.934680137
ASD	2.5	2.234722289
ASD	2.5	2.462285846
ASD	2.5	2.565051084
ASD	2.5	2.826727351
ASD	2.5	3.132576088
ASD	2.5	3.706315006
ASD	2.6	1.542560885
ASD	2.6	1.595070732
ASD	2.6	1.753531106
ASD	2.6	1.780306071
ASD	2.6	1.805664947
ASD	2.6	1.894824741
ASD	2.6	1.909931041
ASD	2.6	1.955851225
ASD	2.6	3.133341616
ASD	2.6	3.173000092
ASD	2.6	3.229318281
ASD	2.6	3.257407069
ASD	2.6	3.455729425
ASD	2.6	3.796997905
ASD	2.6	3.883443524
ASD	2.6	3.891048426
ASD	2.7	1.730023456
ASD	2.7	1.7549687
ASD	2.7	1.768088582
ASD	2.7	1.907207444
ASD	2.7	2.988800014
ASD	2.7	4.382687517
ASD	2.8	1.944459197
ASD	2.8	2.286573842
ASD	2.8	2.662176127
ASD	2.8	2.818519699
ASD	2.8	3.087934721

2.11	-0.75185922
2.11	-0.653970516
2.11	-0.277202098
2.11	0.160247543
2.11	0.287855263
2.11	0.580366281
2.11	0.891548699
2.11	3.863399312
2.11	5.139408564
2.2	-3.277944053
2.2	-2.508143147
2.2	-1.71084678
2.2	0.274377586
2.2	0.280232072
2.2	0.358813252
2.2	0.448160307
2.3	-1.870749474
2.3	-1.857949314
2.3	-1.855818368
2.3	-1.848566219
2.3	-1.685517752
2.3	-1.667653992
2.3	-1.64929055
2.3	-1.643503421
2.3	-1.286011047
2.3	-0.754633566
2.3	-0.676050813
2.3	-0.518653982
2.3	-0.070074887
2.4	-2.458006948
2.4	-2.317962595
2.4	-1.771870471
2.4	-1.770210436
2.4	-1.757547028
2.4	-1.722845476
2.4	-0.683608975
2.4	-0.59447495
2.4	0.301737642
2.4	-2.063985994
2.5	-3.206557459
2.5	-1.890254857
2.5	-1.518464894
2.5	-1.503341403
2.5	-1.501927253
2.5	-1.012955545
2.5	-0.642100515
2.5	-0.474626401
2.5	-0.048178683
2.5	0.450255882
2.5	1.385264835
2.6	-2.140954864
2.6	-2.05538079
2.6	-1.797141609
2.6	-1.753507072
2.6	-1.712180303
2.6	-1.566878663
2.6	-1.542260277
2.6	-1.338984049
2.6	0.28453038
2.6	0.339211174
2.6	0.416862246
2.6	0.455590845
2.6	0.729036139
2.6	1.199574417
2.6	1.318764953
2.6	1.329250532
2.7	-1.650353588
2.7	-1.615959283
2.7	-1.597869694
2.7	-1.406053708
2.7	0.085237564
2.7	2.007118617
2.8	-0.10017412

ASD	2.8	3.439633355
ASD	2.8	3.463212465
ASD	2.8	4.314469988
ASD	2.8	1.941287967
ASD	2.9	0.877654367
ASD	2.9	2.942514612
ASD	2.9	2.982310828
ASD	2.9	3.120210965
ASD	2.9	3.316236645
ASD	2.9	3.393061275
ASD	2.9	3.492670981
ASD	2.9	3.881833807
ASD	3	0.279977415
ASD	3	1.69775407
ASD	3	1.812554288
ASD	3	1.889455816
ASD	3	1.913101432
ASD	3	1.920273411
ASD	3	1.926564964
ASD	3	1.933868368
ASD	3	1.950875952
ASD	3	1.997979799
ASD	3	2.223934968
ASD	3	2.229050172
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NT	2.9	6.450540684
NT	3	0.377826796
NT	3	1.824284114
NT	3	1.961698974
NT	3	2.137636724
NT	3	2.492011984
NT	3	2.572670693
NT	3	2.769971304
NT	3	3.098932323
NT	3	3.15361811
NT	3	3.167651931
NT	3	3.236924353
NT	3	3.261732787
NT	3	3.301943511
NT	3	3.640047714
NT	3	3.691994583
NT	3	3.769852338
NT	3	3.877041072
NT	3	3.96430516
NT	3	4.263707514
NT	3	4.280934647
NT	3	4.319455935
NT	3	4.352751109
NT	3	4.395606078
NT	3	4.515759575
NT	3	4.904170632
NT	3	5.252478488
NT	3	5.546847984
NT	3	5.776309565
NT	3	6.566344306
NT	3	6.60513629
NT	3	6.795485713
NT	3	6.949182436
NT	3	8.423214816
NT	3	11.76847551
NT	3	74.8685423
NT	3	1.948320635
NT	3	3.587889103
NT	3.1	1.245208373
NT	3.1	1.933497847

NT	3.1	2.331238031
NT	3.1	3.349932934
NT	3.1	4.492395793
NT	3.11	4.002659778
NT	3.11	62.6083836
NT	3.2	2.2017919
NT	3.2	3.542524228
NT	3.2	6.327465698
NT	3.3	3.354333599
NT	3.3	3.737872988
NT	3.4	2.191095663
NT	3.4	2.403486411
NT	3.5	3.176157179
NT	3.5	3.674623157
NT	3.5	3.729098498
NT	3.5	3.863118668
NT	3.5	4.013171598
NT	3.5	52.14014418
NT	3.6	3.988985859
NT	3.6	22.85844216
NT	3.7	1.956676533
NT	3.7	2.488558769
NT	3.7	3.062405003
NT	3.7	3.21963121
NT	3.7	4.121153521
NT	3.8	2.180745684
NT	3.8	3.578295693
NT	3.9	1.804770965
NT	3.9	1.969185866
NT	3.9	3.13980416
NT	3.9	4.017326708
NT	4	1.97518754
NT	4	2.155281343
NT	4	2.312532534
NT	4	2.369955901
NT	4	3.001632142
NT	4	3.035910229
NT	4	3.162297562
NT	4	3.165910577
NT	4	3.209253803
NT	4	3.268787247
NT	4	3.330264295
NT	4	3.334068127
NT	4	3.446656781
NT	4	3.449996278
NT	4	3.453196764
NT	4	3.500549273
NT	4	3.538178541
NT	4	3.549589742
NT	4	3.560902866
NT	4	3.576465419
NT	4	3.595601079
NT	4	3.663242355
NT	4	3.727917468
NT	4	3.919293816
NT	4	3.931256002
NT	4	3.934049957
NT	4	3.942381628
NT	4	4.03955931
NT	4	4.04155467
NT	4	4.182377597
NT	4	4.305044762
NT	4	4.320458772
NT	4	4.336846167
NT	4	4.568312784
NT	4	4.761193131
NT	4	5.319044143
NT	4	5.360238492
NT	4	5.460635416
NT	4	5.911139101
NT	4	6.00018324
NT	4	6.325710133
NT	4	6.393501289

NT	4	6.46400138
NT	4	6.539415531
NT	4	6.542432331
NT	4	6.77397484
NT	4	7.381714843
NT	4	7.642111227
NT	4	26.83158226
NT	4	31.18423432
NT	4	31.93567471
NT	4	32.77252145
NT	4	34.94060116
NT	4	42.65409714
NT	4.11	3.530142893
NT	4.2	40.49379522
NT	4.3	2.531207666
NT	4.4	3.081995469
NT	4.5	3.855618992
NT	4.5	4.558662497
NT	4.6	3.729066908
NT	4.6	3.941108805
NT	4.8	8.198543519
NT	5	1.779374799
NT	5	1.960054005
NT	5	2.23192553
NT	5	2.274562796
NT	5	2.318702236
NT	5	2.529072717
NT	5	2.59340738
NT	5	2.720803256
NT	5	3.545759516
NT	5	3.551165591
NT	5	3.566991558
NT	5	3.753826963
NT	5	3.779157468
NT	5	3.835106653
NT	5	3.838940274
NT	5	3.843121613
NT	5	3.855016116
NT	5	3.884759476
NT	5	3.885291206
NT	5	3.929529776
NT	5	3.957240167
NT	5	3.986315841
NT	5	4.120403337
NT	5	4.225559412
NT	5	4.303574183
NT	5	4.457678686
NT	5	4.474272206
NT	5	4.513108152
NT	5	4.567099875
NT	5	5.280376621
NT	5	6.099407285
NT	5	6.107140258
NT	5	6.315563351
NT	5	6.44383876
NT	5	6.537914693
NT	5	6.566197895
NT	5	7.189236011
NT	5	7.344994649
NT	5	7.407147057
NT	5	7.498388872
NT	5	7.519505322
NT	5	7.744149044
NT	5	7.774069093
NT	5	34.91505537
NT	5	34.99903204
NT	5	35.20352845
NT	5	42.79600634
NT	5	61.22160375
NT	5	67.92561226
NT	5.1	3.438383316
NT	5.1	3.586860816
NT	5.1	3.653374915

NT	5.1	3.719629387
NT	5.1	4.06150803
NT	5.1	6.589319445
NT	5.1	7.576136485
NT	5.11	57.3894909
NT	5.3	3.807884693
NT	5.4	5.576240718
NT	5.4	62.56624103
NT	5.5	3.509460691
NT	5.5	27.16703516
NT	5.6	2.553116535
NT	5.6	7.493157342
NT	5.6	38.61759573
NT	5.7	5.913600937
NT	5.8	42.68576851
NT	5.9	31.83459243
NT	6	0.486279997
NT	6	3.15604235
NT	6	3.309690128
NT	6	3.354013935
NT	6	3.502078725
NT	6	3.532104532
NT	6	3.58487343
NT	6	3.699030104
NT	6	3.715537553
NT	6	3.73593047
NT	6	3.948203666
NT	6	3.950219825
NT	6	3.995464588
NT	6	4.001797477
NT	6	4.117739655
NT	6	4.344349908
NT	6	4.374739226
NT	6	4.453845091
NT	6	4.768686704
NT	6	5.328547433
NT	6	5.429568201
NT	6	5.491458021
NT	6	6.106683865
NT	6	6.25036563
NT	6	6.415077552
NT	6	6.499116704
NT	6	6.671043731
NT	6	6.682326384
NT	6	7.058818936
NT	6	7.162552673
NT	6	7.244620018
NT	6	7.360554837
NT	6	7.42443613
NT	6	7.544490479
NT	6	7.607961401
NT	6	26.81040175
NT	6	31.16697593
NT	6	31.68879889
NT	6	34.90814565
NT	6	38.39183072
NT	6	38.92448997
NT	6	43.26228207
NT	6	47.34735973
NT	6	35.47001575
NT	6.1	3.765263552
NT	6.1	4.023228779
NT	6.1	7.412595868
NT	6.1	8.784032546
NT	6.1	47.95186661
NT	6.11	3.446768507
NT	6.11	4.046571968
NT	6.11	5.103651352
NT	6.11	6.584584005
NT	6.2	5.285838753
NT	6.3	4.009606101
NT	6.3	4.856508374
NT	6.3	6.148170888

NT	6.3	6.226273403
NT	6.3	28.25573952
NT	6.3	82.16958302
NT	6.4	6.503220096
NT	6.4	7.180370361
NT	6.5	23.52753642
NT	6.5	52.54075468
NT	6.6	3.145264802
NT	6.6	3.540997232
NT	6.6	5.519839016
NT	6.6	6.781930033
NT	6.6	8.404903509
NT	6.6	52.70533599
NT	6.7	63.07433881
NT	6.8	4.78792916
NT	7	3.131669355
NT	7	3.82147599
NT	7	3.962634784
NT	7	4.003436729
NT	7	4.437029396
NT	7	4.578022629
NT	7	4.624758559
NT	7	4.790863365
NT	7	5.243742332
NT	7	5.569822319
NT	7	5.887372568
NT	7	6.263387211
NT	7	6.413942993
NT	7	6.676628213
NT	7	7.677997641
NT	7	7.678637169
NT	7.1	3.763747563
NT	7.2	53.84473443
NT	7.3	3.132457415
NT	7.3	3.422572571
NT	7.3	31.56373563
NT	7.4	43.13162628
NT	7.4	57.62435235
NT	7.5	3.112055426
NT	7.5	6.822186589
NT	7.5	7.409681
NT	7.5	7.695746616
NT	7.5	62.74466619
NT	7.6	3.15945105
NT	7.6	6.194381803
NT	7.6	7.247949436
NT	7.6	35.97218405
NT	7.8	47.3497697
NT	8	3.953659018
NT	8	3.991396
NT	8	4.17714576
NT	8	4.558316827
NT	8	4.669973622
NT	8	5.401487619
NT	8	5.94328694
NT	8	6.221544012
NT	8	8.270407736
NT	8	8.567906357
NT	8	38.62195185
NT	8	38.76929824
NT	8	62.63769113
NT	8.11	7.771798279
NT	8.2	47.74887018
NT	8.3	3.822458744
NT	8.3	9.630596191
NT	8.3	35.42342876
NT	8.4	6.724911198
NT	8.4	27.3035804
NT	8.4	35.62927192
NT	8.5	5.718995713
NT	8.5	47.47516915
NT	8.6	2.297761204
NT	8.6	7.415941283

NT	8.6	8.429633021
NT	8.6	35.42397942
NT	8.8	6.422391549
NT	9	4.032637601
NT	9	4.44376656
NT	9	6.264302731
NT	9	7.374531351
NT	9	8.313376736
NT	9	8.451855559
NT	9	8.501022313
NT	9.1	31.8006908
NT	9.4	4.817738314
NT	9.6	3.354520847
NT	9.6	57.06053339
NT	9.7	3.856545771
NT	10	4.343403948
NT	10	4.470079993
NT	10	6.462623708
NT	10	6.6969634
NT	10	7.324179351
NT	10	7.640650049
NT	10	7.810621612
NT	10	8.456500515
NT	10	8.524332032
NT	10	8.622698203
NT	10	8.641267918
NT	10	8.857038129
NT	10	8.986138605
NT	10	14.05543176
NT	10.1	3.207058276
NT	11	8.880952284
NT	12	5.513352126
NT	12	8.393864058
NT	12	15.91727522
NT	12	35.7308249
NT	12	38.67477627
NT	12	23.84917989
NT	14	13.31918075
NT	15	6.639530703
NT-adult	16	6.62644159
NT-adult	16	10.3384876
NT-adult	16	14.81856329
NT-adult	18	35.22878225
NT-adult	18	35.27905016
NT-adult	20	5.115192011
NT-adult	20	7.595294051
NT-adult	20	15.23135571
NT-adult	20	17.47241873
NT-adult	20	35.20237386
NT-adult	21	23.26303528
NT-adult	22	30.60271771
NT-adult	23	3.833688798
NT-adult	23	6.113175362
NT-adult	23	8.396139041
NT-adult	23	24.05636355
NT-adult	23	25.58359872
NT-adult	23	25.69227237
NT-adult	24	22.92748238
NT-adult	24	35.18252164

Table 9 Detection rate in NT and ASD cohort						
taxa	Detection rate in NT	Detection rate in ASD	pvalue	qvalue	Difference	Type
d_Bacteria.p_Firmic	0.997772829	1	0.367430442	0.393410372	0.002227171	Increasing
d_Bacteria.p_Prote	0.997772829	0.998706339	1	1	0.00093351	Increasing
d_Bacteria.p_Bacte	0.997772829	0.997412678	1	1	-0.000360151	Decreasing
d_Bacteria.p_Firmic	0.997772829	0.996119017	1	1	-0.001653812	Decreasing
d_Bacteria.p_Firmic	0.997772829	0.984476067	0.038972124	0.045900502	-0.013296761	Decreasing
d_Bacteria.p_Actin	0.995545657	0.985769728	0.149452142	0.166757127	-0.009775929	Decreasing
d_Bacteria.p_Firmic	0.997772829	0.981888745	0.013942934	0.016987942	-0.015884083	Decreasing
d_Bacteria.p_Firmic	0.995545657	0.980595084	0.040093391	0.046702191	-0.014950573	Decreasing
d_Bacteria.p_Firmic	0.986636971	0.96377749	0.018832868	0.022685046	-0.022859481	Decreasing
d_Bacteria.p_Actin	0.975501114	0.966364812	0.488817317	0.513016194	-0.009136301	Decreasing
d_Bacteria.p_Firmic	0.975501114	0.943078913	0.009395497	0.011716737	-0.0324222	Decreasing
d_Bacteria.p_Firmic	0.964365256	0.943078913	0.10130244	0.115462996	-0.021286343	Decreasing
d_Bacteria.p_Firmic	0.971046771	0.927554981	0.001205833	0.001578006	-0.04349179	Decreasing
d_Bacteria.p_Bacte	0.959910913	0.928848642	0.032666675	0.038906377	-0.031062271	Decreasing
d_Bacteria.p_Firmic	0.935412027	0.940491591	0.712592404	0.733347522	0.005079564	Decreasing
d_Bacteria.p_Firmic	0.966819599	0.915912031	0.000245859	0.000338455	-0.052907568	Decreasing
d_Bacteria.p_Firmic	0.9844098	0.905562743	5.92E-09	1.06E-08	-0.078847057	Decreasing
d_Bacteria.p_Firmic	0.966819599	0.908150065	3.47E-05	5.04E-05	-0.060669534	Decreasing
d_Bacteria.p_Firmic	0.953229399	0.913324709	0.011159374	0.013754577	-0.03990469	Decreasing
d_Bacteria.p_Firmic	0.942093541	0.919793014	0.168495819	0.186047467	-0.022300527	Decreasing
d_Bacteria.p_Firmic	0.93986637	0.919793014	0.210302441	0.229815039	-0.020073355	Decreasing
d_Bacteria.p_Prote	0.95545657	0.909443726	0.002971231	0.003794584	-0.046012844	Decreasing
d_Bacteria.p_Firmic	0.95545657	0.891332471	7.20E-05	0.000101813	-0.064124099	Decreasing
d_Bacteria.p_Firmic	0.966819599	0.880983182	2.42E-08	4.14E-08	-0.087836417	Decreasing
d_Bacteria.p_Firmic	0.926503341	0.899094437	0.121507975	0.137019631	-0.027408903	Decreasing
d_Bacteria.p_Bacte	0.966592428	0.870633894	5.90E-09	1.06E-08	-0.095958534	Decreasing
d_Bacteria.p_Firmic	0.953229399	0.877102199	6.61E-06	9.73E-06	-0.076127199	Decreasing
d_Bacteria.p_Firmic	0.957683742	0.861578266	2.69E-08	4.53E-08	-0.096105475	Decreasing
d_Bacteria.p_Firmic	0.93986637	0.839586028	1.18E-07	1.85E-07	-0.100280341	Decreasing
d_Bacteria.p_Firmic	0.879732739	0.861578266	0.381648535	0.404547447	-0.018154473	Decreasing
d_Bacteria.p_Firmic	0.942093541	0.81759379	1.53E-10	2.95E-10	-0.124499751	Decreasing
d_Bacteria.p_Firmic	0.93986637	0.816300129	3.24E-10	6.14E-10	-0.12356624	Decreasing
d_Bacteria.p_Firmic	0.926503341	0.813712807	2.95E-08	4.89E-08	-0.112790534	Decreasing
d_Bacteria.p_Prote	0.902004454	0.81759379	6.48E-05	9.29E-05	-0.084410664	Decreasing
d_Bacteria.p_Firmic	0.915367483	0.800776197	5.49E-08	8.82E-08	-0.114591287	Decreasing
d_Bacteria.p_Firmic	0.846325167	0.831824062	0.521518481	0.541970186	-0.014501105	Decreasing
d_Bacteria.p_Firmic	0.937639198	0.777490298	1.53E-14	3.97E-14	-0.160148901	Decreasing
d_Bacteria.p_Firmic	0.899777283	0.794307891	1.07E-06	1.60E-06	-0.105469392	Decreasing
d_Bacteria.p_Firmic	0.93986637	0.767141009	1.87E-16	6.19E-16	-0.172725361	Decreasing
d_Bacteria.p_Bacte	0.935412027	0.752910737	3.12E-17	1.14E-16	-0.182501289	Decreasing
d_Bacteria.p_Firmic	0.888641425	0.778783959	1.03E-06	1.55E-06	-0.109857467	Decreasing
d_Bacteria.p_Firmic	0.917594655	0.74385511	1.09E-14	2.89E-14	-0.173739545	Decreasing
d_Bacteria.p_Firmic	0.890868597	0.724450194	1.48E-12	3.21E-12	-0.166418403	Decreasing
d_Bacteria.p_Firmic	0.897550111	0.698576973	9.22E-17	3.15E-16	-0.198973139	Decreasing
d_Bacteria.p_Firmic	0.890868597	0.701164295	3.63E-15	1.04E-14	-0.189704302	Decreasing
d_Bacteria.p_Fusot	0.848552339	0.690815006	3.82E-10	7.10E-10	-0.157773732	Decreasing
d_Bacteria.p_Firmic	0.917594655	0.650711514	4.61E-28	5.42E-27	-0.266883141	Decreasing
d_Bacteria.p_Firmic	0.881959911	0.668822768	1.02E-17	3.99E-17	-0.213137142	Decreasing
d_Bacteria.p_Firmic	0.853006682	0.675291074	3.32E-12	6.76E-12	-0.177715608	Decreasing
d_Bacteria.p_Firmic	0.795100223	0.706338939	0.000715978	0.000948667	-0.088761284	Decreasing
d_Bacteria.p_Firmic	0.861915367	0.663648124	5.47E-15	1.53E-14	-0.198267243	Decreasing
d_Bacteria.p_Firmic	0.86636971	0.64683053	1.07E-17	4.07E-17	-0.21953918	Decreasing
d_Bacteria.p_Desul	0.848552339	0.64683053	9.39E-15	2.55E-14	-0.201721808	Decreasing
d_Bacteria.p_Firmic	0.832962138	0.630012937	1.73E-14	4.38E-14	-0.202949201	Decreasing
d_Bacteria.p_Firmic	0.841870824	0.608020699	1.59E-18	7.34E-18	-0.233850125	Decreasing
d_Bacteria.p_Firmic	0.752783964	0.655868158	0.000406791	0.000552819	-0.096897807	Decreasing
d_Bacteria.p_Firmic	0.861915367	0.591203105	1.57E-24	1.11E-23	-0.270712263	Decreasing
d_Bacteria.p_Firmic	0.812917595	0.619663648	7.43E-13	1.64E-12	-0.193253947	Decreasing
d_Bacteria.p_Firmic	0.772828508	0.627425614	1.17E-07	1.85E-07	-0.145402893	Decreasing
d_Bacteria.p_Actin	0.795100223	0.593790427	3.05E-13	7.36E-13	-0.201309796	Decreasing
d_Bacteria.p_Prote	0.844097996	0.564036223	5.08E-25	3.85E-24	-0.280061773	Decreasing
d_Bacteria.p_Firmic	0.839643653	0.553686934	1.25E-25	1.02E-24	-0.285956719	Decreasing
d_Bacteria.p_Firmic	0.815144766	0.562742561	6.02E-20	3.04E-19	-0.252402205	Decreasing
d_Bacteria.p_Verru	0.779510022	0.576972833	3.80E-13	8.57E-13	-0.202537189	Decreasing
d_Bacteria.p_Firmic	0.857461024	0.522639069	1.78E-34	3.78E-33	-0.334821956	Decreasing
d_Bacteria.p_Firmic	0.797327394	0.53686934	1.34E-20	7.49E-20	-0.260458054	Decreasing
d_Bacteria.p_Firmic	0.812917595	0.517464424	5.24E-26	5.55E-25	-0.29545317	Decreasing
d_Bacteria.p_Firmic	0.788418708	0.473479948	4.38E-28	5.42E-27	-0.31493876	Decreasing
d_Bacteria.p_Firmic	0.706013363	0.504527814	4.17E-12	8.35E-12	-0.201485549	Decreasing
d_Bacteria.p_Bacte	0.766146993	0.433376455	1.66E-30	2.94E-29	-0.332770538	Decreasing
d_Bacteria.p_Firmic	0.710467706	0.459249677	9.25E-18	3.77E-17	-0.251218029	Decreasing
d_Bacteria.p_Firmic	0.694877506	0.437257439	2.33E-18	1.03E-17	-0.257620067	Decreasing
d_Bacteria.p_Firmic	0.65701559	0.455368693	1.02E-11	2.00E-11	-0.201646897	Decreasing
d_Bacteria.p_Firmic	0.563474388	0.460543338	0.00057499	0.000771506	-0.10293105	Decreasing
d_Bacteria.p_Firmic	0.708240535	0.375161708	9.10E-30	1.38E-28	-0.333078827	Decreasing
d_Bacteria.p_Firmic	0.632516704	0.385510996	6.56E-17	2.32E-16	-0.247005708	Decreasing
d_Bacteria.p_Firmic	0.599109131	0.39068564	1.95E-12	4.13E-12	-0.208423491	Decreasing
d_Bacteria.p_Firmic	0.605790646	0.363518758	2.33E-16	7.48E-16	-0.242271888	Decreasing
d_Bacteria.p_Bacte	0.576837416	0.342820181	1.86E-15	5.48E-15	-0.234017235	Decreasing
d_Bacteria.p_Bacte	0.603563474	0.323415265	2.08E-21	1.30E-20	-0.280148209	Decreasing
d_Bacteria.p_Bacte	0.659242762	0.253557568	2.95E-44	3.13E-42	-0.405685194	Decreasing
d_Bacteria.p_Desul	0.527839644	0.315653299	3.79E-13	8.57E-13	-0.212186345	Decreasing
d_Bacteria.p_Firmic	0.621380846	0.227684347	1.50E-42	7.95E-41	-0.3936965	Decreasing
d_Bacteria.p_Firmic	0.523385301	0.26778784	5.59E-19	2.69E-18	-0.255597461	Decreasing
d_Bacteria.p_Bacte	0.592427617	0.226390686	2.07E-37	7.33E-36	-0.366036931	Decreasing
d_Bacteria.p_Firmic	0.46325167	0.263906856	2.42E-12	5.02E-12	-0.199344814	Decreasing
d_Bacteria.p_Firmic	0.556792873	0.204398448	6.92E-36	1.83E-34	-0.352394425	Decreasing
d_Bacteria.p_Firmic	0.5077951	0.228978008	4.81E-23	3.18E-22	-0.278817092	Decreasing
d_Bacteria.p_Firmic	0.481069042	0.221216041	1.51E-20	8.02E-20	-0.259853001	Decreasing
d_Bacteria.p_Firmic	0.467706013	0.226390686	5.45E-18	2.31E-17	-0.241315328	Decreasing
d_Bacteria.p_Firmic	0.483296214	0.192755498	5.76E-26	5.56E-25	-0.290540716	Decreasing
d_Bacteria.p_Syner	0.354120267	0.250970246	0.000145438	0.000202847	-0.103150021	Decreasing
d_Bacteria.p_Bacte	0.454342984	0.172056921	1.04E-25	9.21E-25	-0.282286063	Decreasing
d_Bacteria.p_Firmic	0.432071269	0.179818887	5.49E-21	3.23E-20	-0.252252382	Decreasing
d_Bacteria.p_Firmic	0.389755011	0.179818887	1.67E-15	5.07E-15	-0.209936124	Decreasing
d_Bacteria.p_Firmic	0.378619154	0.179818887	3.38E-14	8.32E-14	-0.198800266	Decreasing
d_Bacteria.p_Prote	0.369710468	0.160413972	4.37E-16	1.36E-15	-0.209296496	Decreasing
d_Bacteria.p_Firmic	0.265033408	0.135834411	4.02E-08	6.55E-08	-0.129198996	Decreasing
d_Bacteria.p_Verru	0.229398664	0.078913325	3.34E-13	7.87E-13	-0.150485339	Decreasing
d_Bacteria.p_Firmic	0.131403118	0.094437257	0.055536841	0.063988099	-0.036965861	Decreasing
d_Bacteria.p_Actin	0.08908686	0.068564036	0.218265806	0.236083422	-0.020522823	Decreasing
d_Bacteria.p_Bacte	0.042316258	0.091849935	0.00135048	0.001745742	0.049533677	Decreasing
d_Bacteria.p_Cyan	0.113585746	0.029754204	7.75E-09	1.37E-08	-0.083831542	Decreasing
d_Bacteria.p_Bacte	0.111358575	0.029754204	2.41E-08	4.14E-08	-0.08160437	Decreasing
d_Bacteria.p_Firmic	0.026726058	0.06080207	0.008040858	0.010146797	0.034076012	Decreasing
d_Bacteria.p_Desul	0.08908686	0.021992238	1.75E-07	2.68E-07	-0.067094622	Decreasing

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NT0: taxa detection rate in NT cohort under 3(included) years old
 ASD0: taxa detection rate in ASD cohort under 3(included) years old
 NT1: taxa detection rate in NT cohort after 3 years old
 ASD1: taxa detection rate in ASD cohort after 3 years old
 NT0_ASD0_difference: ASD0-NT0
 NT1_ASD1_difference: ASD1-NT1

		Table 12 Enlarging microbial relationship alternation with increasing ASD score									
index	Taxon1	Taxon2	Group 1		Group 2		Group 3		Group 4		R-square
			0	0.030402191	0.177473584	0.414827419	0.98194699	0.192212614	0.449593496	0.258536585	
1_d	Bacteri	d_Bacteri	0	0.030402191	0.177473584	0.414827419	0.98194699	0.192212614	0.449593496	0.258536585	
2_d	Bacteri	d_Bacteri	0.023748621	0	0.110463288	0.39550352	0.913334129	0.185900866	0.639837398	0.258536585	
3_d	Bacteri	d_Bacteri	0.023635102	0	0.108514535	0.38325046	0.914739418	0.179707679	0.473170732	0.309756098	
4_d	Bacteri	d_Bacteri	0	0.052013578	0.34737405	0.40868343	0.874802924	0.178472382	0.334146341	0.25033552	
5_d	Bacteri	d_Bacteri	0.099926855	0	0.101828421	0.456326731	0.754001498	0.178199938	0.494308943	0.258536585	
6_d	Bacteri	d_Bacteri	0.086644323	0.121380717	0	0.431995102	0.824598097	0.17267539	0.565853659	0.318699187	
7_d	Bacteri	d_Bacteri	0	0.01102791	0.014173968	0.338525328	0.751431186	0.158145059	0.369918699	0.28699187	
8_d	Bacteri	d_Bacteri	0.012065936	0.18235507	0	0.327553328	0.997896068	0.157743896	0.369918699	0.258536585	
9_d	Bacteri	d_Bacteri	0.02006529	0.02715604	0.327051117	0	0.767314024	0.153492914	0.42601626	0.31300813	
10_d	Bacteri	d_Bacteri	0	0.069714028	0.113497108	0.364164351	0.866499156	0.149225162	0.531707317	0.257723577	
11_d	Bacteri	d_Bacteri	0	0.001069837	0.06821461	0.282069571	0.916705326	0.140499867	0.742276423	0.309756098	
12_d	Bacteri	d_Bacteri	0.057002013	0.06881672	0.329716111	0	0.782428395	0.136357049	0.403250303	0.369918699	
13_d	Bacteri	d_Bacteri	0	0.060738219	0.178146912	0.329131261	0.91947012	0.134196521	0.363414634	0.293829327	
14_d	Bacteri	d_Bacteri	0.00084512	0	0.056072492	0.25995212	0.901132794	0.1295535	0.449593496	0.423577236	
15_d	Bacteri	d_Bacteri	0.012213275	0.117099432	0	0.270772682	0.988271454	0.129279704	0.663414634	0.25203252	
16_d	Bacteri	d_Bacteri	0.051784664	0.168763272	0.306393477	0	0.997811796	0.127300406	0.574796748	0.468292683	
17_d	Bacteri	d_Bacteri	0	0.008278091	0.150762693	0.256752623	0.992860592	0.124237266	0.28699187	0.271544715	
18_d	Bacteri	d_Bacteri	0.115123806	0.119556459	0.407929303	0.432233013	0.835636456	0.123970046	0.369918699	0.31300813	
19_d	Bacteri	d_Bacteri	0.092391017	0.114711334	0	0.339014151	0.817271958	0.123311567	0.501626016	0.331707317	
20_d	Bacteri	d_Bacteri	0.011881272	0.076745968	0.253252791	0.351257541	0.97164616	0.119463563	0.4	0.369918699	
21_d	Bacteri	d_Bacteri	0.004325414	0.06394526	0.235094524	0	0.927766146	0.115384555	0.411382114	0.332520325	
22_d	Bacteri	d_Bacteri	0.0053728	0	0.065154918	0.233226345	0.929891524	0.113826772	0.404878049	0.371544715	
23_d	Bacteri	d_Bacteri	0	0.05196818	0.087604647	0.278473112	0.864631674	0.113252466	0.318699187	0.258536585	
24_d	Bacteri	d_Bacteri	0.027066119	0.104629283	0	0.245828533	0.972567831	0.109381207	0.899186992	0.25203252	
25_d	Bacteri	d_Bacteri	0	0.116599337	0.268246561	0.330462256	0.944921176	0.10693146	0.359349593	0.309756098	
26_d	Bacteri	d_Bacteri	0.002354659	0	0.073293788	0.215095638	0.964335824	0.106370088	0.336385366	0.309756098	
27_d	Bacteri	d_Bacteri	0.032505371	0.046971201	0	0.240278073	0.801946854	0.103886351	0.722764228	0.565853659	
28_d	Bacteri	d_Bacteri	0.117006024	0	0.147996596	0.319150603	0.861876097	0.101017289	0.359349593	0.334146341	
29_d	Bacteri	d_Bacteri	0.0154471881	0.130907556	0.215845452	0	0.992326945	0.100198705	0.576422764	0.251219512	
30_d	Bacteri	d_Bacteri	0.003376937	0.079387598	0	0.202788849	0.981521756	0.099705956	0.276422764	0.257723577	
31_d	Bacteri	d_Bacteri	0.081460367	0.12470783	0.279834188	0	0.904139076	0.09918691	0.814634146	0.252845528	
32_d	Bacteri	d_Bacteri	0.041914865	0	0.099050416	0.239401589	0.944121829	0.098743312	0.825203252	0.269105691	
33_d	Bacteri	d_Bacteri	0	0.058389053	0.08340845	0.253551838	0.844369203	0.097581392	0.358536585	0.25203252	
34_d	Bacteri	d_Bacteri	0.0111752145	0.295358974	0.306649989	0.793295496	0.097448922	0.336585366	0.251219512		
35_d	Bacteri	d_Bacteri	0.029409561	0	0.087155548	0.23218857	0.948679144	0.096904498	0.722764228	0.258536585	
36_d	Bacteri	d_Bacteri	0	0.048883358	0.17326723	0.239705352	0.970179351	0.095410997	0.359349593	0.252845528	
37_d	Bacteri	d_Bacteri	0.022268124	0.094254489	0.211206385	0	0.981053202	0.094762572	0.42601626	0.252845528	
38_d	Bacteri	d_Bacteri	0	0.14254242	0.205135171	0.330065385	0.964473646	0.093761472	0.460162602	0.369918699	
39_d	Bacteri	d_Bacteri	0	0.038125728	0.06607506	0.214382938	0.865477583	0.088128605	0.288617886	0.278861789	
40_d	Bacteri	d_Bacteri	0.076582864	0	0.158717037	0.251335956	0.998801539	0.087376546	0.62195122	0.258536585	
41_d	Bacteri	d_Bacteri	0.074137919	0	0.105454162	0.248811134	0.879395819	0.087336008	0.62195122	0.309756098	
42_d	Bacteri	d_Bacteri	0.056668778	0	0.109921633	0.230653646	0.952252858	0.086992434	0.641463415	0.297560976	
43_d	Bacteri	d_Bacteri	0.039755924	0.14422583	0.206873837	0.307395873	0.991491458	0.086556785	0.473170732	0.269105691	
44_d	Bacteri	d_Bacteri	0.032874903	0.041753922	0	0.20110612	0.78949793	0.084115609	0.508943089	0.4	
45_d	Bacteri	d_Bacteri	0.128021265	0.186585795	0.266986772	0.371486405	0.985299159	0.08170164	0.370731707	0.369918699	
46_d	Bacteri	d_Bacteri	0.044619261	0	0.066441916	0.207194647	0.848620797	0.081287693	0.38699187	0.255284553	
47_d	Bacteri	d_Bacteri	0.042536155	0	0.185733871	0.204514319	0.835655744	0.080989082	0.460162602	0.332520325	
48_d	Bacteri	d_Bacteri	0.032411456	0.074644448	0.107523267	0.73287022	0.855344802	0.075555552	0.9	0.302882927	
49_d	Bacteri	d_Bacteri	0.015889585	0.034772553	0.038108974	0.245495837	0.679573089	0.069215518	0.473170732	0.334146341	
50_d	Bacteri	d_Bacteri	0.093511349	0.184044756	0	0.224981329	0.954709739	0.06573499	0.403250303	0.25203252	
51_d	Bacteri	d_Bacteri	0.106764786	0.122792022	0	0.228528293	0.846031977	0.061382254	0.517073171	0.297560976	
52_d	Bacteri	d_Bacteri	0.135826107	0	0.220416102	0.24961324	0.926788298	0.056893566	0.52601626	0.371544715	
53_d	Bacteri	d_Bacteri	0.184601111	0	0.256590113	0.291608441	0.961733295	0.053503865	0.54796748	0.334146341	
54_d	Bacteri	d_Bacteri	0.145877766	0.171130851	0.179044719	0.311646317	0.766421797	0.050521952	0.369918699	0.334146341	

Taxon1 relationship node
Taxon2 relationship node
Group 1: PM score of ASD cohort with ASD score 1 to 3 compared to NT
Group 2: PM score of ASD cohort with ASD score 4 to 5 compared to NT
Group 3: PM score of ASD cohort with ASD score 7 compared to NT
Group 4: PM score of ASD cohort with ASD score 7 to 9 compared to NT
R-square: the linear regression R-square
coefficient: the linear regression coefficient
Taxon1 detection rate: detection rate of taxon1
Taxon2 detection rate: detection rate of taxon2

Table13 Summary of the statistical results of the 20 significant changed microbial taxa across age																											
Row.names	1			2			3			4			5			6			7			8			9		
	mean NT	mean ASD	p-value	mean NT	mean ASD	p-value	mean NT	mean ASD	p-value	mean NT	mean ASD	p-value	mean NT	mean ASD	p-value	mean NT	mean ASD	p-value	mean NT	mean ASD	p-value	mean NT	mean ASD	p-value			
g_Lachnospiraceae UCG-004							0.44%	0.77%	0.025199	0.45%	0.95%	0.020612															
g_Escherichia-Shigella																5.40%	7.38%	0.001517				6.77%	2.27%	0.025688			
g_Butyricoccus																											
g_Faecalibacterium	11.98%	4.12%	0.03829					17.55%	13.00%	0.013482																	
g_Parabacteroides																											
f_Enterobacteriaceae																											
g_Lachnospiraceae NK4A136_group																4.99%	5.13%	0.004266	7.21%	3.53%	1.13724E-05	1.75%	2.48%	0.011476			
g_Bifidobacterium																											
g_Haemophilus								0.29%	0.83%	0.047153																	
g_Veillonella	10.25%	19.99%	0.032053	1.69%	10.47%	5.7118E-05		3.60%	8.03%	0.003709	0.80%	5.48%	8.03E-05	0.76%	6.52%	0.001105	0.66%	3.87%	0.030332								
f_Ruminococcaceae	0.03%	0.14%	0.030366					0.16%	0.09%	0.015762																	
g_Anaerostipes																											
g_Lachnospira								1.87%	3.58%	0.014366																	
g_Blautia																1.56%	3.01%	0.024705	1.65%	2.58%	0.028512499						
g_Granulicatella																											
g_Romboutsia																											
g_Actinomyces																											
g_Roseburia																											
g_Incertae Sedis																											
g_Hungatella				0.13%	0.80%	0.000636712										0.22%	0.21%	0.025056									

Total proportion of the 20 microbial taxa in different age brackets									
age bracket	1	2	3	4	5	6	7	8	9
NT	65.91%	40.10%	50.53%	40.04%	37.16%	38.59%	40.97%	33.41%	35.09%
ASD	62.42%	55.85%	50.09%	45.66%	46.73%	46.45%	39.02%	37.09%	35.70%

Table 14 Summary of the statistical results of the 39 significant changed GBM across age																												
No.	Row name	1			2			3			4			5			6			7			8			9		
		mean NT	mean ASD	p-value	mean NT	mean ASD	p-value	mean NT	mean ASD	p-value	mean NT	mean ASD	p-value	mean NT	mean ASD	p-value	mean NT	mean ASD	p-value	mean NT	mean ASD	p-value	mean NT	mean ASD	p-value			
1	MGB004 Kynurenine synthesis	1092.797	297.3598	0.021074				1160.483	423.7885	0.014966																		
2	MGB025 Nitric oxide synthesis I (NO synthase)	48.63158	3.12	0.00303				196.8714	2.631285	3.06E-07												3	2.586207	0.007224				
3	MGB045 Acetate synthesis III				78271.83	94693.84	0.039124	92155.87	86980.81	0.02902						76626.3	83367.28	0.018106										
4	MGB047 Acetate degradation				34185.86	33968.58	0.035049																					
5	MGB050 Glutamate degradation I				5282.641	14521.4	5.68E-05						4234.183	11689.16	0.028959													
6	MGB028 Nitric oxide degradation II (NO reductase)				1300.1	6200.144	0.142984	1576.387	4628.157	0.023838			715.2168	4315.926	0.012537													
7	MGB009 Histamine synthesis				6216.852	5725.541	0.171932																					
8	MGB031 17- β -Estradiol degradation							56799.02	52701.34	0.044281						46593.32	49709.97	0.039599										
9	MGB055 Propionate synthesis III							35100.37	47227.68	0.015738						33119.66	40996.46	0.022824					34050.77	53703.55	0.000129			
10	MGB044 Acetate synthesis II							69193.54	44421.63	0.033508																		
11	MGB034 Isovaleric acid synthesis I (KADH pathway)							21377.53	29567.96	0.001956														19892.22	33138.89	0.01256		
12	MGB024 DOPAC synthesis							19395.03	16628.75	0.001334																		
13	MGB051 Glutamate degradation II							6278.562	3765.424	0.021857	5264.89	4397.712	0.008437			4380.876	3094.99	0.000249	6386.679	3447.318	0.037749							
14	MGB015 p-Cresol synthesis																								83274.66	118679.3	0.000268	
15	MGB056 Propionate degradation I										2259.034	247.979	5.97E-06	1231.779	2904.984	0.042978	1478.688	88.24674	5.86E-06	1161.887	870.508	0.001545				1859.713	1104.982	0.000152
16	MGB023 Dopamine degradation																2386.973	1767.557	0.048214							397.3296	506.3824	0.012873
17	MGB021 GABA synthesis II																			5050.97	3532.61	0.011751	5159.807	2860.345	0.000162	4398.596	5065.86	0.00462
18	MGB020 GABA synthesis I																											
19	MGB016 p-Cresol degradation																2979.323	3440.453	0.002932	2770.88	1635.698	0.005035						
20	MGB048 Propionate synthesis I							1687.501	1012.833	0.033167	398.9685	2864.111	0.041861			1099.75	629.1248	0.003041	828.4491	368.7868	0.00438				320.7989	78.82941	0.003953	
21	MGB010 Histamine degradation																1478.989	1721.478	0.001436	2286.921	1605.805	0.004333						
22	MGB026 Nitric oxide synthesis II (nitrite reductase)										696.2562	653.8108	0.043096							1096.704	1232.576	0.030099	1481.074	651.145	0.000279			
23	MGB052 Butyrate synthesis I																								555.3852	805.9735	0.031165	
24	MGB007 Glutamate synthesis II																								23393.96	30034.2	0.003496	
25	MGB038 Inositol degradation															7386.579	7494.644	0.035399							101169.1	122042.4	0.006389	
26	MGB006 Glutamate synthesis I																									100197.7	118560.9	0.004365
27	MGB033 Quinolinic acid degradation																79722.27	85805.27	0.005075							84853.1	98243.76	0.046034
28	MGB035 Isovaleric acid synthesis II (KADC pathway)																											
29	MGB036 S-Adenosylmethionine (SAM) synthesis													90423.15	105440.7	0.035302												
30	MGB049 Tryptophan degradation													10332.87	9637.566	0.025768												
31	MGB037 Inositol synthesis										33992.74	39414.31	0.014555													35428.22	45181.6	0.002122
32	MGB027 Nitric oxide degradation I (NO dioxygenase)															4375.189	10843.07	0.00012										
33	MGB019 GABA degradation																						11257.43	6400.679	0.041149			
34	MGB005 Tryptophan synthesis																57626.33	65420.19	0.008114							56928.03	77007.84	0.000511
35	MGB040 Menaquinone synthesis (vitamin K2) I													23046.35	35277.73	0.014219										25949.26	37159.62	0.001383
36	MGB029 ClpB (ATP-dependent chaperone protein)																82244.97	86773.31	0.009216									
37	MGB032 Quinolinic acid synthesis																70137.28	72081.29	0.036633							69053.07	82871.76	0.018522
38	MGB039 g-Hydroxybutyric acid (GHB) degradation																						3822.884	6090.431	0.003407	4356.258	4179.57	0.012649
39	MGB043 Acetate synthesis I													78948.7	93955.13	0.013526	80907.01	86281.85	0.006756									

Table 15 Summary of the statistical results of the 286 significant changed METACYC cross age

No	Row name	Higher level functional annotation	1			2			3			4			5			6			7			8			9		
			mean NT	mean ASD	p-value	mean NT	mean ASD	p-value	mean NT	mean ASD	p-value	mean NT	mean ASD	p-value	mean NT	mean ASD	p-value	mean NT	mean ASD	p-value	mean NT	mean ASD	p-value	mean NT	mean ASD	p-value			
1	PWV-647 Kido transfer to lipid IVA III-chemistry	Carbohydrate metabolism	46011.33389	47668.7195	0.00000002																								
2	PWV-738 superpathway of UDP-glucose-derived G-antigen building blocks biosynthesis	Carbohydrate metabolism	26788.34839	26974.52765	0.04328018																								
3	GC-CHANNAN-1-PWV superpathway of mannose-4-biosynthesis	Carbohydrate metabolism	44001.3117	45765.9178	0.00000000																								
4	HEXITOL-DEPHER-PWV superpathway of hexitol degradation bacteria	SECONDARY METABOLITE-metabolism	28863.45515	14266.4074	0.00000000																								
5	PWV-534 superpathway of sulfur endonucleic Acidium aminoibios	Noncarbon-Nutrients	6327.49473	304.4477	0.00000000																								
6	PWV-634 superpathway of mannose-4-biosynthesis	Carbohydrate metabolism	46011.33389	47668.7195	0.00000000																								
7	PWV-737.4-4-oxo-4-naphthoate biosynthesis II	Carbohydrate metabolism	6418.03448	425.14336	0.00000000																								
8	PWV-586.1-phenylacetate degradation VII in propionamide	Ammonio-Amino-metabolism	162.20110	204.19336	0.00178999																								
9	PWV-737.4-4-oxo-4-naphthoate biosynthesis II	Carbohydrate metabolism	6418.03448	425.14336	0.00000000																								
10	METH-GALATE-DEGRADATION-PWV methyllactate degradation	AMINOACID-COMPOUND-metabolism	5833.92411	1.90248	0.00101133																								
11	GALATE-DEGRADATION-PWV galate degradation II	AMINOACID-COMPOUND-metabolism	584.4443763	13.14322	0.0451838																								
12	GALATE-DEGRADATION-PWV galate degradation I	AMINOACID-COMPOUND-metabolism	462026.6241	17.7468	0.00000000																								
13	PWV-75271-methionine salvage cycle II	Ammonio-Amino-metabolism	26.0793634	1.212224	0.00099171	441.82331	7.68091436	0.02849386	181.38345	5.38755419	1.04497107	5.18999133	6.40639568	0.03204662	10.77205972	5.0683989	0.00140574	599.3186333	5.7962475	5.13208297	7.95784964	6.18314379	0.01885789	68.78314286	1.70657749	0.005770311			
14	PWV-586.1-methionine salvage cycle I	Ammonio-Amino-metabolism	13.07435842	6.75238	0.00536504	132.2413	14.52329	0.00000000	12.7308253	3.59158713	0.13122106	29.740364	40.911933	0.00483177	27.25243589	40.62542705	0.003054421	321.9710113	38.68418135	0.007659712	40.64338571	36.43005	0.029768927						
15	PWV-75271-methionine salvage cycle II	Ammonio-Amino-metabolism	26.0793634	1.212224	0.00099171	441.82331	7.68091436	0.02849386	181.38345	5.38755419	1.04497107	5.18999133	6.40639568	0.03204662	10.77205972	5.0683989	0.00140574	599.3186333	5.7962475	5.13208297	7.95784964	6.18314379	0.01885789	68.78314286	1.70657749	0.005770311			
16	PWV-586.1-methionine salvage cycle I	Ammonio-Amino-metabolism	13.07435842	6.75238	0.00536504	132.2413	14.52329	0.00000000	12.7308253	3.59158713	0.13122106	29.740364	40.911933	0.00483177	27.25243589	40.62542705	0.003054421	321.9710113	38.68418135	0.007659712	40.64338571	36.43005	0.029768927						
17	PWV-75271-methionine salvage cycle II	Ammonio-Amino-metabolism	26.0793634	1.212224	0.00099171	441.82331	7.68091436	0.02849386	181.38345	5.38755419	1.04497107	5.18999133	6.40639568	0.03204662	10.77205972	5.0683989	0.00140574	599.3186333	5.7962475	5.13208297	7.95784964	6.18314379	0.01885789	68.78314286	1.70657749	0.005770311			
18	PWV-586.1-methionine salvage cycle I	Ammonio-Amino-metabolism	13.07435842	6.75238	0.00536504	132.2413	14.52329	0.00000000	12.7308253	3.59158713	0.13122106	29.740364	40.911933	0.00483177	27.25243589	40.62542705	0.003054421	321.9710113	38.68418135	0.007659712	40.64338571	36.43005	0.029768927						
19	PWV-75271-methionine salvage cycle II	Ammonio-Amino-metabolism	26.0793634	1.212224	0.00099171	441.82331	7.68091436	0.02849386	181.38345	5.38755419	1.04497107	5.18999133	6.40639568	0.03204662	10.77205972	5.0683989	0.00140574	599.3186333	5.7962475	5.13208297	7.95784964	6.18314379	0.01885789	68.78314286	1.70657749	0.005770311			
20	PWV-586.1-methionine salvage cycle I	Ammonio-Amino-metabolism	13.07435842	6.75238	0.00536504	132.2413	14.52329	0.00000000	12.7308253	3.59158713	0.13122106	29.740364	40.911933	0.00483177	27.25243589	40.62542705	0.003054421	321.9710113	38.68418135	0.007659712	40.64338571	36.43005	0.029768927						
21	PWV-75271-methionine salvage cycle II	Ammonio-Amino-metabolism	26.0793634	1.212224	0.00099171	441.82331	7.68091436	0.02849386	181.38345	5.38755419	1.04497107	5.18999133	6.40639568	0.03204662	10.77205972	5.0683989	0.00140574	599.3186333	5.7962475	5.13208297	7.95784964	6.18314379	0.01885789	68.78314286	1.70657749	0.005770311			
22	PWV-586.1-methionine salvage cycle I	Ammonio-Amino-metabolism	13.07435842	6.75238	0.00536504	132.2413	14.52329	0.00000000	12.7308253	3.59158713	0.13122106	29.740364	40.911933	0.00483177	27.25243589	40.62542705	0.003054421	321.9710113	38.68418135	0.007659712	40.64338571	36.43005	0.029768927						
23	PWV-75271-methionine salvage cycle II	Ammonio-Amino-metabolism	26.0793634	1.212224	0.00099171	441.82331	7.68091436	0.02849386	181.38345	5.38755419	1.04497107	5.18999133	6.40639568	0.03204662	10.77205972	5.0683989	0.00140574	599.3186333	5.7962475	5.13208297	7.95784964	6.18314379	0.01885789	68.78314286	1.70657749	0.005770311			
24	PWV-586.1-methionine salvage cycle I	Ammonio-Amino-metabolism	13.07435842	6.75238	0.00536504	132.2413	14.52329	0.00000000	12.7308253	3.59158713	0.13122106	29.740364	40.911933	0.00483177	27.25243589	40.62542705	0.003054421	321.9710113	38.68418135	0.007659712	40.64338571	36.43005	0.029768927						
25	PWV-75271-methionine salvage cycle II	Ammonio-Amino-metabolism	26.0793634	1.212224	0.00099171	441.82331	7.68091436	0.02849386	181.38345	5.38755419	1.04497107	5.18999133	6.40639568	0.03204662	10.77205972	5.0683989	0.00140574	599.3186333	5.7962475	5.13208297	7.95784964	6.18314379	0.01885789	68.78314286	1.70657749	0.005770311			
26	PWV-586.1-methionine salvage cycle I	Ammonio-Amino-metabolism	13.07435842	6.75238	0.00536504	132.2413	14.52329	0.00000000	12.7308253	3.59158713	0.13122106	29.740364	40.911933	0.00483177	27.25243589	40.62542705	0.003054421	321.9710113	38.68418135	0.007659712	40.64338571	36.43005	0.029768927						
27	PWV-75271-methionine salvage cycle II	Ammonio-Amino-metabolism	26.0793634	1.212224	0.00099171	441.82331	7.68091436	0.02849386	181.38345	5.38755419	1.04497107	5.18999133	6.40639568	0.03204662	10.77205972	5.0683989	0.00140574	599.3186333	5.7962475	5.13208297	7.95784964	6.18314379	0.01885789	68.78314286	1.70657749	0.005770311			
28	PWV-586.1-methionine salvage cycle I	Ammonio-Amino-metabolism	13.07435842	6.75238	0.00536504	132.2413	14.52329	0.00000000	12.7308253	3.59158713	0.13122106	29.740364	40.911933	0.00483177	27.25243589	40.62542705	0.003054421	321.9710113	38.68418135	0.007659712	40.64338571	36.43005	0.029768927						
29	PWV-75271-methionine salvage cycle II	Ammonio-Amino-metabolism	26.0793634	1.212224	0.00099171	441.82331	7.68091436	0.02849386	181.38345	5.38755419	1.04497107	5.18999133	6.40639568	0.03204662	10.77205972	5.0683989	0.00140574	599.3186333	5.7962475	5.13208297	7.95784964	6.18314379	0.01885789	68.78314286	1.70657749	0.005770311			
30	PWV-586.1-methionine salvage cycle I	Ammonio-Amino-metabolism	13.07435842	6.75238	0.00536504	132.2413	14.52329	0.00000000	12.7308253	3.59158713	0.13122106	29.740364	40.911933	0.00483177	27.25243589	40.62542705	0.003054421	321.9710113	38.68418135	0.007659712	40.64338571	36.43005	0.029768927						
31	PWV-75271-methionine salvage cycle II	Ammonio-Amino-metabolism	26.0793634	1.212224	0.00099171	441.82331	7.68091436	0.02849386	181.38345	5.38755419	1.04497107	5.18999133	6.40639568	0.03204662	10.77205972	5.0683989	0.00140574	599.3186333	5.7962475	5.13208297	7.95784964	6.18314379	0.01885789	68.78314286	1.70657749	0.005770311			
32	PWV-586.1-methionine salvage cycle I	Ammonio-Amino-metabolism	13.07435842	6.75238	0.00536504	132.2413	14.52329	0.00000000	12.7308253	3.59158713	0.13122106	29.740364	40.911933	0.00483177	27.25243589	40.62542705	0.003054421	321.9710113	38.68418135	0.007659712	40.64338571	36.43005	0.029768927						
33	PWV-75271-methionine salvage cycle II	Ammonio-Amino-metabolism	26.0793634	1.212224	0.00099171	441.82331	7.68091436	0.02849386	181.38345	5.38755419	1.04497107	5.18999133	6.40639568	0.03204662	10.77205972	5.0													

[illegible]

[illegible]

Table 16 Summary of the statistical results of correlations between bacterial taxa and clinical phenotypes according to spearman's rank correlations analysis

[illegible]

Lou M, *et al.* *Gut* 2021;0:1–12. doi: 10.1136/gutjnl-2021-325115

Lou M, *et al.* Gut 2021;0:1–12. doi: 10.1136/gutjnl-2021-325115

Lou M. *et al.* Gut 2021;0:1–12. doi: 10.1136/gut-2021-325115

within_3_months

Lou M, *et al.* *Gut* 2021;0:1–12. doi: 10.1136/gutjnl-2021-325115

Lou M, *et al.* *Gut* 2021;0:1–12. doi: 10.1136/gutjnl-2021-325115

	FASN-ELONG-PWT fatty acid elongation - saturated										0.00112881						0.0042711	0.0072821			0.00811991						
	ACo	0.1142301	0.108721	0.102420	0.105922	0.097449					0.0034281						0.007121	0.002891			0.009121						
	ACo	0.1142301	0.108721	0.102420	0.105922	0.097449					0.0034281						0.007121	0.002891			0.009121						
	ACo	0.1142301	0.108721	0.102420	0.105922	0.097449					0.0034281						0.007121	0.002891			0.009121						
DENOVOPURINE2-PWT superpathway of purine nucleotides de novo biosynthesis II	ACo	0.1142301	0.108721	0.102420	0.105922	0.097449					0.0034281						0.007121	0.002891			0.009121						
	ACo	0.1142301	0.108721	0.102420	0.105922	0.097449					0.0034281						0.007121	0.002891			0.009121						
	ACo	0.1142301	0.108721	0.102420	0.105922	0.097449					0.0034281						0.007121	0.002891			0.009121						
	ACo	0.1142301	0.108721	0.102420	0.105922	0.097449					0.0034281						0.007121	0.002891			0.009121						
COXII-PWT inducible acetyl coenzyme A pathway	ACo	0.1142301	0.108721	0.102420	0.105922	0.097449					0.0034281						0.007121	0.002891			0.009121						
	ACo	0.1142301	0.108721	0.102420	0.105922	0.097449					0.0034281						0.007121	0.002891			0.009121						
	ACo	0.1142301	0.108721	0.102420	0.105922	0.097449					0.0034281						0.007121	0.002891			0.009121						
	ACo	0.1142301	0.108721	0.102420	0.105922	0.097449					0.0034281						0.007121	0.002891			0.009121						
ARGIN-PWT L-arginine biosynthesis Lys L-ornithine	ACo	0.1142301	0.108721	0.102420	0.105922	0.097449					0.0034281						0.007121	0.002891			0.009121						
	ACo	0.1142301	0.108721	0.102420	0.105922	0.097449					0.0034281						0.007121	0.002891			0.009121						
	ACo	0.1142301	0.108721	0.102420	0.105922	0.097449					0.0034281						0.007121	0.002891			0.009121						
	ACo	0.1142301	0.108721	0.102420	0.105922	0.097449					0.0034281						0.007121	0.002891			0.009121						

Supplementary methods

ASD cohort

A total of 773 participants with clinically diagnosed ASD were recruited (aged between 16 months and 19 years) (online supplemental tables S1 and S2). ASD was diagnosed based on the Diagnostic and Statistical Manual of Mental Disorders Fifth Edition (DSM-V) criteria by two or three psychiatrists in a face-to-face observation and interviewing the guardian(s). In the case of children < 2 years of age, the Childhood Autism Rating Scale (CARS) and Gesell Developmental Schedules (GDS) schedules were conducted. For children > 2 years of age, the Autism Diagnostic Observation Schedule (ADOS), Autism Diagnostic Interview-Revised (ADI), or ADOS+ADI were used. Most participant diagnoses were confirmed with ADI-R (197/773), ADOS (354/773), or both (ADOS + ADI, 14/773), natively or locally. All participants will be reassessed in our outpatient or ward and obtained continuous clinical observations based on diagnostic criteria in the follow-up clinical intervention. Given the economic acceptability, convenience, and consistency in clinical practice, the severity of ASD was scored by accumulating the severity scores of clinical manifestations by psychiatrists. The patients' clinical manifestations were clinically evaluated as mild (score 1), moderate (score 2), and severe (score 3) according to DSM-IV and DSM-V¹; the online supplemental table S3 presents the detailed clinical evaluation standard.

Control cohort

A total of 429 neurotypical (NT) children (aged between 11 months and 15 years) and 20 unrelated healthy adults (aged 16–24 years) were recruited (online supplemental tables S1 and S2). All healthy participants were from schools or companies that cooperated with our hospital for routine examinations. The 429 relatively young participants were mainly from the provinces of Hunan, Shandong, Zhejiang, Shanghai, and Beijing (online supplemental table S4) and were employed in the prospective, multiregional, and observational cohort study. The metadata used in this study are detailed in online supplemental table S1. For the information registration, each sample was scored as 1 (yes) or 0 (no) for each factor. Comorbidities, such as gastrointestinal (GI) problems, sleep complaints, and immune abnormalities, indicated the body conditions during the past two weeks before sampling. The summaries of age, demographic, clinical, and district characteristics are provided in online supplemental tables S2 and S4. No participant took any drug, such as antibiotics, opioids, metformin and statins, or dietary supplements, such as probiotics or prebiotics, which have been proven to impact gut microbiota, in the month before sampling. The use of antibiotics in

the three months prior to sampling was recorded in detail (online supplemental table S1).

Validation cohort

Full age brackets involving the appended cohort were conducted as validation cohort 1, which was recruited by our outpatient department from 2019-2021. This cohort included 73 subjects with ASD and 32 age-matched NT subjects (online supplemental tables S1 and S2).

The unrelated cohort reported by Dan et al. 2020² was utilized as validation cohort 2 and included 143 subjects with a clinical diagnosis of ASD (average age, 4.937 ± 0.155) and 143 age- and sex-matched NT individuals (average age, 5.189 ± 0.170) in China.

The unrelated cohort reported by Cao et al. 2021³ was employed as validation cohort 3 and included 45 subjects with ASD (average age 6.80 ± 3.79) and 41 NT subjects (average age 5.16 ± 0.99) in China.

Stool sample collection and DNA extraction

All of the stool samples from participants were collected by themselves or their trained guardians at home/outpatient/ward within 3 minutes after defecation. After sampling, the container was labeled and transferred ($<20^\circ\text{C}$) to GUHE Laboratories (Hangzhou, China) within 3 days and stored at -80°C until further processing. Stool samples were excluded if any organic changes were detected. Total bacterial genomic DNA was extracted using the GHFDE100 DNA isolation kit (Zhejiang Hangzhou Equipment Preparation: 20190952) in accordance with the manufacturer's instructions. Incidentally, the genomic extraction method employed by the kit has obtained a Chinese national invention patent (NO: ZL201511009389.7). The quantity and quality of the extracted DNA were measured using NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, USA) and agarose gel electrophoresis, respectively.

16S rRNA gene sequencing

For each sample, we amplified variable V4 regions of the 16S rRNA gene using the forward primer 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and the reverse primer 806R (5'-GGACTACHVGGGTWTCTAAT-3'). The PCR components contained 25 μl of Phusion High-Fidelity PCR Master Mix, 3 μl (10 μM) of each Forward and Reverse primer, 10 μl of DNA Template, 3 μl of DMSO, and 6 μl of ddH₂O. The following cycling conditions were used: initial denaturation at 98°C for 30s followed by 25 cycles of

denaturation at 98 °C for 15 s, annealing at 58 °C for 15 s, and extension at 72°C for 15 s and a final extension of 1 min at 72°C. PCR amplicons were purified with Agencourt AMPure XP Beads (Beckman Coulter, Indianapolis, IN) and quantified using the PicoGreen dsDNA Assay Kit (Invitrogen, Carlsbad, CA, USA).

Bioinformatics and statistical analysis

Briefly, raw sequences with exactly matched barcodes were assigned to their unique corresponding samples and identified as valid sequences. Inferior sequences were filtered out according to the following criteria: (i) sequences with <150 bp length or <20 average Phred score, (ii) sequences that contained ambiguous bases or >8 bp mononucleotide repeats^{4 5} and the average of clean reads from each sample was 126340. Qualified paired-end reads were blasted, dereplicated (--derep_fulllength), clustered (--cluster_unoise), and chimera detected (--uchime3_denovo) using VSEARCH (V2.4.4)⁶ against the SILVA138 database⁷ followed by assembly into operational taxonomic units (OTUs) with sequence similarity $\geq 97\%$ using the Quantitative Insights Into Microbial Ecology (QIIME2, v2020.6) pipeline. OTUs containing <0.001% of the total sequences across all samples were discarded. To minimize the difference in sequencing depth across samples, an average, rounded rarefied OTU table was generated by averaging 100 evenly resampled OTU subsets under 90% of the minimum sequencing depth for further analysis. OTU-level alpha diversity, such as the Chao1, richness, abundance-based coverage estimator, Shannon, and Simpson index of each sample were calculated using the OTU table in QIIME. Beta diversity analysis was performed using UniFrac distance metrics^{8 9} and visualized via principal component analysis (PCA) based on the OTU-level compositional profiles¹⁰. The significance in the differentiation of microbiota structure among groups was assessed by permutational multivariate analysis of variance (PERMANOVA) using the R package “vegan”. For taxa, the relative abundance changes between groups were statistically analyzed using the Kruskal-Wallis test from the R stats package at the phylum, class, order, family, genus, and species levels. Based on the normalized OTU tables, functional modules predicted by PICRUSt2.3 were used to predict metagenomic functions (MetaCYC)¹¹. Human gut-brain modules (GBMs) were profiled using the Omixer-RPM version 1.0 (<https://github.com/raeslab/omixer-rpm>) with default parameters. Co-occurrence analysis was performed by calculating Spearman’s rank correlations between microbial taxa/function and clinical phenotype. The *Veillonella* correlation network was performed using the Pearson correlation. Correlations with $p < 0.05$, were validated as pre-significant co-features. Microbial taxa pre-co-feature are visualized in Figure 4B.

For each functional pre-co-feature, we calculated the perturbed score, defined as

$$S = -\log_{10}(P) * |RHO|$$

where p-value was the paired t-test between functional 'pre-co-feature' and clinical phenotype. Values of the perturbed score of each functional 'pre-co-feature' (mainly to METACYC) at different age brackets were written into the same box and then averaged as

$$R_S = \frac{\sum_{t=1}^T -\log_{10} P * |RHO|}{T}$$

All functional pre-co-features were ranked by Rsscore for further analysis. The correlation network was drawn using Cytoscape according to the perturbed score, and the circos plot was plotted using the R package.

The multivariate linear modeling system was used to calculate the association between selected microbial features and factors for the fixed effects of potentially confounding covariates (other factors from metadata). Associations from MaAslin^{21 23} generally represented the causal relationship between the abundance change of microbial features and metadata (usually the categorical data). Significance values across all associations were then adjusted using the Benjamini–Hochberg False Discovery Rate (FDR) method.

EnvFit^{14 15} was performed using the 'vegan' R package. The covariates and significance of each factor were determined using EnvFit based on NMDS with Bray-Curtis dissimilarity. A total of 33 factors were included in the effect size calculation (Online Supplemental Table S6). The significance value of each factor was determined based on 10,000 permutations and adjusted using FDR adjustment (Benjamini–Hochberg procedure).

Random forest analysis was performed to discriminate samples from different groups using the R package "randomForest" with 1,000 trees, and all default settings turned off^{16 17}. The generalization error was estimated using 10-fold cross-validation. The expected "baseline" error was also included, which was obtained using a classifier that simply predicts the most common category label. The SHAP (SHapley Additive exPlanations) value was regarded as the sum of all quantitative impacts of potential influencing factors on the feature. The feature with equal importance in the ASD or NT group typically indicated that individual factors induced the feature with the lowest bias.

Deep neural network for microbiota age quantification.

Feature selection models were trained using a full list of OTU-level features, which

included 7,573 microbial taxa. Training and validation sets were separated to contain 90% and 10% of the profiles in all cases. The regressors were built using taxonomic profiles derived from individual samples (sample-based models). The model was trained as a regressor using five-fold cross-validation. After completing the grid search for various model configurations, the best-performing model was selected based on the maximal R^2 score. The model contained three hidden layers with 512 nodes in each, with a PReLU activation function, Adam optimizer, dropout fraction of 0.5, and a learning rate of 0.001. To verify the importance of features derived from the sample-based DNN model, gradient boosting was used, as implemented in the XGBoost Python library. The best-performing XGBoost model was trained using the following parameters: linear_nthread = 35, max_depth = 6, max_delta_step = 2, lambda = 0, gamma = 0.1, eta = 0.1, and alpha = 0.5. The performance of the XGBoost models was evaluated using the MAE.

Microbial relationship alteration analysis

The PM score was used to quantify the relationship alteration to remove the potential impact of changes on microbial abundance. A threshold FDR q-value < 0.05, p-value < 0.05, PM score > 0.25, and taxa detection rate > 0.25 were used to filter significant relationship alterations.

In addition to the PM2RA analysis, changes in the occurrence network were calculated. Pearson's correlation was used to describe the relationships between taxa and build the occurrence network. After obtaining the two occurrence networks for NT and ASD cohorts, Pearson correlation differences were calculated pairwise. Pearson correlation alterations with difference > 0.7 or difference < -0.7 and taxa detection rate > 0.25 were used to build the final altered occurrence network.

Identification of microbial relationship alteration with increasing ASD score

The clinical symptoms of children with ASD were clustered into four groups: group 1 with ASD scores 1-3 (included), group 2 with ASD scores 4-5 (included), group 3 with ASD score 6, and group 4 with ASD scores 7-9 (included). The paired microbial relationship alteration between these four groups and the NT cohort was quantified using PM2RA. We performed linear regressions on each microbial relationship to identify changes in the microbial relationship with increasing ASD scores. Several thresholds were used to filter the expanded relationship alterations. The linear regression R-square should be >0.7, and the regression coefficients should be > 0.05. Compared with NT, the PM score in groups 3 and 4 should be >0.2. The corresponding

detection rate of taxa should be >0.25 . The information regarding the 54 identified microbial relationship alterations is shown in online supplemental table S11.

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