Original research

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 emoloyed 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. Since Leo Kanner first described early infantile autism clinically, the 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

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.
variants and environmental factors (eg, perinatal events) are potential triggers of ASD. An encouraging hypothesis recently proposed that gut microbiota may be an important factor in a broad range of neurological and psychiatric disorders and diseases. 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. Accordingly, whether there is a real difference in the gut microbiota between healthy individuals and those with ASD has been questioned. 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 or the gut microbiota-brain axis in the postpartum microbiota involved in host development. 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. 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 1–310 are shown in online supplemental methods 1.

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; 2×150 bp pair-end sequencing was performed using the Illumina NovoSeq6000 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 USEARCH (V2.4.4) against the SILVA138 database. 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. MaAslin 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. 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 Lee to interpret the kind of host factor that affected the selected feature. The decision tree was visualised using treebeaR R package.

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. 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. All deep neural networks (DNNs) were implemented using the Python V3.6 Keras library with Tensorflow backend. The detail process of mode 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. 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). 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. province | ASD | NT | ASD | NT | ASD | NT | ASD | NT | ASD | NT | ASD | NT | ASD | NT | ASD | NT | ASD | NT |
| Hunan    | 18  | 15 | 71  | 26 | 63  | 35 | 45  | 16 | 37  | 32 | 14  | 28 | 15  | 17 | 9    | 17 | 13  | 11 |
| Guangdong| 0   | 0  | 12  | 2  | 17  | 0  | 18  | 0  | 26  | 0  | 6   | 0  | 9   | 0  | 4    | 0  | 12  | 0  |
| Shandong | 1   | 0  | 10  | 1  | 19  | 21 | 10  | 35 | 17  | 34 | 4   | 25 | 0   | 3  | 4    | 1  | 0   | 0  |
| Beijing  | 0   | 0  | 9   | 0  | 14  | 11 | 13  | 0  | 2   | 0  | 2   | 0  | 2   | 0  | 0    | 0  | 5   | 0  |
| Sichuan  | 0   | 0  | 4   | 0  | 7   | 0  | 6   | 0  | 8   | 0  | 1   | 0  | 3   | 0  | 1    | 0  | 6   | 1  |
| Hubei    | 0   | 0  | 3   | 2  | 10  | 5  | 4   | 2  | 5   | 3  | 1   | 0  | 2   | 2  | 4    | 2  | 4   | 1  |
| Shanghai | 0   | 2  | 1   | 0  | 5   | 2  | 4   | 2  | 2   | 0  | 4   | 4  | 2   | 2  | 1    | 3  | 1   | 1  |
| Zhejiang | 0   | 2  | 0   | 9  | 7   | 6  | 7   | 6  | 4   | 2  | 1   | 7  | 1   | 4  | 1    | 3  | 2   | 1  |
| Jiangsu  | 1   | 0  | 2   | 0  | 10  | 0  | 1   | 0  | 3   | 0  | 0   | 0  | 1   | 1  | 2    | 1  | 0   | 0  |
| Jiangxi  | 2   | 0  | 1   | 0  | 2   | 0  | 3   | 0  | 9   | 0  | 0   | 0  | 0   | 2  | 0    | 0  | 0   | 0  |
| Shandong | 0   | 0  | 4   | 0  | 7   | 0  | 6   | 0  | 8   | 0  | 1   | 0  | 3   | 0  | 1    | 0  | 6   | 1  |
| Hebei    | 1   | 0  | 1   | 0  | 5   | 0  | 4   | 0  | 2   | 0  | 0   | 0  | 1   | 0  | 0    | 0  | 2   | 0  |
| Chongqing| 0   | 0  | 3   | 0  | 1   | 0  | 6   | 0  | 0   | 0  | 1   | 0  | 0   | 0  | 0    | 0  | 0   | 0  |
| Tianjin  | 0   | 1  | 0   | 3  | 0   | 2  | 0   | 4  | 0   | 1  | 0   | 0  | 0   | 0  | 0    | 0  | 0   | 0  |
| Xinjiang | 0   | 0  | 0   | 2  | 0   | 0  | 1   | 0  | 2   | 0  | 2   | 0  | 3   | 1  | 0    | 0  | 0   | 0  |
| Anhui    | 0   | 0  | 5   | 0  | 2   | 0  | 3   | 0  | 0   | 0  | 0   | 0  | 1   | 0  | 0    | 0  | 1   | 0  |
| Henan    | 0   | 0  | 2   | 0  | 1   | 0  | 3   | 0  | 0   | 0  | 0   | 0  | 0   | 0  | 3    | 0  | 0   | 0  |
| Gansu    | 0   | 0  | 0   | 0  | 1   | 0  | 5   | 0  | 0   | 0  | 0   | 0  | 0   | 0  | 0    | 0  | 0   | 0  |
| Guangxi  | 2   | 0  | 1   | 0  | 0   | 0  | 1   | 0  | 0   | 0  | 0   | 0  | 0   | 0  | 1    | 0  | 0   | 0  |
| Fujian   | 0   | 0  | 1   | 0  | 1   | 0  | 2   | 0  | 0   | 0  | 0   | 0  | 0   | 0  | 0    | 0  | 0   | 0  |
| Hainan   | 0   | 0  | 1   | 0  | 0   | 0  | 2   | 0  | 0   | 0  | 0   | 0  | 1   | 0  | 0    | 0  | 1   | 0  |
| Liaoning | 0   | 0  | 0   | 0  | 3   | 0  | 2   | 0  | 0   | 0  | 0   | 0  | 0   | 0  | 0    | 0  | 0   | 0  |
| Neimeng  | 0   | 0  | 1   | 0  | 1   | 0  | 2   | 0  | 0   | 0  | 0   | 0  | 0   | 0  | 0    | 0  | 0   | 0  |
| Guizhou  | 0   | 0  | 0   | 0  | 1   | 0  | 2   | 0  | 0   | 0  | 0   | 0  | 0   | 0  | 0    | 0  | 0   | 0  |
| Jilin    | 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 multilocus analysis of variance (providing 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.
The 20 adults were observational cohort, mainly for monitoring the development of both alpha diversity and gut microbiota age.

Consistent with previous studies, 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 healthy adults.
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.\(^\text{27-28}\) 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,\(^\text{29}\) 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 Erysipelatoclostridium (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 taxon-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,\(^\text{30-31}\) and the spatiotemporal dislocation of functional bacterial groups indicates immaturity.\(^\text{23-32}\) Using the random forests machine learning algorithm,\(^\text{23}\) 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,\(^\text{23,33}\) 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,\(^\text{23}\) 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 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.\(^\text{15}\) 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 SSC, 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.\(^\text{34-36}\) Revealing the relationship alteration (RA) of gut microbiota between groups of subjects provides additional ecological perspective.\(^\text{36-37}\) To reveal the microbial RA between two groups with age, using our newly developed analysis tooling called PM2RA,\(^\text{26}\) we quantified the
Gut microbiota

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 microbes 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 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.
Gut microbiota 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) siraeum and Lactobacillus 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). Interestingly, 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, 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-functional 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 XICMET2-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 ‘Methods’ 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 treebeat (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...
ASD from NT reached 81% (GBM model in the current set), 64%–82% (GBM model in validation sets), 85% (METACYC model in current set) and 62%–90% (METACYC model in the validation sets). We then mixed all features and evaluated their detection effectiveness. The accuracies of all features reached 85% and 82% in the current and validation set 2, respectively. 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.
By deconstructing the AUC from our random-forest-based models across different age brackets, we found that the efficiency of both all-features and the top 20 features-based models fluctuated (figure 5E). Impressively, before 6 years of age, the diagnostic efficiency of our top 20 features was appreciable, especially in age brackets of 3–6 years (AUC 0.93–0.97) (figure 5E).

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. 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. 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 and cystic fibrosis, 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. 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. However, both OTUs were significantly disturbed in children with ASD, suggesting that both OTUs play an important role in establishing 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. 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, 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 treebeat and SHAP. 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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REFERENCES
1. Battle DE. Diagnostic and statistical manual of mental disorders (DSM). Codas 2013;25:191–2.
2. Kann L. Autistic disturbances of affective contact. *Nervous Child* 1943;2:217–50 https://psycnet.apa.org/record/1943-0362-001
3. Baner AI, Brugha TS, Erskine HE, et al. The epidemiology and global burden of autism spectrum disorders. *Psychol Med* 2015;45:601–13.
4. Patrick ME, Shaw KA, Dietz PM, et al. Prevalence of intellectual disability among children and teens with autism spectrum disorder. *Nat Commun* 2019;10:1517.
5. Wang T, Guo H, Xiong B, et al. De novo genic mutations among a Chinese autism spectrum disorder cohort. *Nat Commun* 2016;7:13316.
6. Modabbernia A, Velthorst E, Reichenberg A. Environmental risk factors for autism: an evidence-based review of systematic reviews and meta-analyses. *Mol Autism* 2017;8:13.
7. Lord C, Brugha TS, Chanm N, et al. Autism spectrum disorder. *Nat Rev Dis Primers* 2020;6.
8. Rogers GB, Keating DJ, Young RL, et al. From gut dysbiosis to altered brain function and mental illness: mechanisms and pathways. *Mol Psychiatry* 2016;21:738–48.
9. Zhang M, Chu Y, Meng Q, et al. A quasi-paired cohort strategy reveals the impaired detoxifying function of microbes in the gut of autistic children. *Sci Adv.* 2020;6.
10. Dan Z, Mao X, Liu Q, et al. Altered gut microbial profile is associated with abnormal metabolism activity of autism spectrum disorder. *Gut Microbes* 2020;11:1246–67.
11. Vukovic-Cvijin I, Sklar J, Jiang L, et al. Host variables confound gut microbiota studies of human disease. *Nature* 2020;587:448–54.
12. Kim S, Kim H, Yim YS, et al. Maternal gut bacteria promote neurodevelopmental abnormalities in mouse offspring. *Nature* 2017;549:528–32.
13. Choi GB, Yim YS, Wong H, et al. The maternal interleukin-17A pathway in mice promotes autism-like phenotypes in offspring. *Science* 2016;351:933–9.
14. Cryan JF, O’Riordan KJ, Sandhu K, et al. The gut microbiome in neurological disorders. *The Lancet Neurology* 2020;19:179–94.
15. Rosswall J, Olsson LM, Kovatcheva-Datchary P, et al. Developmental trajectory of the healthy human gut microbiota during the first 5 years of life. *Cell Host Microbe* 2021;29:765–76.
16. Cao X, Liu X, Liu J, et al. Dysbiotic gut microbiota and dysregulation of cytokine profile in children and teens with autism spectrum disorder. *Front Neurosci* 2021;15:639256.
17. Quast C, Pruesse E, Yilmaz P, et al. The Silva ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res* 2013;41:0590–6.
18. Mallick H, Rahnavard A, McIver LJ, et al. Multivariable association discovery in omics studies. *Plos Comput Biol* 2017;13:e1004942.
19. Breiman L. Random forests. *Mach Learn* 2001;45:5–32.
20. Svetnik V, Liaw A, Tong C, et al. Random forest: a classification and regression tool for compound classification and QSAR modeling. *J Chem Inf Comput Sci* 2003;43:1947–58.
21. Lundberg S, Lee S-J. A unified approach to interpreting model predictions. arXiv.org 2017 https://arxiv.org/abs/1705.07874
22. Le TT, Moore JH. treehearth: an R package for interpretable decision tree visualizations. *Bioinformatics* 2021;37:282–4.
23. Subramanian S, Hug S, Yatsunenko T, et al. Persistent gut microbiota immunity in malnourished Bangladeshi children. *Nature* 2014;510:417–21.
24. Galik F, Mamoshina P, Alipr A, et al. Human gut microbiome aging clock based on taxonomic profiling and deep learning. *iScience* 2020;23:101199.
25. Lin H, Peddada SD. Analysis of compositions of microbiomes with bias correction. *Nat Commun* 2020;11:19514.
26. Liu Z, Mi K, Xu ZZ, Zech Xu Z, et al. PM2RA: a framework for detecting and quantifying relationship alterations in microbial community. *Genomics Proteomics Bioinformatics* 2019;19:154–67.
27. He Y, Wu W, Zheng H-M, et al. Regional variation limits applications of healthy gut microbiome reference ranges and disease models. *Nat Med* 2018;24:1532–5.
28. Foulis F, Watkins C, Hill CJ, et al. Perinatal factors affect the gut microbiota up to four years after birth. *Nat Commun* 2019;10:1517.
29. Yan W, Zhu T, Xu Z, et al. Underdevelopment of the gut microbiota and bacteria species as non-invasive markers of prediction in children with autism spectrum disorder. *Gut* 2022;71:910–8.
30. Rao C, Coley KZ, Bainter W, et al. Multi-kingdom ecological drivers of microbiota assembly in preterm infants. *Nature* 2021;591:633–8.
31. Stewart CJ, Ajiama NJ, O’Brien JL, et al. Temporal development of the gut microbiome in early childhood from the TEDDY study. *Nature* 2018;562:533–8.
32. Raman AS, Gehrig JL, Venkatesh S, et al. A sparse covarying unit that describes healthy and impaired human gut microbiota development. *Science* 2019;365:eaau4735.
33. Shao Y, Forster SC, Tsaliki E, et al. Stunted microbiota and opportunistic pathogen colonization in caesarean-section birth. *Nature* 2019;574:117–21.
34. Yatsunenko T, Rey FE, Maney MJ, et al. Human gut microbiome viewed across age and geography. *Nature* 2012;486:222–27.
35. Bergstrom A, Skov TH, Bahl M, et al. Establishment of intestinal microbiota during early life: a longitudinal, explorative study of a large cohort of Danish infants. *Appl Environ Microbiol* 2014;80:2889–900.
36. Flint HJ, Duncan SH, Scott KP, et al. Interactions and competition within the microbiotal community of the human colon: links between diet and health. *Environ Microbiol* 2007;9:1101–11.
37. Faust K, Raes J. Microbial interactions: from networks to models. *Nat Rev Microbiol* 2012;10:538–50.
38. Valles-Colomer M, Falony G, Darai Y, et al. The neuroactive potential of the human gut microbiota in quality of life and depression. *Nat Microbiol* 2019;4:623–32.
39. Golubeva AV, Joyce SA, Moloney G, et al. Microbiota-related Changes in Bile Acid & Tryptophan Metabolism are Associated with Gastrointestinal Dysfunction in a Mouse Model of Autism. *EbmMedicine* 2017;24:166–78.
40. Milani C, Duranti S, Boccaccini F, et al. The first microbial colonizers of the human gut: composition, activities, and health implications of the infant gut microbiota. *Microbiol Mol Biol Rev* 2017;81.
41. MadFabe DF. Short-chain fatty acid fermentation products of the gut microbiome: implications in autism spectrum disorders. *Microbial Ecology in Health & Disease* 2012;23.
42. Aldinger KA, Lane C, Veenstra-VanderWeele J, et al. Patterns of risk for multiple Co-Occurring medical conditions replicate across distinct cohorts of children with autism spectrum disorder. *Austism Research* 2015;8:771–81.
43. Fadini CC, Lamónica DA, Fett-Conte AC, et al. Influence of sleep disorders on the behavior of individuals with autism spectrum disorder. *Front Hum Neurosci* 2015;9:347.
44. Li H, Liu H, Chen X, et al. Association of food hypersensitivity in children with the risk of autism spectrum disorder: a meta-analysis. *Eur J Pediatr* 2021;180:999–1008.
45. Dinan TG, Cryan JF. Gut instincts: microbiota as a key regulator of brain development, ageing and neurodegeneration. *J Physiol* 2017;579:489–503.
46. Hayden HS, Eng A, Pope CE, et al. Fecal dysbiosis in infants with cystic fibrosis is associated with early linear growth failure. *Nat Med* 2020;26:215–21.
47. Needham BD, Kaddurah-Daouk R, Mazmanian SK. Gut microbial molecules in behavioural and neurodegenerative conditions. *Nat Rev Neurosci* 2020;21:717–31.
48. Yachida S, Mizutan S, Shrima H, et al. Metagenomic and metabolic analyses reveal distinct stage-specific phenotypes of the gut microbiota in colorectal cancer. *Nat Med* 2019;25:968–76.
49. Zhu F, JY, Wang W, et al. Metagenome-wide association of gut microbiome features for schizophrenia. *Nat Commun* 2020;11:1612.