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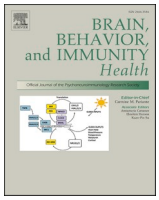

Oral microbiota in autistic children: Diagnosis-related differences and associations with clinical characteristics

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ABSTRACT

Similar to the gut microbiome, oral microbiome compositions have been suggested to play an important role in the etiology of autism. However, empirical research on how variations in the oral microbiome relate to clinical-behavioral difficulties associated with autism remains sparse. Furthermore, it is largely unknown how potentially confounding lifestyle variables, such as oral health and nutrition, may impact these associations. To fill this gap, the current study examined diagnosis-related differences in oral microbiome composition between 80 school-aged autistic children (8–12 years; 64 boys, 16 girls) versus 40 age-matched typically developing peers (32 boys, 8 girls). In addition, associations with individual differences in social functioning (SRS-2), repetitive behavior (RBS-R) and anxiety (SCARED) were explored, as well as the impact of several lifestyle variables regarding nutrition and oral health. Results provide important indications that the bacterial genera *Solobacterium*, *Stomatobaculum*, *Ruminococcaceae UCG.014, Tannerella* and *Campylobacter* were significantly more abundant in autistic compared to non-autistic children. Furthermore, the former four bacteria that were significantly more abundant in the autistic children showed significant associations with parent-reported social difficulties, repetitive and restrictive behavior and with parent-reported anxiety-like behavior. Importantly, associations among oral microbiome and quantitative diagnostic characteristics were not significantly driven by differences in lifestyle variables. This exploratory study reveals significant differences in oral microbiome composition between autistic and non-autistic children, even while controlling for potential confounding lifestyle variables. Furthermore, the significant associations with clinical characteristics suggest that individual differences in microbiome composition might be involved in shaping the clinical phenotype of autism. However, these associations warrant further exploration of the oral microbiome's potential beyond the oral cavity and specifically with respect to neuropsychiatric conditions.

1. Introduction

Autism spectrum disorder is a neurodevelopmental condition characterized by difficulties with social communication and interaction, combined with expressions of restricted and repetitive behaviors and interests. Aside from these core symptoms, individuals with autism often display a broad range of co-occurring conditions, including attachment difficulties (Heather, 2010), (social) stress and anxiety (McVey, 2019), as well as gastro-intestinal (GI) problems and poor oral health (Como et al., 2021).

In the last decade, increasing evidence showed that the gut microbiota, i.e. the collective entity of microorganisms inhabiting the GI tract (Cryan et al., 2019), may play a role in the etiology of autism (Mangiola et al., 2016). Specifically, recent studies have pointed toward altered gut

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microbiome compositions, referred to as 'dysbiosis', indicating altered microbial diversity and/or overgrowth of particular bacteria that are linked to autism symptom severity, increased repetitive behavior, and GI problems (Adams et al., 2011). Overall, these associations between gut microbiome and autism characteristics are suggested to be linked to alterations in communication along the gut-brain axis, which allows bidirectional interaction between the microbiome and neural processes (Mayer et al., 2019).

In recent years, not only the role of the gut microbiome has gained increasing interest. A handful of studies have also started to explore dysbiosis in oral microbiome compositions in individuals with autism, and how variations in oral microbiome composition may mediate neural processing (Bowland and Weyrich, 2022). For example, compelling evidence suggests that similar bidirectional interactions between the oral microbiome and neural processes exist, thereby linking oral microbiota to the expression of distinct neuropsychiatric as well as neurological conditions, such as autism and Alzheimer's disease (Bowland and Weyrich, 2022; Qiao et al., 2018). However, the precise mechanism how oral microbiome dysbiosis could influence brain functioning remains unclear (Bowland and Weyrich, 2022). Compared to the general population, autistic individuals are known to display an increased risk of oral health problems (Como et al., 2021), resulting in higher risks of periodontal problems, caries, and alteration in mouth microbiome compositions (Qiao et al., 2018). In line with these observations, previous studies have reported significant differences in oral microbiome composition between children with and without autism (4–8 years old, 53 autism and 27 controls (Ragusa et al., 2020); 7–14 years old, 32 autism and 27 controls (Qiao et al., 2018); 2–6 years old, 180 autism and 106 controls (Hicks et al., 2018); 7–12 years old, 25 autism and 38 controls (Abdulhaq et al., 2021)), as well as between autistic children/adolescents and their parents/siblings (7–25 years old, 20 autism and 19 controls (Kong et al., 2019)). Also associations between variations in oral microbiota and behavioral difficulties in autism have been identified. In rodent models, Qiao et al. (2022) demonstrated that a transfer of oral microbiota obtained from autistic children into mice could evoke autism-like behaviors in the animals (e.g. deficits in sociability, anxiety-like behavior and compulsive and repetitive behavior), as well as differences in both oral and gut microbiome compositions and neurosignaling activities. Furthermore, in young children with autism, Ragusa et al. (2020) reported significant associations between oral microbiome compositions and anomalies in neurodevelopment, verbal intelligence as well as qualitative anomalies in social interaction, communication and repetitive and restricted behavior. Moreover, Hicks et al. (2018) reported associations of oral microbiome compositions with autism symptom severity, as assessed using the Autism Diagnostic Observation Schedule (ADOS) observation scale.

Together, these studies provided important initial insights on the potential link between the oral microbiome and autism characteristics. However, lifestyle variables, such as oral health and nutrition, can potentially impact these associations (Sharma et al., 2018), but thus far, no study has considered these potentially confounding variables.

Within the current study, diagnosis-related differences in oral microbiome compositions were explored in a representative sample of 80 autistic children and 40 age- and gender-matched non-autistic peers. The cohort was thoroughly characterized using distinct clinical-behavioral questionnaires, assessing autism-related differences in social and repetitive behaviors as well as anxiety-like behaviors. In addition, we also inquired a series of lifestyle variables relating to oral health and nutrition that could potentially impact microbiome compositions. These assessments allow exploring whether variations in oral microbial compositions also dimensionally relate to inter-individual variations in clinical-behavioral autism characteristics, and importantly, how lifestyle variables may impact on these associations.

To obtain an in-depth characterization of oral microbiota, the following hierarchically structured analysis approach was applied. First, we examined several general microbiome outcome measures, including alpha- and beta-diversity. Alpha-diversity refers to the diversity of bacteria within a sample (microbiota richness) as well as the homogeneity in abundance of different bacteria in a sample (microbiota evenness). Beta-diversity refers to the difference in overall microbiome composition between two groups. Second, exploratory analyses (using MaAsLin2 (Mallick et al., 2021)) were performed, allowing to examine differences in the relative abundance of all the different taxa (e.g. bacteria) in the dataset between the autistic and non-autistic children. In this exploratory analysis, MaAsLin2 was also able to take confounding variables into account. Third, LEfSE analyses were performed allowing to specifically analyze diagnostic group differences in terms of the specific amplicon sequence variants (ASVs), which are the sequences (i.e., order of bases forming the genetic code of an organism, e.g. bacteria) differing from each other by a single nucleotide and representing the different bacteria. Accordingly, these analyses allow identifying discriminative features between the autistic and non-autistic children, while accounting for the taxonomic classification of bacteria.

2. Methods

The study was approved by the Ethical Committee for Biomedical Research at the University of Leuven, KU Leuven (S61358) in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki). Written informed consent from the parents and assent from the children were obtained prior to study enrolment. Microbial data collections were part of a larger clinical study, also including neurophysiological and endocrinological assessments (Moerkerke et al., 2023).

2.1. Participants

Eighty children with a formal diagnosis of autism, aged between 8 and 12 years with a 4/1 boys/girls' ratio were recruited through the Leuven Autism Expertise Centre at the Leuven University Hospital between July 2019 and January 2021. Alongside, forty age- and gender-matched typically developing peers were recruited (see Table 1).

For the autistic children, a clinical diagnosis of autism spectrum disorder was established by a multidisciplinary team (child psychiatrist and/or expert neuropediatrician, psychologist, speech/language pathologist and/or physiotherapist) based on the strict criteria of the DSM-5 (Diagnostic and Statistical Manual of Mental Disorders) (American Psychiatric Association, 2013). Children without autism were screened to not display any neuropsychiatric condition. Shared main inclusion criteria comprised intelligence quotient above 70 (scored by using the four subtests of the Wechsler Intelligence Scale for Children, Fifth Edition, Dutch version (Wechsler, 2018)), premenstrual girls and native Dutch speaker. Main exclusion criteria comprised a history of any neurological disorder (stroke, concussion, epilepsy etc.), any significant physical disorder (liver, renal, cardiac pathology) and the use of antibiotics within the last three months. In addition, for the autistic children, the Autism Diagnostic Observation Schedule, second edition was obtained (Lord et al., 2012).

2.2. Questionnaires assessing behavioral characteristics and dental and nutritional lifestyle variables

Core autism characteristics were assessed using the parent-reported versions of the Social Responsiveness Scale, second edition (SRS-2) (Constantino and Gruber, 2012; Roeyers et al., 2015) and the Repetitive Behavior Scale-Revised (RBS-R) (Bodfish et al., 2000). Anxiety was evaluated using both the child- and the parent-reported version of the Screen for Anxiety Related Emotional Disorders (SCARED) (Behrens et al., 2019) (see **Supplementary Methods** for a detailed description of the adopted clinical-behavioral scales).

Furthermore, the children's parents were asked to fill in an extensive questionnaire that was used in previous studies (Falony et al., 2016;

Table 1
Participants' characteristics.

	Autistic children		Non-autistic children		Independent t-test	
	N	Mean ± SD	N	Mean ± SD	t-value	*p*-value
Age (years)	80	10.5 ± 1.3	40	10.3 ± 1.3	0.790	0.431
IQ						
Verbal IQ	78	107.7 ± 15.2	40	117.3 ± 12.2	−3.441	<0.001**
Performance IQ	79	102.3 ± 14.1	40	107.8 ± 12.2	−2.093	0.039*
Gender						
Girl	16 (20%)		8 (20%)			
Boy	64 (80%)		32 (80%)			
Handedness						
Left	10 (12%)		6 (15%)			
Right	70 (88%)		34 (85%)			
ADOS-2						
Social affect	65	7.3 ± 3.7		/		
Restricted and repetitive behavior	65	1.9 ± 1.2		/		
Total	65	9.4 ± 4.1		/		
					MWU-test	
					Z	*p*-value
Clinical characteristics						
Social responsiveness						
SRS-2	80	89.2 ± 21.3	40	21.9 ± 12.7	8.833	<0.001**
Repetitive/restrictive behavior						
RBS-R	80	27.4 ± 15.7	40	2.5 ± 4.7	8.376	<0.001**
Anxiety						
SCARED - Child report	80	40.1 ± 21.8	40	26.9 ± 15.3	3.394	<0.001**
SCARED - Parent report	80	43.1 ± 20.1	40	15.2 ± 12.7	6.776	<0.001**

Data are shown as mean ± standard deviation. IQ Intelligence Quotient, ADOS-2 Autism Diagnostic Observation Schedule, SRS-2 Social Responsiveness Scale, RBS-R Repetitive Behavior Scale-Revised, SCARED Screen for Child Anxiety Related Emotional Disorders. *p < 0.05 **p < 0.001.

Zhernakova et al., 2016), which was a combination of some validated questionnaires, including confounding variables for gut microbiome data within adults, as well as variables linked before to gut-microbiome and health (Tigchelaar et al., 2015). This questionnaire was complemented with a set of questions on oral health and questions that were not relevant for children were removed (on menopause among others). In line with prior literature demonstrating a significant impact of nutrition and oral health on mouth microbiome compositions (Calderon et al., 2021; Sedghi et al., 2019), we here specifically assessed how these metadata lifestyle variables could impact oral microbiome compositions. The full list of assessed nutrition and oral health lifestyle variables can be found in Supplementary Tables S5–6.

2.3. Oral microbiome DNA extraction and illumina sequencing

Oral samples were collected using a standardized protocol. More specifically, parents were instructed to sample the mid-tongue dorsum of their child in circular movements with Floqswabs® in the morning within 30 minutes after awakening, before tooth brushing and breakfast. Tongue swabs were immediately frozen and stored at −20 °C at home, until frozen transport to the lab where they were stored at −80 °C. DNA was isolated from frozen tongue swabs using RNeasy PowerMicrobiome kit (Qiagen) according to the manufacturer's instructions. Yet, the DNase steps (steps 12–16) were not performed and an additional heating step of 95 °C for 10 minutes after step 4 was added to increase the DNA yield (Falony et al., 2016). Isolated DNA was subsequently sent to BaseClear BV (Leiden, The Netherlands) for 16S rRNA gene (V3–V4 region) amplicon sequencing using the 341F/785R primers. The amplified DNA was sequenced with the Illumina MiSeq sequencing platform (Illumina, San Diego, CA) to generate 2 x 300 base-pair (bp) paired-end reads. More detailed information regarding the transport of the samples is provided in **Supplementary methods**.

2.4. Bioinformatics and statistical analyses

DADA2 pipeline (version 1.16) was used for data cleaning and trimming of the raw microbial reads, merging of the forward and reverse reads and assigning taxonomy, using the Silva database (version 138.1) (Quast et al., 2013). Upon taxonomic assignment, an overview of the abundances of different bacteria within each sample was obtained and adopted in subsequent analyses using R (version 4.2.1).

Alpha-diversity. Microbial alpha- (bacterial diversity within a group) and beta-diversities (bacterial diversity between two groups) were calculated using the Phyloseq package within R. Specifically in terms of microbial alpha-diversity, microbial richness (Chao1), evenness (Pielou), observed richness, and diversity (Shannon and Simpson) were assessed. Microbial richness reflects the amount of different kind of bacteria within a sample and evenness the homogeneity in abundance (equality in amount of abundance) of the different bacteria within a sample. Diversity takes into account both the microbiota richness and evenness, with Shannon diversity providing a higher weight to microbiota richness and Simpson to microbiota evenness. To examine diagnosis-related differences in alpha-diversity, non-parametric Mann-Whitney U tests were performed. Associations with clinical characteristics, independent of diagnosis, were examined using Spearman correlation analyses, with Benjamini-Hochberg correction (BH-correction) for multiple testing (see **Supplementary results**) (Benjamini and Hochberg, 1995), using IBM SPSS Statistics 28.0 (IBM Corp. Released, 2021. Armonk, NY: IBM Corp).

Beta-diversity. Bray-Curtis distances were used for assessing beta-diversity indices, which were visualized using non-metric multidimensional scaling (NMDS) plots to examine the difference in overall oral microbiome composition between the autistic and the non-autistic children. Diagnostic-related group differences in beta-diversity examining overall microbiome composition were analyzed using the ANOSIM (Analysis Of Similarities) package within R.

Relative abundance. To examine diagnosis-related differences in the relative abundance of distinct bacterial taxa, and their associations with clinical characteristics, the R package MaAsLin2 was used, with BH-correction for multiple testing (Benjamini and Hochberg, 1995), resulting in q values, i.e., the p-values after correction for multiple testing, of which a q value < 0.25 was a significant result. The default of MaAsLin2 was used, including a standardization, log-transformation and a prevalence threshold of 0.1. Note that all significant results emerging from the MaAsLin2 microbiome analyses were corrected for confounding lifestyle variables that were identified to associate with overall microbial compositions. Besides correcting for these confounding variables, corrections for diagnosis were performed, as all associations were determined independent of diagnosis.

LEfSe analysis. Finally, discriminative microbial features between the two groups were identified using linear discriminant effect size (LEfSe). LEfSe makes use of linear discriminant analyses, resulting in a LDA score. The higher the LDA score, the more these bacteria drive differences between both groups. A threshold of LDA score >2.0 and $p <$ 0.05 were used within our overview. The cladogram was presented by LEfSe algorithm via the online platform Galaxy (http://huttenhower.sph

.harvard.edu/galaxy). Discriminative features without taxonomic identification were identified using the NCBI databank (National Center for Biotechnology Information) and the Human Oral Microbiome database (version 3.1) (HOMD Human Oral Microbiome Database). To do so, the Basic Local Alignment Search Tool was used to search for similar sequences within other databases.

Metadata lifestyle variable. Diagnosis-related differences in lifestyle variables were examined using Mann-Whitney U test for ordinal data (with >5 categories) and a Chi-square test for categorical data, with multiple testing correction (BH-correction). We examined the impact of lifestyle variables related to nutrition and oral health on the overall microbiome composition (beta-diversity) by the use of a Principal Component Analysis (PCA). The lifestyle variables having a significant impact on beta-diversity ($p < 0.05$) were taken into account into the diagnosis-related differences and association analyses.

3. Results

3.1. Cohort description

A total of 80 children with autism (16 girls, 64 boys) and 40 children without autism (8 girls, 32 boys) participated in the study (Table 1).

As anticipated, compared to the non-autistic group, autistic children displayed significantly higher scores on the parent-reported SRS-2 and RBS-R, indicating more social difficulties and more frequent expressions of restricted and repetitive behavior ($p < 0.001$, Table 1). Also, questionnaires assessing anxiety-like behavior (SCARED, reported by either the parents or self-reported by the child), showed diagnosis-related effects, indicating more severe expressions of anxiety in the autistic, compared to the non-autistic children ($p < 0.05$, Table 1).

3.2. Diagnosis-related differences in microbial diversity

Upon running the DADA2 pipeline, taxonomy was assigned to ASVs. The total amount of 1 669 064 reads (i.e., the DNA sequence from one fragment of DNA) could be assigned to 2130 ASVs. After removing ASVs assigned to eukaryotes, mitochondria and chloroplasts, a total amount of 1870 ASVs remained.

Alpha-diversity. Compared to the non-autistic children, the autistic children displayed a non-significant trend towards higher microbial alpha-diversity (bacterial diversity within one group), indicating tentatively higher microbial richness (Chao1: $Z = 1.82$; $p = 0.069$), evenness (Pielou: $Z = 1.27$; $p = 0.204$), observed ($Z = 1.84$; $p = 0.066$) and diversity (Shannon: $Z = 1.80$; $p = 0.072$; and Simpson: $Z = 1.33$; $p = 0.185$) (see Fig. 1, **panel A**).

Beta-diversity. Examining the overall oral microbial diversity between two groups showed no significant difference between the autistic and the non-autistic children (using Analysis Of Similarities test (ANOSIM): $p = 0.866$). As visualized in Fig. 1, **panel B**, no specific clustering of samples was evident within the group of children with or without

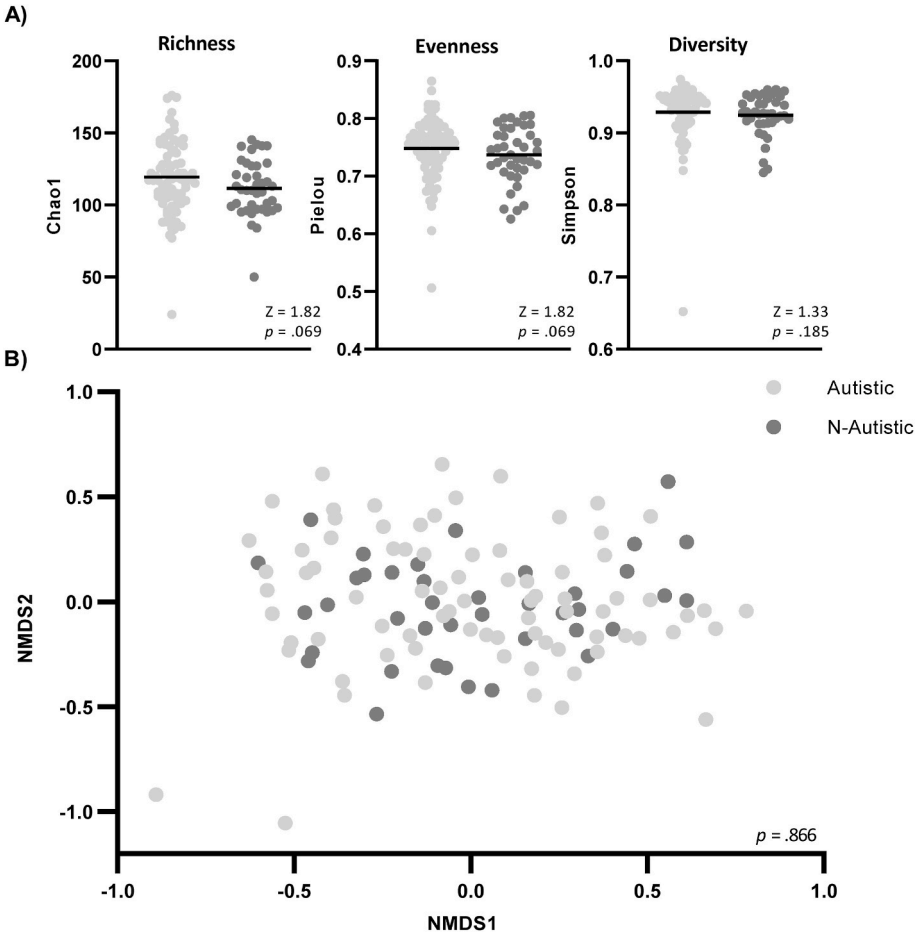

Fig. 1. Overall oral microbiome-based diversity differences between autistic and non-autistic children. Panel A. Differences in alpha-diversity indices between autistic and non-autistic children depicted for microbial richness (Chao1), evenness (Pielou), and diversity (Simpson). Only non-significant trends of higher microbiota richness, evenness, observed richness and diversity within the autistic versus the non-autistic children were observed. **Panel B.** Non-metric multidimensional scaling plot (NMDS) of pairwise Bray-Curtis distances visualized no significant differences in overall microbiome composition between autistic and non-autistic children. One dot represents the summary of the microbiome composition of one sample (light grey dots autistic children, black dots non-autistic children). The larger the distance between two dots, the more different their microbiome compositions.

autism.

3.3. Diagnosis-related differences in bacterial relative abundance

After performing MaAsLin2 analyses with multiple testing correction, five genera displayed a higher relative abundance in the autistic children, namely *Solobacterium*, *Campylobacter*, *Ruminococcaceae UCG.014*, *Tannerella* and *Stomatobaculum* (see Fig. 2). In contrast, no significant higher abundances of specific oral bacteria were found in the non-autistic children compared to the autistic children ($q > 0.25$). A detailed overview of these results (e.g. coefficiënt, q-values, prevalence) can be found in Supplementary Table S3.

Notably, the aforementioned results were also consistently found at family level, indicating the robustness of the identified microbial alterations in autism. A detailed description hereof can be found in the **Supplementary results.**

Furthermore, subsequent LefSE analyses, allowing the identification of discriminative features in microbiome composition taking into account the taxonomic classification of all bacteria, also further confirmed the aforementioned genus-level results. Specifically, of the five genera that were identified above by MaAsLin2 as being significantly more abundant within the autistic children in comparison with the non-autistic children, *Ruminococcaceae UCG.014, Stomatobaculum* and *Tannerella* were also confirmed by LEfSE as discriminative oral microbiome features of the autistic children (see Supplementary Fig. S5).

3.4. Associations between oral microbiome and clinical characteristics

Genus level x clinical characteristics. Upon correlating microbiota relative abundances with all clinical characterizations, MaAsLin2 analyses with multiple testing correction revealed significant positive associations between parent-reported SRS-2 scores and the relative abundance of *Ruminococcaceae UCG.014, Solobacterium, Stomatobaculum, Tannerella, Selenomonas 3* and *Leptotrichia*, indicating that children with more social difficulties showed a higher abundance of those bacteria in their oral samples. Furthermore, a significant negative association was evident between parent-reported SRS-2 scores and the relative abundance of *Actinobacillus*, indicating that children with more social difficulties display a lower abundance of *Actinobacillus* (see **panel A** Fig. 3 and **panel A** Supplementary Fig. S6).

Significant positive associations were also evident between parent-reported RBS-R scores and the relative abundance of *Solobacterium, Megasphaera* and *Ruminococcaceae UCG.014*, indicating that children with a higher expression of repetitive and restrictive behavior showed a higher abundance of them in their oral samples. Furthermore, a significant negative association was evident between RBS-R scores and the relative abundance of *Actinobacillus*, indicating that children with more repetitive and restrictive behavior display a lower abundance of *Actinobacillus* (see **panel B** Fig. 3 and **panel B** Supplementary Fig. S6).

Regarding parent-reported anxiety, a significant positive association was evident between parent-reported SCARED scores and the relative abundance of *Selenomonas 3, Leptotrichia, Tannerella, Ruminococcaceae UCG.014, Johnsonella, Solobacterium* and *Megasphaera*, indicating that children with higher levels of anxiety show a higher abundance of those bacteria in their oral samples. Furthermore, a significant negative association was evident between parent-reported SCARED scores and the relative abundance of *Rothia* and *Actinobacillus,* indicating that children with higher levels of anxiety have a lower abundance of *Rothia* and

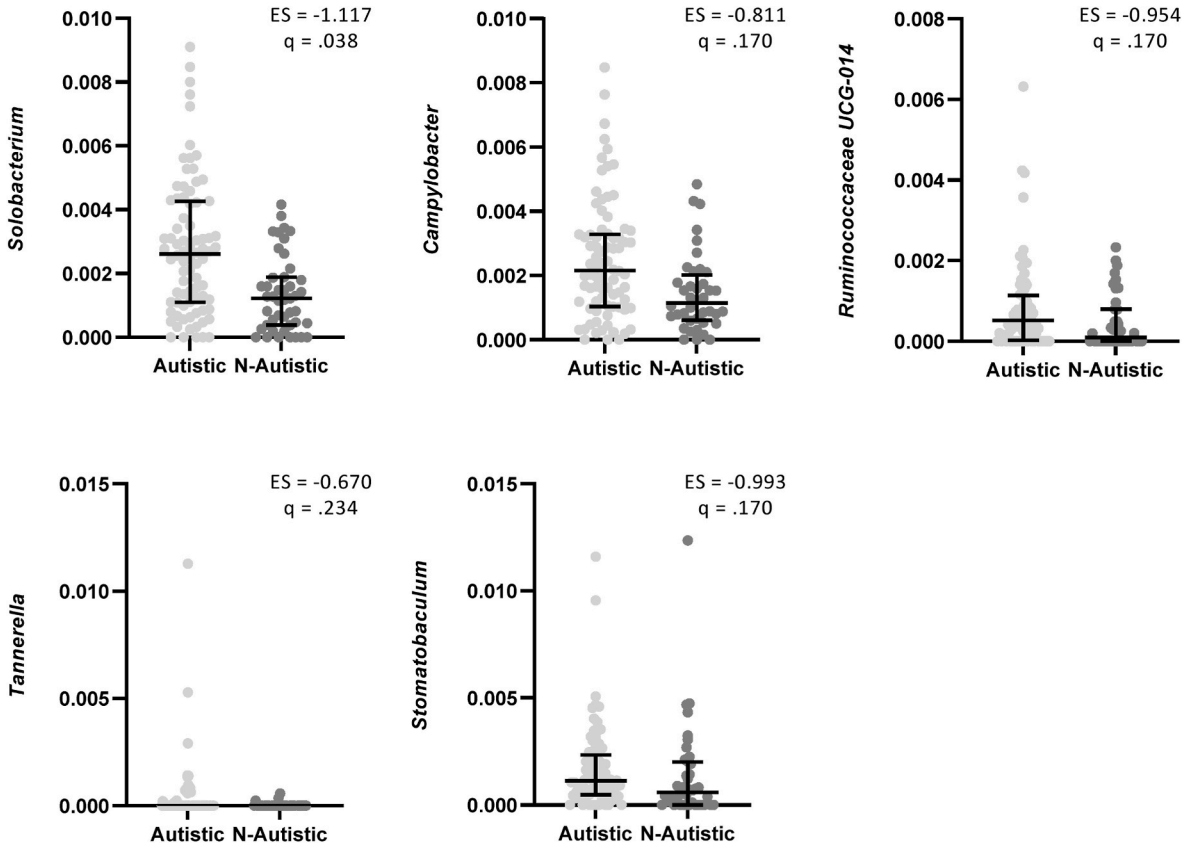

Fig. 2. Genus level differences in abundances of oral bacteria between autistic and non-autistic children. Data are represented as median with vertical error bars representing interquartile range. For each significant diagnosis-related difference in oral bacteria identified using MaAsLin2 analyses, the effect size (ES) is indicated, with a negative ES representing a higher abundance in the autistic children, compared to the non-autistic children. As visualized, higher relative abundances of the bacterial genera *Solobacterium, Campylobacter, Ruminococcaceae UCG.014, Tannerella* and *Stomatobaculum* in oral microbiota were evident in the group of autistic children, compared to the group of non-autistic children.

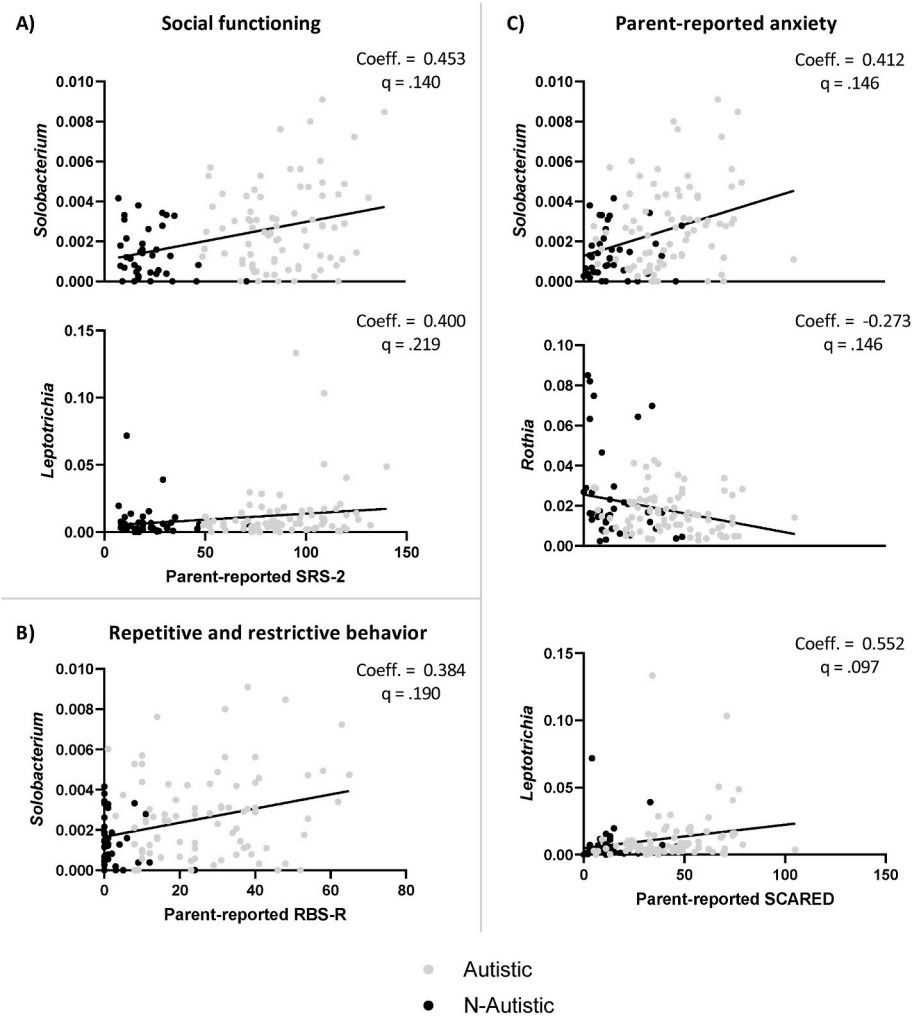

Fig. 3. Overview of associations between the relative abundance of highly prevalent oral bacteria at genus level and clinical characteristics in children with and without autism. Significant associations are visualized of highly prevalent bacteria (>100/120 participants) Linear regression lines are plotted across diagnostic groups (autistic: grey dots; non-autistic: black dots). **Panel A** visualizes the relationship between social functioning (SRS-2, with higher scores indicating more impairment) and the relative abundance of the genera *Solobacterium* and *Leptotrichia*. **Panel B** visualizes the relationship between repetitive and restrictive behavior (RBS-R, with higher scores indicating more impairment) and the relative abundance of the genus *Solobacterium*. **Panel C** visualizes the relationship between parent-reported anxiety (SCARED, with higher scores indicating more impairment) and the relative abundance of the genera *Solobacterium, Rothia* and *Leptotrichia*. SRS-2 Social Responsiveness Scale, RBS-R Repetitive Behavior Scale-Revised, SCARED Screen for Child Anxiety Related Emotional Disorders.

Actinobacillus (see **panel C** Fig. 3 and **panel D** Supplementary Fig. S6).

Finally, in terms of self-reported anxiety, a positive significant association was observed between child-reported SCARED scores and the relative abundance of *Selenomonas 3*, indicating that children with higher self-reports of anxiety have a higher abundance of *Selenomonas 3* (see **panel C** Supplementary Fig. S6). A detailed overview of these results (e.g. coefficient, q-values, prevalence) is presented in Supplementary Table S4. Note that the majority of the reported relationships were robust to correction for diagnostic group (see Supplementary Table S4), indicating that cross-diagnostic dimensional variations in anxiety, social and repetitive behaviors, rather than diagnosis-related features per se drove the identified associations.

An overview of the consistency of these results over the different analyses thus far is summarized in Table 2 and in Supplementary Fig. S7.

Table 2
Overview consistency results.

	Diagnosis-related differences MaAsLin2	Discriminative features LefSE	Associations clinical characteristics MaAsLin2
Stomatobaculum	☑	☑	☑
Ruminococcaceae UCG.014	☑	☑	☑
Tannerella	☑	☑	☑
Solobacterium	☑	☒	☑
Campylobacter	☑	☒	☒

3.5. Metadata lifestyle variables related to oral health and nutrition

3.5.1. Diagnosis-related differences in metadata lifestyle variables

Examining diagnosis-related differences in the assessed lifestyle variables revealed that the autistic children went signifcantly more to the dentist for filling a tooth (**OH10a**, $X^2 = 20.000$; $p = <0.001$; BH-threshold = <0.001) and prevention (**OH10b**, $X^2 = 12.727$; $p = <0.001$; BH-threshold = 0.002) compared to the non-autistic children (see Supplementary Tables S5–6, all reported with BH correction). In terms of nutrition, no significant differences between the autistic and non-autistic children were evident after BH correction (see Supplementary Tables S5–6).

3.5.2. Impact of metadata lifestyle variables on overall microbiome compositions

After performing a PCA to examine whether any of the assessed lifestyle variables impacted on the overall microbiome composition (beta-diversity), a significant impact of five confounding variables was observed, namely, (i) 'Did your child already go to the dentist for filling a tooth?' (**OH10a**) (score of 1 = 'yes', score of 0 = 'no'; significantly more in autistic children (p = <0.001); significant impact on beta-diveristy (p = 0.037)); (ii) 'Did your child already go to the dentist for esthetical reasons?' (**OH10c**) (score of 1 = 'yes', score of 0 = 'no'; no significant group difference; significant impact on beta-diveristy (p = 0.041)); (iii) 'How often as well as how much does your child eat salty/sweet snacks?' (**N24**) (Composition score: How often scored as: 0 = 'never', 5 = 'daily'; How much scored as: 0 = '0g', 4 = '100g'; no significant group differences; significant impact on beta-diveristy (p = 0.008)); (iv) 'How often as well as how much does your child eat eggs?' (**N15**) (Composition score: How often scored as: 0 = 'never', 5 = 'daily'; How much scored as: 0 = 'zero eggs', 5 = 'five eggs'; no significant group difference, significant impact on beta-diveristy (p = 0.024)) and (v) 'Is your child suffering at the moment of a loose tooth?' (**OH7a**) (score of 1 = 'yes', score of 0 = 'no'; no significant group differences; significant impact on beta-diveristy (p = 0.005)) (see Supplementary Fig. S8). These five confounding variables were taken into account into the diagnosis-related differences and association analyses. A detailed overview of all the included lifestyle variables can be found in Supplementary Table S5.

3.5.3. Impact of metadata lifestyle variables on diagnosis-related in differences in bacterial relative abundance and associations with clinical characteristics

All discriminative features identified at genus, as well as associations with clinical characteristics remained significant after correction for these lifestyle variables. A detailed overview of these results (e.g. coefficiënt, q-values, prevalence) can be found in Supplementary Tables S3–4. This indicates that the identified diagnosis-related differences and clinical associations with microbial abundances were not driven by external factors relating to oral health or nutrition.

4. Discussion

In the current study, we investigated diagnosis-related differences in oral microbiome compositions and associations with clinical characteristics in school-aged children with and without autism, which were matched based on age and biological sex. Given the limited number of studies in the field, our analyses are currently devoid of specific hypotheses and were mostly of an exploratory, hypothesis-generating nature.

4.1. Diagnosis-related differences in oral microbiome

Despite the relatively small sample size comprising 80 autistic and 40 non-autistic children, the unprecedented combination of meticulous patient-control, sample and extensive metadata selection, allowed to reveal robust and consistent diagnosis-related differences that were concordant across various taxonomic levels.

At genus level, we found that ***Solobacterium***, ***Campylobacter***, ***Stomatobaculum***, ***Ruminococcaceae UCG.014*** and ***Tannerella*** were significantly more abundant in autistic children, compared to the non-autistic children. The latter three were also identified as discriminative features of the autistic children via LefSe analysis. After correction for the five identified lifestyle variables with an overall impact on microbial compositions as indexed using beta-diversity, all these results remained evident. This indicates that even though specific lifestyle variables were shown to display a significant effect on overall microbiome composition (beta-diversity), they were not driving the identified diagnosis-related differences in bacterial abundances or their associations with clinical characteristics.

While an association between ***Solobacterium*** in the oral cavity and autism is novel, previous data on the gut microbiome already suggested a role of the bacterial family Erysipelotrichaceae, to which the genus *Solobacterium* belongs, as being more abundant within autistic children, in comparison with non-autistic children (Ding et al., 2020). This concordant observation is highly relevant, considering the increasing evidence that oral bacteria can translocate to the gut (Olsen and Hicks, 2020). Furthermore, prior research also showed a link between Erysipelotrichaceae and symptom severity, indicating higher abundance in children with higher autism symptom severity (Ding et al., 2020). Importantly, prior research has also demonstrated a connection between the presence of the family Erysipelotrichaceae in the gut and the development of colorectal cancer, as well as various inflammatory disorders affecting the GI tract (Kaakoush, 2015).

Similarly, for ***Campylobacter***, no established link between their presence in the oral cavity and autism has been reported before. However, increased abundances of *Campylobacter* species in the mouth have been linked to GI problems, including Inflammatory Bowel Disease (IBD) (Zhang, 2015). Given that autistic individuals are more likely to develop IBD (Zhang, 2015), our findings on the increased abundance of *Solobacterium* and *Campylobacter* in autistic children, in comparison with non-autistic children, could relate to this association with IBD and prospective follow-up of the current cohort might be of interest.

Stomatobaculum has also not previously been linked to autism but has been identified as a metagenomic biomarker for oral cancer (Eun et al., 2021). Furthermore, this bacterial genus has been related to prediabetic conditions (Rungrueang et al., 2021) and reflux (Liang et al., 2021).

In line with our results, the family Ruminococcaceae, to which the genus ***Ruminococcaceae UCG.014*** belongs, has been reported before as being overrepresented in autistic children in comparison with non-autistic children when looking at gut microbiome compositions (Ding et al., 2021). In particular, this bacterial family has been shown to be more abundant in autistic children with GI problems, in comparison with non-autistic children with GI problems (Rose et al., 2018).

Finally, regarding the genus ***Tannerella***, prior research reported a relative higher abundance in oral samples of non-autistic versus autistic children (Ragusa et al., 2020). However, our results are in line with recent rodent models of autism, describing *Tannerella* to be more abundant, both within mice receiving oral microbiota from autistic donor children, as within the autistic donor children themselves (Qiao et al., 2022). Interestingly, *Tannerella* has also been linked to periodontal diseases previously (Chukkapalli et al., 2015).

4.2. Associations between oral microbiome and clinical characteristics

Examination of the associations with clinical characteristics showed that of these five bacterial genera, the oral abundance of ***Solobacterium*** was significantly associated with more social difficulties, more repetitive and restrictive behavior and with more anxiety-like behavior. Furthermore, ***Stomatobaculum***, which emerged as a discriminative feature for the autistic children, was associated with more social difficulties. Moreover, ***Ruminococcaceae UCG.014***, which also emerged as a discriminative feature for the autistic children, was significantly associated with more social difficulties, more repetitive and restrictive behavior and more anxiety-like behavior. Lastly, ***Tannerella***, which also emerged as a discriminative feature for the autistic children, was significantly associated with more social difficulties and more anxiety-like behavior.

Also here, all the aforementioned associations remained evident after correcting for diagnosis as well as for several oral health and nutritional factors, known to impact overall microbial beta-diversity compositions. This indicates that variations in these bacteria were not particularly driven by variations in these lifestyle variables, but intrinsically related to variations in the clinical-behavioral constructs related to the autism phenotype, i.e. displaying heightened problems with social

and repetitive behaviors as well as anxiety. In line with our results, previous literature in rodent models demonstrated a significant positive association between the relative abundance of *Tannerella* and social difficulties, measured by the Sociability Index (Qiao et al., 2022). Note that this same study, in contrast to ours, also reported a positive association among the relative abundance of *Tannerella* and repetitive and restricted behavior in rodents, as measured by the Marble Burying test (Qiao et al., 2022).

In autistic children, Ragusa et al. (2020) reported a significant binominal regression between the relative abundance of *Tannerella* and social interaction and communication (measures by the ADOS), which is in line with our results, but they also reported a significant regression with restrictive and repetitive behavior (measured by the Autism Diagnostic Interview–Revised). The other four bacteria were not previously associated with oral microbiome compositions in autistic children.

Together, the identification of diagnostic and dimensional associations between oral microbiota and autistic-like behaviors suggests that oral microbiota characterizations may leverage important (biological) markers for diagnostic or treatment-evaluation purposes. Furthermore, while the exact mechanisms by which microbiota interact with behavior are not fully clear, the development of e.g., probiotic-based interventional approaches may be envisaged for enhancing or diminishing particular bacterial compositions, also in the oral cavity.

4.3. The oral microbiome-brain axis

At the moment, the precise mechanism how oral microbiome dysbiosis could influence brain functioning remains unclear. However, Bowland and Weyrich et al. (2022) have described some potential biological/anatomical mechanisms of the oral microbiome-brain axis. A first potential mechanism is that oral microbiome causes an increased permeability of the blood-brain barrier and a build-up of toxins in the brain via the production of pro-inflammatory cytokines. Secondly, oral microorganisms can reach the brain through systemic circulation and upon increased permeability of the blood-brain barrier, have a direct local impact. Thirdly, there could be a direct interaction between the oral microbiota and the central nervous system. Here, similar to the important attributed role of the nervus vagus in the gut-brain axis, predominantly the trigeminal nerve is deemed important for the innervation and the relaying of signals between the oral and nasal cavities and the brain. Furthermore, the oral microbiota can also directly affect certain pathways in the brain, and so the functional network connectivity, by influencing the production of neurotransmitters. Lastly, neurohormones, like cortisol, are known to modulate oral bacterial gene expression. Increased levels of cortisol may thereby cause changes in oral microbiome compositions, which in turn could lead to increased inflammation associated with periodontal diseases.

4.4. Limitations

While the current study provides important new insights into oral microbiome compositions within autistic children and the link with behavioral characteristics, the following limitations and recommendations are noted. First, due to the use of amplicon sequencing, diagnosis-related differences and associations were limited up to genus level. Increased taxonomic precision would allow looking at diagnosis-related differences at species to strain level and the association with behavioral outcome measures.

Second, the children included reflect a rather homogenous group of high-functioning children within a narrow pre-pubertal age range, rendering generalizability of the identified effects to more heterogeneous younger or older autistic individuals uncertain. Also, due to the high comorbidity of autism and intellectual disability, further research is needed within this subgroup of autistic children.

Third, we asked retrospectively to the children's food habits. As prospective food logs would be required to provide a more accurate overview of the children's food habits, these results need to be interpreted with caution.

While the included number of boys and girls in our sample reflected the well-documented four-to-one boys/girls ratio in autism prevalence (Fombonne, 2009), future research is warranted to examine the observed effects also in larger samples of girls with autism.

Lastly, given the limited number of existing studies in the field, our analyses are currently devoid of specific hypotheses and are therefore of a more exploratory nature.

5. Conclusion

This exploratory study underscores the potential role of the oral microbiome in diagnosis-related differences between autistic children and age and biological sex-matched controls. Consistent results provided important indications that the bacterial genera *Stomatobaculum, Ruminococcaceae UCG.014* and *Tannerella* were significantly more abundant in the oral cavity of autistic children, compared to non-autistic children and were identified as discriminative oral microbiome features of the autistic children. All these results remained evident even after correction for potentially confounding dental and nutritional variables. Further, these three bacteria were also significantly associated with more social difficulties, repetitive and restrictive behavior and anxiety-like behavior, even after correction for lifestyle confounding variables. However, the identified associations with clinical characteristics warrant further exploration of the oral microbiome's potential beyond the oral cavity and specifically with respect to neuropsychiatric conditions.

CRediT authorship contribution statement

Margaux Evenepoel: Formal analysis, Funding acquisition, Investigation, Methodology, Writing – original draft, Writing – review & editing. **Nicky Daniels:** Conceptualization, Data curation, Project administration. **Matthijs Moerkerke:** Conceptualization, Data curation, Project administration. **Michiel Van de Vliet:** Formal analysis. **Jellina Prinsen:** Conceptualization, Data curation, Funding acquisition. **Elise Tuerlinckx:** Project administration, Visualization. **Jean Steyaert:** Conceptualization, Funding acquisition, Project administration, Supervision. **Bart Boets:** Conceptualization, Funding acquisition, Project administration, Supervision. **Kaat Alaerts:** Conceptualization, Funding acquisition, Investigation, Project administration, Writing – review & editing. **Marie Joossens:** Conceptualization, Investigation, Methodology, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

the corresponding author will provide this answer

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbih.2024.100801.

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