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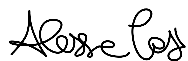
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**The oral microbiota in Autism Spectrum Disorders: an intra-familial clinical
study on associations with clinical and behavioral parameters**

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Table of contents

1. Introduction.....	1
1.1 Epidemiology	4
1.2 Etiopathogenesis	6
1.3 Clinical management of ASD	11
2. ASD and oral health	13
2.1 Dental caries.....	15
2.2 Periodontal status and oral hygiene.....	15
2.3 Parafunctional habits	15
2.4 Dental trauma and oral lesions	16
2.5 Malocclusions and oral abnormalities	16
2.6 Access to dental care	17
2.7 Behavioral approach in dentistry.....	17
2.8 Methodological limitations and future perspectives	20
3. Microbiome and Microbiota	21
3.1 Microbiota and Microbiome: definitions and general concepts.....	21
3.2 Development and modulation of the human microbiota	22
3.3 Microbiota in different body sites	24
3.4 Gut microbiota.....	24
3.5 Oral microbiota	25
3.6 Functions of the microbiota	27
3.7 Sequencing technologies and characterization of the human microbiota.....	29
4. Gut microbiota in Autism Spectrum Disorder	31
4.1 Techniques for characterization of the gut microbiota.....	33
4.2 Microbiota-Targeted therapeutic strategies in ASD	34
5. Oral microbiota and Autism Spectrum Disorder.....	36
5.1 Metagenomic studies and clinical associations	38
5.2 Artificial Intelligence and the oral microbiota	39
5.3 Limitations of current literature and future perspectives	40
6. Saliva and salivary diagnostics.....	42
7. Aim of the study	47
8 - Materials and methods	48
8.1 Study design and population	48
8.2 Study protocol.....	51
8.3 Clinical procedures.....	52

8.4 Salivary sample collection.....	53
8.6 Statistical analysis.....	54
8.6.1 Sample size considerations.....	54
9. Results	56
9.1 Descriptive analysis of the sample.....	57
9.2 Clinical and dental variables of the sample	58
9.3 Dietary variables of the sample	59
9.4 Perinatal and medical history variables of the sample	60
9.5 Distribution of F/B ratio and Proteobacteria in ASD and Caregiver samples	61
9.6 Distribution of clinical, microbiological, dental, Oral hygiene, and dietary variables according to dentition.....	62
9.6.1 Stratified analysis according to dentition of clinical, microbiological, and dental variables	62
9.6.2 Stratified analysis according to sex of clinical, microbiological, and dental variables	64
9.6.3 Stratified analysis according to ASD support level of clinical, microbiological and dental variables	66
9.7 Analysis of microbiological parameters	69
9.8 Descriptive statistics of microbiological variables.....	71
9.9 Descriptive analysis of quantitative microbiological parameters stratified according to dentist.....	72
9.10 Descriptive analysis of quantitative microbiological parameters stratified according to sex.....	73
9.11 Descriptive analysis of quantitative microbiological parameters according to ASD support level.....	74
9.12 Association Between F/B Ratio in ASD Subjects and Caregivers.....	75
9.13 Association Between Proteobacteria in ASD Subjects and Caregivers.....	75
10 - Discussion	77
11 - Conclusion	85
References	87

1. Introduction

Autism Spectrum Disorder (ASD) has attracted increasing attention in recent years not only in the field of neuropsychiatry, but also across several other medical disciplines, including dentistry. This growing interest is mainly related to the fact that autism, beyond affecting behavioral and communicative domains, may also influence multiple aspects of health and overall quality of life.

From a dental perspective, sensory difficulties, behavioral alterations, food selectivity, and reduced cooperation may considerably complicate oral health management in patients with ASD. These factors may contribute to poorer oral conditions, lower participation in preventive dental programs and, consequently, a reduced quality of life in these individuals.

In recent years, increasing attention has also been directed toward the human microbiota and its possible role in several physiological and pathological conditions. In particular, the oral microbiota represents a complex and dynamic ecosystem, influenced by diet, oral hygiene habits, environmental factors, and general health status.

In light of these considerations, the present study was designed to further investigate the microbiological and clinical characteristics of a sample of subjects with Autism Spectrum Disorder, with particular focus on the Firmicutes/Bacteroidetes (F/B) ratio and the relative abundance of Proteobacteria within the oral microbiota, analyzing their possible associations with clinical, dietary, and oral health variables.

At the same time, this research project also aims to highlight the importance of a personalized, gradual, and prevention-oriented dental approach for patients with ASD, emphasizing how specific and continuous professional training, appropriate behavioral management strategies, and a proper familiarization process may often result in levels of patient cooperation higher than expected.

Autism Spectrum Disorder

The term “autism” was first introduced by Eugen Bleuler in 1911 to describe the social withdrawal and self-centered behavior frequently observed in patients with schizophrenia.

In 1943, Leo Kanner used the term autism to describe children presenting language abnormalities, hypothesizing that extreme social isolation and insistence on sameness represented the core features of the syndrome [1].

In 1944, Hans Asperger described children with social deficits and above-average language abilities [2].

Autism Spectrum Disorder (ASD) is a multifactorial neurodevelopmental disorder characterized by deficits in social communication and the presence of restricted interests and repetitive behaviors [3].

According to the *Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition, Text Revision* (DSM-5-TR) [4], the diagnostic criteria for Autism Spectrum Disorder include:

- Persistent difficulties in social communication across multiple contexts:
 - Deficits in social-emotional reciprocity, ranging from abnormal social approach and inability to sustain a normal conversation, to reduced sharing of interests, emotions, or affect, up to failure to initiate or respond to social interactions.
 - Deficits in nonverbal communicative behaviors used for social interaction,
 - Deficits in developing, maintaining, and understanding relationships, including difficulties adapting behavior to different social contexts, sharing imaginative play, and lack of interest in peers [5],
- Restricted and repetitive patterns of behavior, interests, or activities, as manifested by at least two of the following:
 - Stereotyped or repetitive motor movements, use of objects, or speech,
 - Excessive adherence to routines or ritualized patterns of behavior,
 - Highly restricted and abnormal interests,
 - Hyper- or hyporeactivity to sensory input or unusual interest in sensory aspects of the environment [6].

The diagnosis of ASD requires the presence of at least three symptoms within the domain of social communication deficits and at least two symptoms within the domain of restricted and repetitive behaviors.

Another important change introduced in the DSM-5 was the replacement of the DSM-IV criterion requiring symptom onset before 36 months of age with the following definition: “Symptoms must be present in the early developmental period but may not become fully manifest until social demands exceed limited capacities” [4].

Symptoms must cause clinically significant impairment in social, occupational, or other important areas of current functioning and cannot be better explained by intellectual disability or global developmental delay [7].

The DSM-5 introduced the concept of ASD as a “spectrum”, combining the previous diagnoses included among pervasive developmental disorders (PDDs) into a single diagnostic category. These therefore include autistic disorder, Asperger syndrome, childhood disintegrative disorder, and pervasive developmental disorder not otherwise specified (PDD-NOS) [8].

Conversely, Rett syndrome is no longer included within ASD in the DSM-5, as it is considered a distinct neurological disorder.

Furthermore, the latest DSM version introduced Social (Pragmatic) Communication Disorder (SPCD) as a separate condition for individuals presenting difficulties in social communication without restricted or repetitive behaviors; therefore, these subjects do not meet the diagnostic criteria for ASD [9,10].

Severity level descriptors were also added to categorize the degree of support required by individuals with ASD (Table 1) [11].

An important aspect of the DSM-5-TR diagnostic criteria is the use of specifiers regarding current severity, level of intellectual impairment, language impairment, association with known medical or genetic conditions or environmental factors, and the presence of catatonia, allowing clinicians to provide a more detailed clinical characterization of ASD diagnosis [12].

Table 1. ASD severity levels

ASD Level	Description	Support Needs
Level 1 – Requiring Support	Individuals present difficulties in social communication and interaction, with reduced behavioral flexibility interfering with functioning in one or more contexts. They may appear interested in social interaction but experience difficulties initiating or maintaining reciprocal communication.	Requires support
Level 2 – Requiring Substantial Support	Marked deficits in verbal and nonverbal social communication skills are evident even with supports in place. Social impairments are more pronounced, and restricted/repetitive behaviors are frequent and clearly observable. Difficulty coping with change is commonly present.	Requires substantial support
Level 3 – Requiring Very Substantial Support	Severe deficits in verbal and nonverbal social communication cause major impairment in functioning. Initiation of social interactions is very limited, and responses to social approaches from others are minimal. Restricted and repetitive behaviors markedly interfere with all areas of daily functioning.	Requires very substantial support

1.1 Epidemiology

The World Health Organization (WHO) estimates the international prevalence of ASD at 0.76%; however, this figure currently represents only approximately 16% of the global pediatric population [13].

In recent years, the global prevalence of ASD has markedly increased. The 2023 *Community Report on Autism* published by the Centers for Disease Control and Prevention (CDC) reported an increase in diagnostic rates from 1 child in 88 (1.1%) in 2008 to 1 child in 36 (2.76%) in 2020, corresponding to a 151% increase over 12 years [14].

ASD affects approximately 1 child in 100 worldwide; however, prevalence rates vary considerably across countries and are influenced by diagnostic methods, study populations, and available healthcare resources [19].

A recent global meta-analysis reported a pooled ASD prevalence of 1.01% in North America, 0.73% in Europe, and 0.41% in Asia. The highest prevalence rates were observed in the United States (1.12%), Sweden (0.90%), and Denmark (0.73%), whereas the lowest rates were reported in Taiwan (0.11%), France (0.32%), and China (0.42%) [15].

The CDC estimates that approximately 1.68% of 8-year-old children in the United States (1 in 59) receive an ASD diagnosis [16]. However, parent-reported ASD diagnoses in the United States were slightly higher in 2016, reaching 2.5%, while the prevalence among adults was estimated at 2.2% [16].

The estimated prevalence of ASD in the United States increased from 1.1% in 2008 to 2.3% in 2018, likely in association with changes in diagnostic criteria, improvements in screening and diagnostic tools, and increased public awareness [17]. Nevertheless, the full impact of DSM-5 diagnostic criteria has not yet been completely observed [18].

Following the transition from DSM-IV to DSM-5, some studies predicted a reduction in ASD prevalence [14]. A systematic review suggested that only 50%–75% of individuals retained the diagnosis under the new criteria [19].

Overall, DSM-5 provides greater specificity, but lower sensitivity compared with DSM-IV; therefore, although individuals receiving an ASD diagnosis are more likely to actually present the disorder, a higher number of children may remain undiagnosed. The introduction of SPCD as a separate diagnosis is also expected to influence ASD prevalence estimates, although the magnitude of this effect remains unclear [20].

ASD occurs across all racial, ethnic, and socioeconomic groups, although diagnosis is not uniformly distributed among these populations. For example, White children are more frequently identified with ASD compared with Black or Hispanic children [21].

Although these differences appear to be gradually decreasing, the persistent disparity may still be related to stigma, limited access to healthcare services, and language barriers when English is not the patient's primary language. Nevertheless, while ASD prevalence was previously estimated to be approximately 50% higher among White children than among Black or Hispanic children, this gap has progressively narrowed with improvements in diagnostic equity [22].

ASD is more common in males [23]; however, a recent meta-analysis suggested that the actual male-to-female ratio may be closer to 3:1 rather than the previously estimated 4:1. Females meeting ASD diagnostic criteria are at greater risk of remaining undiagnosed, are more likely to mask social deficits through a process known as “camouflaging”, and may be affected by gender bias and persistent stereotypes describing ASD as a predominantly male disorder [24,25].

1.2 Etiopathogenesis

ASD is a neurobiological disorder influenced by both genetic and environmental factors acting on the developing brain. Current research continues to investigate the potential etiological mechanisms involved in ASD, although no single unifying cause has yet been identified.

Genetic factors play a major role in ASD susceptibility. Siblings of affected individuals show a significantly increased risk of diagnosis compared with the general population, and concordance rates are substantially higher among monozygotic twins, with heritability estimates around 50% [26].

The underlying mechanisms of ASD, which remain only partially understood, may involve immune dysregulation, mitochondrial dysfunction, oxidative stress, alterations of the gut microbiome, and hormonal abnormalities [27].

The etiology of ASD is considered multigenic and highly heterogeneous, with hundreds of risk genes identified to date [28].

Genome-wide association studies and whole-exome sequencing techniques have considerably expanded current knowledge regarding ASD susceptibility genes, contributing to a better understanding of the possible biological mechanisms involved. Candidate genes include those associated with brain development, neurotransmitter function, and regulation of neuronal excitability [29].

Many of the genetic defects associated with ASD encode proteins involved in synapse formation, transcriptional regulation, and chromatin remodeling [26].

Potential converging “networks” of genetic risk in ASD also include pathways related to neurotransmission and neuroinflammation. Alterations in transcriptional and splicing mechanisms, as well as epigenetic modifications, may also play a relevant role [30].

Particular attention has been directed toward genes involved in epigenetic modulation, synaptic transmission, immune function, ion transport, and GABAergic signaling [28].

Overall, ASD remains one of the most genetically heterogeneous neuropsychiatric disorders, with rare de novo and inherited variants identified in more than 700 genes [31].

Although genetics clearly plays a major role in ASD etiology, the phenotypic expression of genetic susceptibility remains extremely variable [32].

Several genetic syndromes are associated with an increased frequency of ASD compared with the general population, including Fragile X syndrome, tuberous sclerosis complex, Down syndrome, and Rett syndrome [33].

Studies involving children with sex chromosome aneuploidies have described a specific social functioning profile in males, suggesting increased vulnerability to autism [23]. Several theories also propose the existence of intrinsic protective factors in females.

A partial overlap between the genetic basis of ASD and that of other neuropsychiatric disorders, including schizophrenia, attention-deficit/hyperactivity disorder, intellectual disability, and bipolar disorder, has also been described [28].

A recent study additionally identified 16 novel genes associated with ASD, suggesting the involvement of new potential mechanisms, including cellular cytoskeleton structure and ion transport pathways [34].

The marked heterogeneity among available studies makes it difficult to quantify the exact contribution of etiological factors; however, in some individuals, genetic risk may also be modulated by prenatal, perinatal, and postnatal environmental factors [35].

Potential risk factors described in the literature include: prenatal exposure to thalidomide and valproic acid [36], advanced maternal and paternal age [37], maternal history of autoimmune diseases, such as diabetes, thyroid disorders, psoriasis, or maternal obesity [38], maternal infections during pregnancy, including rubella, influenza, cytomegalovirus infection, and fever, as well as maternal immune activation during gestation [39], particularly short or excessively long interpregnancy intervals [40], obstetric factors such as gestational diabetes, uterine bleeding, cesarean delivery, low birth weight, prematurity, and low Apgar scores [41], prenatal exposure to phthalates, air pollutants, and heavy metals such as lead and mercury, as well as possible exposure to pesticides [27].

Despite the concern generated by the well-known article published in *The Lancet* in 1998, which was later retracted, there is currently no scientific evidence supporting an association between vaccines, thimerosal, mercury, and ASD [42].

Available neuropathological studies remain limited; however, they have reported differences in cerebellar architecture and connectivity, abnormalities involving the limbic system, cortical alterations affecting the frontal and temporal lobes, together with other subtle malformations [43].

A small exploratory study investigating neocortical architecture in young children demonstrated focal disorganization of cortical laminar architecture in most analyzed subjects, suggesting abnormalities in cortical layer formation and neuronal differentiation [44].

Increased brain growth, both in terms of cortical size and extra-axial fluid volume, has also been described in children with ASD and is currently under investigation both for its potential role in ASD etiopathogenesis and as a possible biomarker [45].

Overall, research continues to identify factors associated with ASD risk; however, definitive causal relationships have not yet been established.

Diagnosis, Management, Prognosis and Complications

The diagnosis of ASD involves the identification and assessment of behavioral clinical symptoms according to DSM-5-TR diagnostic criteria by an experienced professional.

Early warning signs of ASD include poor eye contact, reduced response to name, lack of pointing or shared attention, absence of communicative gestures by 12 months of age, loss of language or social skills, and absence of symbolic play by 18 months. More general warning signs include a tendency to avoid eye contact and prefer solitude, difficulties understanding others' emotions or expressing one's own feelings, delayed language and communication skills, repetition of words or phrases, unrelated responses to questions, irritability in response to minor changes, restricted or obsessive interests, repetitive movements, and unusual reactions to sensory stimuli [47].

During preschool age, warning signs may include limited symbolic play, unusual or intensely focused interests, and behavioral rigidity. School-age children may instead exhibit concrete or literal thinking, difficulties understanding emotions, and impairments in conversational skills and social interaction.

ASD evaluation generally begins with pediatric population screening programs aimed at identifying children at risk or presenting suspicious signs, such as language, cognitive, or motor delays [48], followed by a comprehensive diagnostic assessment [49]. In fact, the American Academy of Pediatrics (AAP) recommends ASD screening for all children since early intervention may significantly influence outcomes.

An ASD assessment should include:

- complete medical, developmental, social, and family history,

- comprehensive physical examination, including growth parameters, detailed neurological examination, and evaluation of dysmorphic features or skin manifestations associated with neurocutaneous disorders,
- hearing and vision testing,
- structured behavioral observation, requiring specific specialist training of the clinician [47].

The gold standard for ASD diagnosis consists of a comprehensive evaluation performed by a multidisciplinary clinical team, based on direct observation of the child's behavior and interviews with parents/caregivers regarding developmental history and behavioral characteristics, using standardized instruments such as the *Autism Diagnostic Observation Schedule – Second Edition* (sensitivity 91%, specificity 76%) and the *Autism Diagnostic Interview* (sensitivity 80%, specificity 72%) [50]. Screening tools commonly used include the *Modified Checklist for Autism in Toddlers, Revised, with Follow-up* (M-CHAT-R/F) and the *Survey of Wellbeing of Young Children* (SWYC) [51].

Clinicians should also refer the child to a specialist for comprehensive evaluation and definitive diagnosis, which currently remains based on descriptive criteria in the absence of clear biomarkers or definitive diagnostic measures [52].

Guidelines from the American College of Medical Genetics and Genomics (ACMG) recommend chromosomal microarray analysis for all children, Fragile X testing in males, and additional genetic analyses, including *PTEN* and *MECP2* testing in selected patients, as first-line genetic investigations in ASD evaluation [53]. Owing to major advances in genetics, recent studies suggest that whole-exome sequencing may become the most appropriate method for clinical genetic testing in individuals with ASD [54].

In addition to genetic testing, further investigations may be appropriate in patients presenting specific signs or risk factors, such as developmental regression with loss or arrest of motor skills, hypotonia, recurrent vomiting episodes, lethargy, hypoglycemia, microcephaly, poor growth, suspected involvement of other organs, seizures, or ataxia.

Neuroimaging is not routinely indicated for all patients with ASD [55], but it may be recommended in subjects with suspected tuberous sclerosis, other neurocutaneous disorders, microcephaly, or neurological abnormalities.

Given the increasing prevalence of ASD worldwide, the development of efficient, accurate, and cost-effective screening and early diagnostic tools represents a major global challenge. Currently, ASD diagnosis is still mainly based on behavioral indicators and standardized

assessments, both of which present limitations related to subjectivity, the need for highly specialized professionals, and the time required for diagnostic procedures. Consequently, interest in objective biological biomarkers capable of supporting early diagnosis, patient stratification, and clinical monitoring in ASD is steadily increasing.

For symptom improvement, intensive behavioral interventions such as the *Early Start Denver Model* are recommended and appear beneficial in children aged five years or younger, particularly regarding language development, play, and social communication [17]. Pharmacological treatment is instead indicated for associated psychiatric conditions, such as emotional dysregulation or attention-deficit/hyperactivity disorder. Risperidone and aripiprazole may improve irritability and aggressive behaviors. Psychostimulants are effective in the treatment of attention-deficit/hyperactivity disorder. However, these medications are also associated with adverse effects, including changes in appetite, body weight, and sleep patterns [47].

The prognosis of ASD varies considerably among individuals, making outcomes difficult to predict, especially at the time of diagnosis. Although some children may show improvement in core symptoms, 9 out of 10 children younger than 6 years with ASD continue to meet diagnostic criteria for at least one year following diagnosis [56].

Favorable prognostic factors include better cognitive abilities at 2 years of age, reduction of repetitive behaviors, increased verbal intelligence quotient, and the presence of family and community support systems. Difficulties in executive functioning are associated with poorer outcomes [47].

In addition to the marked clinical and prognostic heterogeneity, individuals with ASD frequently present concomitant medical and psychiatric conditions. Whenever behavioral changes occur, physical causes of discomfort and medical conditions should always be considered.

Approximately 74% of individuals with ASD present at least one comorbidity [57], significantly more frequently than in the general population. These comorbidities represent a clinically relevant aspect in the overall management of the patient [58], particularly because symptoms may present atypically, making diagnosis itself more challenging.

Compared with individuals without ASD, subjects with ASD show higher rates of depression (20% vs 7%), anxiety (11% vs 5%), sleep disturbances (13% vs 5%), and epilepsy (21% in subjects with associated intellectual disability vs 0.8%) [17].

These conditions contribute not only to the complexity of the clinical picture, but also to worsening behavioral symptoms.

Another important aspect that should not be underestimated is gastrointestinal symptomatology (10–12% of cases) [6], including constipation, diarrhea, gastroesophageal reflux, abdominal pain, and food intolerances. The mechanisms underlying these manifestations appear to involve gut microbiota dysbiosis, chronic inflammation, and immune system dysfunction, suggesting a potential role of the microbiota–gut–brain axis in the pathogenesis and modulation of ASD symptoms [59].

In addition to core ASD symptoms, disruptive behaviors such as wandering, behavioral crises, self-injurious behavior, and aggression during medical settings are frequently associated with higher levels of anxiety in individuals with ASD. Deficits in attention and hyperactivity (ADHD), sleep disorders, and behavioral problems are also commonly observed [60].

More generally, ASD is associated with a significantly increased risk of mortality from all causes, including suicide [61].

Early recognition and treatment of comorbidities therefore remain essential to improve overall patient outcomes, quality of life, and well-being [58].

1.3 Clinical management of ASD

Families of children affected by ASD are constantly required to cope with neurodevelopmental challenges, frustration, anxiety, relational difficulties, and economic burden. At the same time, patients often encounter barriers in accessing care, making a personalized and structured approach particularly important.

One of the most widely proposed models to facilitate healthcare assistance is the SPACE model (sensory, predictability, acceptance, communication, empathy), which provides practical guidance for healthcare professionals caring for children with special needs and complex health conditions.

Sensory stimuli are often poorly tolerated by individuals with ASD and may become physically difficult to manage. These patients frequently require predictable routines to avoid generating anxiety and distress related to changes in the environment, while involuntary sensory behaviors in an unfamiliar setting may contribute to particularly

challenging situations. Stereotyped behaviors may also become highly demanding, requiring healthcare professionals to develop appropriate adaptation skills and empathy.

Under stressful conditions, individuals with ASD may experience difficulties in verbal communication and expression of their emotional needs. Excessive environmental stimulation, such as background noise or crowded settings, may contribute to sensory hyper-reactivity and emotional overload; therefore, healthcare professionals should avoid constantly drawing attention to the patient [62].

Individuals with ASD frequently experience difficulties accessing complex and coordinated healthcare services; therefore, strategies including family-centered and coordinated care models remain particularly important.

2. ASD and oral health

The complex relationship between ASD and oral health has been extensively documented in the scientific literature. Oral health conditions in individuals with ASD are now considered a significant issue requiring further investigation and clinical attention.

The relationship between ASD and oral health may be influenced by several variables, including sensory sensitivity, communication difficulties, and behavioral problems. However, available findings remain highly heterogeneous across studies, making it difficult to establish definitive conclusions regarding certain clinical outcomes [63].

Methodological differences represent one of the major limitations of the currently available literature. Studies vary considerably regarding participants' age, ASD diagnostic criteria, sample size, clinical assessment tools, socioeconomic variables, dietary habits, and caregiver involvement [64].

This topic involves several different aspects. Considering the high and continuously increasing prevalence of ASD, a growing number of dentists encounter children with ASD in daily clinical practice and are required to manage them appropriately. For this reason, understanding how ASD symptoms and severity may influence oral health, and how interdisciplinary approaches may improve healthcare assistance for this population, has become increasingly important [65].

According to several studies, oral health in individuals with ASD is influenced by selective food preferences, high carbohydrate intake, frequent consumption of soft and sugary foods, chronic medication use, resistance to oral hygiene procedures, and dependence on caregivers for daily oral hygiene practices. These habits may significantly increase the risk of dental caries, plaque accumulation, gingivitis, and alterations of the oral microbiota.

Furthermore, some medications frequently prescribed in ASD patients may negatively affect oral health, causing xerostomia, salivary alterations, increased appetite, and behavioral changes [66].

It should also be considered that ASD is frequently associated with self-injurious behaviors, anger, and behavioral crises, often triggered by daily environmental stimuli, increased activation, or stressful situations. More than 60% of children with ASD reportedly engage in physical self-injurious behaviors [67]. Specifically, regarding the oral cavity, bruxism,

tongue thrusting, gingival picking, and lip biting are common harmful habits that may increase susceptibility to periodontal disease, dental trauma, and other oral conditions [68].

Sensory alterations represent one of the major challenges during dental treatment in subjects with ASD. Patients may present hypersensitivity to noise, light, odors, physical contact, and dental instruments, resulting in reduced cooperation, oppositional behaviors, behavioral crises, and inability to complete dental treatment in an outpatient setting [69].

The difficulties experienced by individuals with ASD in accessing dental care are further aggravated by the complex interaction between behavioral problems, concomitant disabilities, and economic issues. Nevertheless, many individuals with ASD can successfully receive dental treatment in an outpatient setting using specific behavioral management strategies implemented by trained dental professionals. In more severe cases, conscious or deep sedation under general anesthesia in a hospital setting may become necessary [70].

Numerous studies in the field of ASD have investigated the prevalence and severity of dental caries, periodontal status, oral hygiene indices, oral diseases, malocclusions, oral habits, dental trauma, therapeutic approaches, use of general anesthesia, and the influence of ASD-related behaviors on oral health outcomes.

Overall, current evidence suggests that individuals with ASD frequently present poorer oral hygiene conditions, increased plaque accumulation, and greater gingival inflammation. However, oral health in the ASD population should not be interpreted exclusively through clinical parameters, but rather within a broader context including behavioral functioning, cognitive aspects, family environment, and accessibility to healthcare services [71].

Several studies underline the importance of caregiver involvement, as it significantly influences oral hygiene practices, toothbrushing frequency, access to dental care, and therapeutic compliance. Consequently, educational interventions directed toward caregivers may substantially improve oral health outcomes in these patients.

Another relevant aspect concerns sensory difficulties. Many individuals with ASD present tactile, auditory, and visual hypersensitivity, which may compromise treatment tolerance, feasibility of preventive procedures, and cooperation during dental care [64].

2.1 Dental caries

The relationship between ASD and dental caries remains not completely clarified.

Some studies report higher DMFT/dmft values in subjects with ASD compared with neurotypical controls, whereas others did not identify significant differences.

The pooled prevalence of dental caries reported in the literature ranges from 60.6% to 67.3% (95% CI = 44.0–75.19). These differences may be associated with dietary habits, sensory preferences, consumption of cariogenic foods, difficulties in oral hygiene practices, and disparities in access to dental care [72].

Some studies even reported a lower prevalence of dental caries in children with ASD compared with controls, possibly due to greater parental supervision of dietary habits [73]. Other studies, on the contrary, described an increased caries risk associated with reduced cooperation during oral hygiene procedures, limited motor abilities, sensory sensitivity, chronic pharmacological therapies, and behavioral difficulties [74].

2.2 Periodontal status and oral hygiene

The pooled prevalence of periodontal disease ranged from 59.8% to 69.4% (95% CI = 47.6–85.0) [72].

Several studies reported significantly higher levels of dental plaque, gingivitis, and gingival bleeding in individuals with ASD. These subjects frequently present poor oral hygiene, difficulties with toothbrushing, reduced tolerance to oral hygiene procedures, and greater bacterial biofilm accumulation. According to the authors, these problems may be associated with the same variables influencing dental caries prevalence [75].

2.3 Parafunctional habits

Several studies reported a higher prevalence of parafunctional habits such as bruxism, tongue thrusting, and lip biting, all of which may contribute to gingival inflammation, oral trauma, and occlusal alterations [76].

Bruxism represents one of the parafunctional habits most frequently observed in children with Autism Spectrum Disorder. It may occur both during sleep and wakefulness and,

according to several studies reported in the literature, individuals with ASD show a significantly higher prevalence of this condition compared with neurotypical peers. The exact causes underlying this increased prevalence are still not completely understood; however, several authors suggest a possible association with neurological alterations, sensory dysregulation, anxiety, stress, stereotyped behaviors, sleep disorders, and the use of specific pharmacological therapies [77].

Authors emphasize that bruxism may result in several oral complications, including dental wear, dentin hypersensitivity, dental fractures, muscle pain, temporomandibular disorders, and masticatory muscle fatigue. Management of this condition in individuals with ASD is often challenging because of reduced patient cooperation and associated behavioral difficulties. For this reason, treatment should be highly individualized and require a multidisciplinary approach including clinical monitoring, behavioral management, neurological evaluation, and stress control. In selected cases, intraoral devices may also be used, provided they are adequately tolerated by the patient.

2.4 Dental trauma and oral lesions

Several studies reported a higher frequency of dental trauma in individuals with ASD, associated with motor alterations, behavioral difficulties, behavioral crises, and self-injurious behaviors [73–75].

2.5 Malocclusions and oral abnormalities

Some studies reported a higher prevalence of malocclusions, crowding, open bite, and crossbite in individuals with ASD. However, findings remain inconsistent [78].

Regarding malocclusions, the review by Zerman et al. reported that individuals with ASD frequently present increased prevalence of dental crowding, open bite, crossbite, and dental protrusion. However, the authors also highlighted the heterogeneity and inconsistency of findings available in the literature. According to the review, several factors may contribute to the development of occlusal alterations in children with ASD, including parafunctional habits, tongue thrusting, bruxism, lip biting, oral breathing, muscular alterations, and atypical postures.

The presence of malocclusions may further compromise oral hygiene, mastication, speech, quality of life, and overall dental management of the patient. The authors also reported that orthodontic treatment in individuals with ASD may be particularly difficult because of limited cooperation, sensory sensitivity, difficulties adapting to orthodontic appliances, and associated behavioral problems. For this reason, orthodontic management should be individualized, gradual, multidisciplinary, and adapted to the patient's specific abilities [79]. The review also emphasized the importance of multidisciplinary approaches and personalized preventive programs aimed at improving quality of life, reducing the need for invasive treatments, decreasing the use of general anesthesia, and increasing accessibility to dental care [80].

2.6 Access to dental care

One of the most relevant findings concerns difficulties in accessing dental care.

Several studies reported poor cooperation during dental treatment, high anxiety levels, sensory alterations, and communication difficulties in patients with ASD. These factors frequently contributed to the need for sedation and general anesthesia. However, dental treatment under general anesthesia represents a significant clinical issue in the ASD population because of long waiting times, reduced therapeutic possibilities, and postoperative management difficulties [64].

The need for sedation or general anesthesia is significantly more frequent in individuals with ASD compared with healthy controls, mainly because of the behavioral and sensory difficulties associated with the disorder [80].

In addition, children with neurodevelopmental disorders generally face higher healthcare costs than their peers, and dental care is often more expensive because of the frequent need for behavioral management techniques, sedation, or general anesthesia [81].

2.7 Behavioral approach in dentistry

To perform dental procedures in children diagnosed with ASD, the use of basic behavioral approaches such as tell-show-do, positive reinforcement, and desensitization is essential. Visual pedagogy, social stories, video modeling, illustrated sequences, and applied behavior

analysis are also specialized educational and therapeutic approaches that have shown promising results in facilitating dental assessments and basic preventive treatments in the ASD population [64].

To ensure appropriate dental care, it is essential to recognize the importance of multidisciplinary approaches, structured preventive programs, caregiver support, environmental adaptations, behavioral techniques, and specific training of dental professionals. According to the authors, many preventive programs remain insufficient or are initiated too late, when dental problems are already established.

In children with ASD, early introduction of oral hygiene practices is considered essential. Authors emphasize that toothbrushing should be introduced gradually, using behavioral approaches, positive reinforcement, repetitive routines, visual supports, and progressive desensitization.

Visual support-based strategies may also improve cooperation and participation. These include visual schedules, visual representations of activities divided into step-by-step sequences, and visual timers to help the child understand the duration of the procedure [83]. In several settings, video tutorials have been developed to improve toothbrushing techniques in children with ASD through virtual modeling of target behaviors. Video modeling showing a child successfully undergoing a dental visit, as well as watching a favorite movie as a distraction technique, may also represent useful interventions to reduce fear and non-cooperative behaviors during dental appointments [84].

Interventions based on high-technology Augmentative and Alternative Communication (AAC) techniques have also been shown to improve cooperation during dental treatment when used within digital communication support systems and personalized ICT (Information and Communication Technology) applications [85].

Another strategy that may improve compliance in patients with ASD is the creation of “sensory-friendly” environments, aimed at modifying the sensory experiences perceived by children with ASD during dental care through adjustment of sensory stimuli present in the treatment area or waiting room [86]. These adaptations may include relaxing music, reduction of bright lights, and minimization of noise levels, with the goal of reducing anxiety and stress.

Active caregiver involvement is also essential for the success of preventive programs. Caregivers should receive appropriate education regarding correct toothbrushing techniques,

fluoride use, dietary habits, oral hygiene practices, and the importance of regular dental visits [87].

In the Indian study conducted by Dave et al., a self-administered questionnaire completed by parents to assess their knowledge regarding oral health showed that most parents were unaware of the relationship between oral health and general health. For this reason, the authors suggested the implementation of oral health promotion programs aimed at increasing awareness of the importance of oral health and, consequently, improving oral health-related quality of life in individuals with autism [88].

Prevention represents the cornerstone of dental management in patients with ASD, with the aim of reducing the need for invasive treatments, decreasing the use of general anesthesia, improving compliance, and promoting a better quality of life.

Another valuable form of support during dental visits and treatment is occupational therapy. Occupational therapists frequently work with children with ASD in different settings, evaluating sensory aspects, motor abilities, cognitive functioning, social skills, and communication abilities that may influence participation in daily activities. By identifying the child's strengths and difficulties, therapists can modify activities to facilitate participation. Consequently, they may support the child during dental treatment directly in the clinic, for example by assisting emotional regulation, or even before the appointment through home-based desensitization strategies and social stories [89].

An increasing body of evidence suggests the need to adapt dental treatment approaches for individuals with special healthcare needs, including patients with ASD. Nelson et al. examined the impact of desensitization on oral care in autistic children and recommended that, during the first dental visit, the patient should simply observe and experience the treatment environment. Adapting the clinical experience to the patient's individual needs and developing familiarity through consistent experiences may significantly improve cooperation [90].

Overall, strategies aimed at improving oral health in autistic individuals are multifactorial and involve modifications by dental professionals, parents or caregivers, as well as changes in healthcare delivery systems and the clinical environment. Further research is needed to better understand the barriers interfering with oral health improvement and access to appropriate dental care, as well as the importance of a multidisciplinary approach to oral health management [91].

Nevertheless, these traditional strategies may not always be sufficient in children with ASD, since some behaviors associated with the condition itself may represent barriers to dental care. For example, certain patients may exhibit repetitive or stereotyped movements, such as rocking or hand flapping, which can interfere with dental treatment.

In addition, many families of children with ASD report considerable difficulty finding dentists willing to treat their child. In fact, only approximately 40% of general dentists reported being willing to treat children with ASD [92].

2.8 Methodological limitations and future perspectives

Currently, studies investigating ASD and oral health present significant methodological limitations, including inconsistencies in oral health assessment methods and potential sources of bias.

Several reviews have reported heterogeneous findings, showing variability in the prevalence of dental caries and periodontal disease among individuals with ASD.

To improve current knowledge and strengthen available evidence in this field, further longitudinal studies with standardized criteria, larger sample sizes, longer follow-up periods, and shared protocols are needed. Future research should further investigate the relationship between ASD and the oral microbiota, the role of dietary habits, the impact of pharmacological therapies, and the potential development of personalized preventive strategies.

3. Microbiome and Microbiota

3.1 Microbiota and Microbiome: definitions and general concepts

The origin of the term “microbiota” dates to the early twentieth century, when it was observed that a vast number of microorganisms, including bacteria, yeasts, and viruses, coexist in different sites of the human body, such as the intestine, skin, lungs, and oral cavity. The human microbiota, also referred to as the “hidden organ”, contributes a quantity of genetic information more than 150 times greater than that of the entire human genome. Indeed, the microbial communities hosted by the human body outnumber human cells, with an estimated ratio of approximately ten bacterial cells for every human cell. Nearly 90% of the cells present on and within the human body are microbial cells [93].

The terms “microbiota” and “microbiome” are often used interchangeably; however, there are important differences between the two concepts. Microbiota refers to the living microorganisms present within a defined environment, such as the oral or gut microbiota. The microbiome, on the other hand, represents a broader concept and refers to the collective genomes of all microorganisms inhabiting a specific environment; it also includes microbial structural elements, metabolites, and associated environmental conditions [94].

The modern concept of the microbiome is progressively moving away from the idea of a “universal” microbiota shared by all individuals, shifting instead toward highly personalized and dynamic ecosystems continuously influenced by host–environment interactions. For this reason, the human microbiome is currently considered extremely variable even among apparently similar individuals.

To better understand the human metagenome, the Human Microbiome Project was launched in 2007 [95].

Each individual hosts different microbial communities localized in distinct body habitats, and these microbiomes differ considerably in both composition and function. Most human-associated bacteria belong to four major phyla: Actinobacteria, Firmicutes, Proteobacteria, and Bacteroidetes, each including numerous different bacterial species. The distribution and relative abundance of these phyla vary substantially depending on the anatomical site [96].

During co-evolution, humans and microbes have developed multiple reciprocal adaptive mechanisms. Understanding this relationship of physiological interdependence and, at the

same time, internal competition with commensal microorganisms is considered fundamental for understanding the transition between health and disease [97].

Studies conducted on germ-free models have demonstrated how profoundly the microbiome influences the development and functioning of the human organism [98]. Animals raised in completely sterile conditions show major immunological, metabolic, neurological, and structural alterations, suggesting that the microbiome should not be considered an accessory element, but rather an essential component of host physiology. These observations have also contributed to the development of the microbiota–gut–brain axis concept. In this context, the distinction between “innate genome” and “adaptive genome” has been proposed: the former corresponds to the human genetic heritage inherited at birth, relatively stable and encoded within human chromosomes, whereas the latter corresponds to the microbiome, an external, dynamic, and adaptable genetic system progressively acquired throughout life [99].

According to this view, the microbiome significantly expands the functional potential of the human organism by providing metabolic, immunological, and adaptive capabilities that are not directly encoded in the human genome itself. This concept introduces the term “meta-host”, according to which the human being is no longer considered an isolated organism, but rather a complex ecosystem composed of human cells and symbiotic microbial communities. The microbiome displays characteristics typical of a functional organ, to the extent that it has been defined as a “microbiotic organ”: it performs specialized metabolic activities, interacts with different physiological systems, contributes to host homeostasis, and participates in pathological processes [97].

Although under physiological conditions there is normally a dynamic balance between host regulation and microbial adaptation, disruption of this equilibrium may lead to dysbiosis, characterized by loss of microbiota ecological stability and development of pathological processes.

3.2 Development and modulation of the human microbiota

In general, microbiota development begins at birth through an initial phase known as “primary succession”, characterized by rapid changes in microbial composition. Subsequently, these variations progressively slow down until a relatively stable community,

referred to as the “climax community”, is reached during adolescence; however, the microbiota continues to fluctuate throughout adult life [100].

Neonatal colonization begins during delivery, and complex bacterial communities are already present by the end of the first week of life. Mode of delivery represents one of the first factors capable of influencing neonatal microbial colonization: following vaginal delivery, newborns predominantly acquire microorganisms derived from the maternal vaginal microbiota, whereas cesarean section is associated with an initial colonization more like the skin microbiota.

The first year of life represents a particularly important window for modulation of the human microbiota. During infancy, microbiota complexity progressively increases until reaching a configuration like that of adults.

Breastfeeding also plays a fundamental role in maturation of the infant microbiota. Human milk contains not only nutrients, but also immunoglobulins, metabolites, oligosaccharides, and microorganisms that directly influence the neonatal gut microbial ecosystem [101].

Diet represents one of the main factors capable of shaping the microbiota throughout life. Different dietary patterns modify both microbial composition and metabolic function. For example, Western, Mediterranean, vegetarian, vegan, or ketogenic diets induce different microbial profiles, influencing bacterial richness, relative abundance of taxa, and production of biologically active metabolites [102].

Perturbing events such as infections or antibiotic treatments, as well as environmental factors including water quality, geography, climate, migration, cohabitation, environmental exposures, and social interactions, may rapidly alter microbial community composition and function, sometimes promoting the establishment of new microbiota equilibria [103].

The human microbiota is characterized by marked individual personalization, relative stability, and resilience. A longitudinal study from the Integrative Human Microbiome Project, which monitored fecal, oral, skin, and nasal microbiota of healthy individuals over six years, demonstrated that oral and gut microbiota are generally more stable than skin and nasal microbiota.

Cohabiting individuals share a significant portion of their microbiota: approximately 12% of gut strains and up to 32% of oral strains may be shared among people living in the same environment. The concept of “social microbiome” describes the interaction among

individual microbiota within human social networks and their possible impact on health and disease [104].

3.3 Microbiota in different body sites

Microbial diversity varies according to anatomical site, and the functional complexity of bacterial communities is closely related to health status, genotype, diet, and hygiene habits [103].

Among the different body sites, the gastrointestinal tract represents the most densely populated bacterial environment, followed by the oral cavity and skin. The lungs, skin, urogenital tract, and other anatomical districts also host specific microbial communities [105].

Although healthy lungs were long considered sterile, several studies have demonstrated the presence of microbiota within pulmonary tissue. The central lung microbiota predominantly includes Actinobacteria, Bacteroidetes, Firmicutes, and Proteobacteria [106].

The skin also hosts different microbial communities. The distribution of glands and hair follicles creates distinct physical and chemical conditions that strongly influence skin microbiota composition [107].

The vaginal microbiome is highly specialized and relatively less diverse compared with other body sites. In most healthy women, *Lactobacillus* species predominate and contribute to maintenance of an acidic vaginal environment.

In recent years, evidence has also emerged regarding the possible presence of microbiota in sites previously considered sterile, such as the urinary tract, breast tissue, gallbladder, and certain vascular regions; however, these findings remain partly controversial [107].

3.4 Gut microbiota

The human gastrointestinal tract contains a microbial community composed of approximately 100 trillion microorganisms and represents the most important microbiological district for maintenance of human health [108].

The gut microbiota is generally considered to consist of six major phyla: Firmicutes and Bacteroidetes, which are predominant, together with Actinobacteria, Proteobacteria, Fusobacteria, and Verrucomicrobia [109].

The gut microbiota performs several physiological functions, including food fermentation, protection against pathogens, stimulation of immune responses, metabolism, and nutrient extraction [110].

Under physiological conditions, the gut microbiota is characterized by high taxonomic diversity, stability, resilience, and symbiotic interaction with the host. Nevertheless, the relative distribution of microorganisms is unique for each individual and may vary throughout life.

Environmental factors strongly influence the human gut microbiota. Fiber-rich diets generally promote the growth of short-chain fatty acid-producing bacteria, whereas Western diets rich in sugars and fats are frequently associated with reduced microbial biodiversity [105].

Medications also significantly influence the gut microbiota. Antibiotics represent the main pharmacological factor capable of profoundly altering intestinal ecosystem stability [111].

In recent years, several studies have highlighted the role of the microbiota–gut–brain axis in modulation of neurological and behavioral functions.

3.5 Oral microbiota

The oral microbiome represents a complex and dynamic microbial community normally residing within the oral cavity. The oral microbiota is considered the second largest microbial community in the human body and contributes to maintenance of host health through different mechanisms.

The oral cavity may be divided into different microbiological habitats, including saliva, tongue, dental surfaces, gingiva, buccal mucosa, palate, supragingival plaque, and subgingival plaque.

Temperature, oxygen availability, pH, nutrients, salivary flow, and surface adhesive properties represent important selective factors for the oral microbiome. Teeth constitute hard surfaces that allow persistent formation of complex microbial biofilms corresponding to dental plaque [112].

In general, the major bacteria present in the oral microbiota belong to the phyla Firmicutes, Proteobacteria, Bacteroidetes, Actinobacteria, Fusobacteria, and Spirochaetes. Despite considerable interindividual variability, these microbial groups constitute the predominant core of the human oral microbiota.

Under physiological conditions, the oral microbiome exists in a state of dynamic equilibrium with the host organism. Resident microbial communities contribute to protection against pathogens through ecological competition for nutrients and adhesion sites, production of antimicrobial substances, and modulation of local immune responses.

Saliva plays a fundamental role in maintenance of oral homeostasis: it contributes to pH regulation, mechanical removal of microorganisms, and transport of antimicrobial and immunological molecules [113].

Oral bacteria predominantly live organized within complex biofilms adherent to oral surfaces. Early colonizers, mainly streptococci, initially adhere to the dental surface and subsequently allow adhesion of secondary and late colonizing species.

A healthy oral microbiome should not be interpreted simply as the absence of pathogenic microorganisms, but rather as a relatively stable ecological equilibrium between microbiota and host organism, continuously influenced by local, environmental, and behavioral factors. Modern sequencing technologies have demonstrated that the oral microbiome possesses important metabolic and functional capabilities and participates in immune regulation, nutrient metabolism, and maintenance of mucosal homeostasis [114].

Alterations in oral microbiome structure resulting from diet, poor oral hygiene, antibiotic use, smoking, immune alterations, and modifications of the oral environment may affect not only oral health, but also systemic health [115].

Dental caries represents one of the main examples of diseases associated with oral dysbiosis. High availability of fermentable carbohydrates promotes proliferation of acidogenic and aciduric bacteria, leading to local pH reduction and progressive demineralization of dental enamel [116].

Periodontitis is also currently considered a complex dysbiotic disease characterized by chronic inflammatory lesions localized within the subgingival sulcus and degradation of periodontal tissues associated with complex subgingival biofilms. Gram-negative anaerobic bacteria such as *Porphyromonas gingivalis*, *Treponema denticola*, and *Tannerella forsythia*

are frequently associated with chronic periodontal lesions and possess virulence factors capable of altering the ecological balance of the oral biofilm [116].

Modern metagenomic technologies are expanding the traditional understanding of oral diseases, which are no longer considered infections caused by single pathogens, but rather multifactorial conditions associated with complex ecological imbalances of the oral microbiome.

Several studies have also associated periodontal diseases with increased risk of cardiovascular diseases, diabetes, rheumatoid arthritis, preterm birth, and other systemic inflammatory conditions, suggesting that alterations of the oral microbiome may have consequences extending far beyond the oral cavity [117].

3.6 Functions of the microbiota

The human microbiome is a dynamic and metabolically active ecosystem that continuously interacts with the host organism, contributing to maintenance of health and modulation of pathological processes.

Microbial communities actively participate in metabolic, immunological, and inflammatory processes through the production of metabolites, signaling molecules, and bioactive factors. The microbiome contributes to both innate and adaptive immune responses, influencing immune maturation, tolerance, and inflammation through continuous interactions among microorganisms, epithelial cells, and the host immune system [118].

Intermicrobial communication is also fundamental: bacteria use quorum sensing systems to coordinate gene expression and modulate the collective behavior of microbial communities [119].

Within the gastrointestinal tract, the microbiota produces metabolic enzymes that enhance the digestive and metabolic capabilities of the human host.

The gut microbiota also contributes to synthesis of vitamin K and most water-soluble B vitamins. Among the most extensively studied metabolites are short-chain fatty acids (SCFAs), involved in immunological, metabolic, and inflammatory regulation.

The oral microbiota also participates in important metabolic processes. Certain nitrate-reducing oral bacteria, including *Veillonella*, *Rothia*, and *Actinomyces*, contribute to nitric

oxide production, which is involved in regulation of vascular tone and systemic hemodynamics [120].

The concept of “acquired microbial immunity” reflects an immune function mediated by the human microbiome through two fundamental mechanisms: “colonization resistance” and “immune modulation”.

“Colonization resistance” refers to the ability of the resident microbiota to prevent colonization by pathogenic microorganisms, whereas “immune modulation” describes the ability of commensal microorganisms to influence development and maturation of the immune system [121].

Loss of these protective functions during dysbiotic conditions may promote infections, immune dysregulation, chronic inflammation, and development of systemic diseases.

Dysbiosis and Pathological Implications

Dysbiosis refers to an alteration in the composition, function, or stability of the microbiota associated with development of different pathological conditions.

In recent years, numerous studies have demonstrated that microbiota dysbiosis is involved in a broad range of human diseases, including metabolic, inflammatory, autoimmune, cardiovascular, neurological, and neoplastic disorders.

According to the “health–disease conversion model”, the transition from health to disease does not depend exclusively on the presence of specific pathogens, but rather on alterations in the ecological balance between the human organism and microbial communities [97].

The main dysbiosis patterns include [122]:

- loss of beneficial microorganisms,
- expansion of pathobionts,
- reduction of microbial diversity,
- functional metabolic alterations,
- ecological instability of microbial communities.

Within the gastrointestinal tract, dysbiosis has been associated with chronic inflammatory bowel diseases, irritable bowel syndrome, obesity, type 2 diabetes mellitus, and different neurological and neuropsychiatric conditions.

The microbiota may contribute to pathological processes through modulation of systemic immune responses and promotion of a chronic low-grade inflammatory state [123].

In recent years, several studies have reported alterations in gut microbiota composition in subjects with Autism Spectrum Disorder. Among the most frequently observed findings are reduced levels of *Bifidobacterium* and *Lactobacillus* and increased abundance of *Clostridium* species.

Some studies have also described alterations in the Firmicutes/Bacteroidetes (F/B) ratio, commonly used as a general index of gut microbiota balance [123].

Despite these findings, the relationship between dysbiosis and disease remains complex, and it is still not completely clear whether microbiota alterations represent a direct cause of disease or a secondary consequence of the pathological condition itself, especially considering that different diseases may exhibit at least partially overlapping microbiota patterns.

In recent years, microbiota-targeted therapies have been proposed as innovative strategies for prevention and treatment of different pathological conditions. These approaches include probiotics, prebiotics, dietary modifications, and fecal microbiota transplantation.

One of the major limitations of current microbiota-targeted therapeutic strategies is the marked interindividual heterogeneity of the human microbiota [125].

3.7 Sequencing technologies and characterization of the human microbiota

With the continuous development of next-generation sequencing technologies, together with gene expression analysis, metaproteomics, and metabonomics, differences in microbial composition and function are progressively being correlated with states of health and disease.

In many complex microbial communities, most microorganisms identified through molecular techniques have resisted cultivation attempts. For this reason, microbial community genomics and culture-independent molecular techniques have provided important insights into the complex diversity of the human microbiota.

Most studies investigating the human microbiota have focused on characterization of microbial communities through comparative analysis of bacterial 16S ribosomal RNA gene sequences. This gene represents a reliable microbial genetic marker because of the presence

of highly conserved regions and hypervariable regions that allow detailed phylogenetic resolution [118].

Modern high-throughput sequencing technologies have enabled simultaneous analysis of thousands of samples derived from large subject cohorts, profoundly changing the experimental design of microbiota studies.

Non-cultivable microorganisms can be identified through 16S rRNA gene sequencing, whereas entire genomes and metabolic pathways may be reconstructed using whole-genome shotgun metagenomics [126].

The Human Microbiome Project has played a fundamental role in identifying new microorganisms and microbial genes, contributing to expansion of databases used for microbial identification and functional annotation.

Bioinformatic analyses and integration of ecological parameters, such as community similarity, richness, and structure, have significantly contributed to understanding human microbial communities.

However, the degree of microbiota variation among different individuals still represents an important confounding factor in comparative studies.

In addition to microbial DNA and RNA sequencing, metaproteomic and metabonomic strategies allow identification of microbial biomarkers potentially associated with different host metabolic and physiological functions.

4. Gut microbiota in Autism Spectrum Disorder

The human intestine hosts trillions of microorganisms that constitute the gut microbiota and play a fundamental role in digestion, metabolism, immune regulation, and maintenance of intestinal barrier integrity. In recent years, increasing attention has been directed toward the concept of the “gut–brain axis”, a bidirectional communication network involving the central nervous system, enteric nervous system, immune system, endocrine system, and gut microbiota. Considering this, alterations in microbiota composition may influence these systems and contribute to the development of different pathological conditions [127].

The gut–brain axis refers to the bidirectional communication between the central nervous system and the gastrointestinal tract, integrating multiple neural, endocrine, immunological, and metabolic pathways. This axis is increasingly recognized for its role in regulation of mood, behavior, and cognitive functions, and its influence appears to extend beyond normal physiological processes, being implicated in several neuropsychiatric and neurodevelopmental disorders, including Autism Spectrum Disorder. Within the context of ASD, it has been hypothesized that alterations of the gut microbiota may interfere with this communication, potentially affecting neurodevelopment and suggesting a possible link between gut health and the disorder [128].

The gut–brain axis includes different pathways of continuous communication between the intestine and the brain. Among these, the vagus nerve plays a central role in transmission of signals from the gut to the brain, carrying sensory information and microbiota-derived signals. In ASD, alterations in vagal signaling related to microbial imbalance may contribute to changes in brain function and behavior [129]. Furthermore, the gastrointestinal tract contains the enteric nervous system (ENS), also referred to as the “second brain”, composed of a very large number of intestinal neurons and glial cells that connect the intestine and brain bidirectionally through the vagus nerve. This neuronal network enables the gastrointestinal tract to maintain part of its functions even in the absence of direct central nervous system input, while still receiving sensory information from the autonomic nervous system and transmitting information back to it [130].

Another important mechanism involved in the gut–brain axis is immune modulation. A substantial proportion of the body’s immune cells resides within gut-associated lymphoid tissue, and dysbiosis may lead to immune dysregulation, chronic inflammation, and release

of pro-inflammatory cytokines such as IL-6 and TNF- α , which may cross the blood–brain barrier and contribute to neuroinflammatory processes frequently observed in ASD. Several studies have indeed reported altered inflammatory cytokine levels in individuals with ASD, suggesting a possible involvement of immune processes in the pathophysiology of the disorder [131].

The gut microbiota also influences production of different hormones, neurotransmitters, and biologically active metabolites capable of modulating the central nervous system through systemic circulation. Among the most extensively studied metabolites are short-chain fatty acids (SCFAs), involved in immune regulation, intestinal barrier integrity, and gut–brain communication. Several studies have reported altered SCFA production in individuals with ASD; altered propionate levels, for example, may influence neuronal function and social behavior [132].

Among the mechanisms through which the gut microbiota may contribute to development or modulation of ASD symptoms are neuroinflammation, altered intestinal permeability, immune modulation, and production of neuroactive metabolites. Alterations of the intestinal barrier represent another possible mechanism involved in ASD: increased intestinal permeability may facilitate passage of microbial metabolites, toxins, and inflammatory mediators into systemic circulation, contributing to development of neuroinflammatory processes [133]. The vagus nerve also represents one of the principal communication pathways between the intestine and brain and, if influenced by signals derived from the gut microbiota, its activity may be altered, potentially modulating different neurological and behavioral functions.

Although the etiology of ASD is not completely understood, it is believed to result from the interaction of multiple genetic, environmental, immunological, and neurological factors. Early-life and environmental factors, including diet, antibiotic use, delivery mode, and environmental exposures during early developmental stages, may also interact with the gut microbiota and contribute to development of the disorder [68]. Children with ASD frequently present food selectivity and restricted dietary preferences, which may further contribute to alterations of the gut microbiota.

Children with ASD frequently present gastrointestinal disorders, including abdominal pain, constipation, diarrhea, gastroesophageal reflux, and bowel habit alterations, with significantly higher prevalence compared with the general population. Indeed, the incidence

of gastrointestinal problems in ASD has been estimated to reach up to 80%. Several studies have reported alterations in gut microbial composition in individuals with ASD compared with neurotypical subjects, highlighting differences in microbial diversity, taxonomic composition, and production of microbial metabolites [134].

Several studies have observed modifications in the ratio between Firmicutes and Bacteroidetes, the predominant phyla of the human gut microbiota. Some investigations reported increased abundance of bacteria belonging to the genera *Clostridium*, *Desulfovibrio*, and *Sutterella*, whereas other species generally considered beneficial, such as *Bifidobacterium* and *Prevotella*, were reduced. Certain *Clostridium* species are also capable of producing toxins and neuroactive metabolites that may influence the central nervous system and contribute to ASD behavioral symptoms [129]. Reduced gut microbial diversity has frequently been associated with ASD, and lower biodiversity may compromise intestinal ecosystem stability and favor proliferation of opportunistic microorganisms. However, currently available findings remain heterogeneous and, in some cases, contradictory.

4.1 Techniques for characterization of the gut microbiota

Different methodological approaches have been used to characterize microbial composition and its biological functions in ASD, including modern sequencing technologies that have significantly expanded understanding of gut microbial communities. The most commonly used methodologies include 16S ribosomal RNA gene sequencing and whole-genome shotgun sequencing applied to fecal samples [125].

16S rRNA gene sequencing represents one of the most widely used methodologies for analysis of the human bacterial microbiota. The 16S rRNA gene, present in all bacteria, encodes a component of the bacterial 30S ribosomal subunit and contains highly conserved regions alternating with hypervariable regions (V1–V9), allowing taxonomic identification of numerous bacterial species [135]. This technique enables characterization of complex microbial communities without the need for traditional microbiological culture and also allows identification of non-cultivable bacterial species.

Analysis based on 16S rRNA sequencing includes collection of the biological sample, extraction of microbial DNA, PCR amplification of hypervariable regions of the 16S rRNA gene using universal primers, and subsequent sequencing through Next Generation

Sequencing (NGS) platforms such as Illumina MiSeq. Obtained sequences are then processed through bioinformatic pipelines aimed at quality control of reads, removal of artifact sequences, and taxonomic assignment through comparison with reference databases such as SILVA, Greengenes, or RDP [135].

Furthermore, 16S rRNA sequencing allows evaluation of relative abundance of bacterial taxa, alpha-diversity, beta-diversity, and compositional differences among different sample groups. This methodology has been extensively used in the study of microbial community composition and diversity in ASD, providing important information regarding microbial shifts potentially associated with the disorder. Several studies have indeed reported consistent alterations in gut microbiota composition in individuals with ASD, including reduced microbial diversity and changes in relative abundance of specific bacterial taxa [136].

Although 16S rRNA sequencing represents a useful and relatively inexpensive technique, it also presents certain limitations: it mainly provides taxonomic information, has limited species-level resolution for some bacterial groups, and does not guarantee complete functional characterization of the microbiome compared with shotgun metagenomic approaches. Nevertheless, because of its relatively low cost, high sensitivity, and rapid execution, 16S rRNA gene sequencing remains one of the most widely used methodologies in human microbiota research.

Shotgun metagenomic techniques, on the other hand, allow analysis of the entire microbial genetic content present in biological samples, enabling not only taxonomic identification but also evaluation of potential metabolic functions of microbial communities. Metabolomic and metatranscriptomic approaches further contribute to understanding functional interactions between microbiota and host organism, including metabolite production and microbial gene expression profiles [126].

4.2 Microbiota-Targeted therapeutic strategies in ASD

Interest in the role of the gut microbiota in ASD has stimulated growing attention toward development of potential therapeutic strategies aimed at modulation of intestinal flora, including dietary interventions, probiotics, prebiotics, antibiotics, and microbiota transfer therapy. Probiotics and Microbiota Transfer Therapy represent emerging approaches aimed

at modulating gut microbiota composition and consequently contributing to improvement of gastrointestinal symptoms and certain behavioral manifestations in children with ASD [137]. Regarding dietary modifications, a diet rich in fiber, fruits, vegetables, and whole grains may promote growth of beneficial bacteria and increase production of microbial metabolites useful for the host organism. Diet indeed represents one of the main factors capable of influencing gut microbiota composition. Children with ASD frequently present food selectivity and restricted dietary preferences, which may contribute to alterations of the gut microbiota.

Among the most extensively studied dietary approaches are gluten-free diet, casein-free diet, dietary fiber supplementation, and nutritional modifications aimed at microbiota modulation. Some studies have reported clinical improvement in subgroups of patients, whereas others did not observe significant benefits; therefore, the efficacy of these interventions remains controversial [138].

In recent years, postbiotics have also been increasingly considered. These are bioactive compounds produced by gut bacteria during metabolism of prebiotics and may possess antibacterial, antioxidant, and immunomodulatory properties [124]. Among them are SCFAs such as acetate, propionate, and butyrate, which are involved in different biological processes including inflammation, hormonal regulation, intestinal barrier integrity, and gut–brain communication.

5. Oral microbiota and Autism Spectrum Disorder

The oral–gut–brain axis represents a complex bidirectional communication system through which the oral and gut microbiome may influence neurological function and behavior. Although most research investigating the microbiota–gut–brain axis has mainly focused on the gut microbiota, growing interest has recently emerged regarding the possible role of the oral microbiota in ASD [139]. The oral cavity is, in fact, one of the most complex microbial ecosystems of the human body and represents an important point of interaction between the external environment and the host organism.

The oral microbiota includes a large community of bacteria, viruses, fungi, and archaea colonizing different anatomical surfaces of the oral cavity, including the tongue, oral mucosa, saliva, dental surfaces, and gingival sulcus [140]. Under physiological conditions, these microbial communities live in dynamic equilibrium with the host and contribute to maintenance of oral homeostasis through mechanisms of competitive exclusion of pathogens and modulation of local immune responses [141].

Given the growing interest in biological biomarkers for ASD, the oral microbiota represents a promising opportunity, mainly because of its accessibility and the simplicity of salivary sampling, which is a non-invasive method easily applicable even in children or subjects with limited cooperation [139]. Saliva indeed represents an important diagnostic tool in oral microbiota research, as it provides a relatively representative reflection of the microbial communities inhabiting different oral habitats.

Modern metagenomic technologies and 16S rRNA gene sequencing now allow identification of microbial species previously not cultivable through conventional microbiological techniques. Recent sequencing studies demonstrated that individuals with ASD present alterations in oral microbiota composition compared with healthy controls, with significant differences in microbial diversity and relative abundance of specific bacterial taxa.

Within the field investigating the relationship between microbiota and ASD, the oral microbiota has also become an important area of research over the last decade, frequently showing alterations in children with ASD. Some studies observed higher abundance of bacteria potentially associated with inflammatory or cariogenic processes in ASD subjects, whereas other investigations reported reductions in species generally considered commensal

or protective [142]. Increased abundance of bacterial species belonging to the genera *Streptococcus*, *Haemophilus*, *Rothia*, and *Cutibacterium* has been described, together with reduced abundance of *Prevotella* and *Actinomyces*, which are more commonly associated with oral health. Reduced *Prevotella* has frequently been reported in both oral and gut microbiomes of subjects with ASD, suggesting a possible shared dysbiotic pattern across different body districts [143].

Another study reported reduced abundance of *Campylobacter concisus* and *Streptococcus vestibularis* in children with ASD. However, many microbial species associated with ASD are normally present within the human oral cavity, suggesting that observed differences mainly reflect ecological alterations of the microbial community rather than the simple presence of specific pathogens [144]. This concept supports the dysbiosis model as an imbalance involving the entire oral bacterial community.

Alterations of the oral microbiota in subjects with ASD may be influenced by multiple factors, including selective dietary habits, difficulties with oral hygiene, medication use, immune alterations, repetitive behaviors, and sensory sensitivities characteristic of ASD. In addition, the difficulties experienced by ASD patients within the dental setting may further contribute to alteration of the oral microbial ecosystem. Children with ASD frequently present food selectivity, restricted dietary habits, and difficulties with oral hygiene, all variables with potential impact on oral microbiological composition [145]. Available studies suggest that both food selectivity and oral hygiene have modest but significant effects on the observed microbiological variability; however, these factors alone do not appear to completely explain the differences associated with ASD.

The oral microbiota may influence systemic health through different biological mechanisms. Oral microorganisms and their metabolic products may reach the gastrointestinal tract through continuous swallowing of saliva, potentially contributing to modulation of the gut microbiota as well. Furthermore, chronic inflammatory conditions of the oral cavity may promote systemic release of inflammatory mediators and bacterial components, contributing to systemic inflammatory processes [146]. Several studies have shown that some oral bacteria are capable of surviving within the gastrointestinal tract and modulating intestinal immunity, microbial metabolism, and mucosal barrier integrity [147].

Alterations of the oral microbiome in ASD therefore do not appear to be limited exclusively to the oral cavity but may exert broader systemic effects through the dynamic interaction

among oral microbiome, gut microbiome, and central nervous system. Imbalances in the oral microbiome may influence production of biologically active microbial metabolites, including lipopolysaccharides (LPS), short-chain fatty acids (SCFAs), and amino acid-derived metabolites, which may enter systemic circulation and contribute to immune activation and chronic low-grade inflammation. Bacterial LPS may stimulate production of pro-inflammatory cytokines and promote microglial activation within the central nervous system, phenomena associated with neuroinflammation, altered neurodevelopment, and synaptic dysfunction observed in individuals with ASD [148].

Metagenomic analyses also allow evaluation of the functional potential of the oral microbiome and have identified different metabolic pathways associated with ASD children compared with neurotypical subjects, involving neurotransmitter metabolism, SCFA production, immune modulation, and production of pro-inflammatory metabolites. These functional differences suggest that the oral microbiome may indirectly contribute to neurobiological processes involved in ASD through production or modulation of neuroactive metabolites. Enrichment of enzymes involved in serotonin metabolism, GABA degradation, and dopamine metabolic pathways has been described; these neurotransmitters play a central role in behavioral regulation, cognitive function, and neurodevelopmental processes [149].

5.1 Metagenomic studies and clinical associations

An important study by Manghi et al. published in 2024 explored the oral microbiome of 2025 American families belonging to the SPARK-WGS cohort through metagenomic analysis [150]. Composition and function of the oral microbiome were examined in ASD children and neurotypical subjects, correlating metagenomic characteristics with IQ, social communication, developmental coordination, restrictive behaviors, and food selectivity. Results demonstrated modest but significant differences in oral microbiome composition between ASD children and neurotypical subjects. In fact, ASD diagnosis was significantly associated with oral microbiome composition through PERMANOVA analysis ($p = 0.001$), whereas alpha diversity did not show significant differences between ASD subjects and controls.

Subsequently, mixed linear models identified the microbial species most strongly associated with ASD, including *Actinomyces hongkongensis*, *Actinomyces johnsonii*, *Cutibacterium*

acnes, *Eikenella* species NML130454, and *Rothia dentocariosa*, whereas *Eubacterium sulci*, *Oribacterium parvum*, *Prevotella pallens*, *Prevotella shaii*, and *Prevotella jejunii* were more strongly associated with neurotypical subjects.

The study also demonstrated that ASD children with $IQ < 70$ showed significantly different oral microbial composition compared both with neurotypical subjects and ASD children with $IQ \geq 70$. The authors observed that greater severity of repetitive behaviors, poorer social communication scores, and altered motor coordination were associated with specific differences in oral microbiome composition.

Furthermore, the study found that ASD children with $IQ < 70$ showed reduced microbial strain sharing with their parents compared both with neurotypical children and ASD children with $IQ \geq 70$. More generally, environmental factors, behavioral habits, food selectivity, and social interactions may influence acquisition and maintenance of the oral microbiome in children with ASD.

Several studies have also provided important preliminary evidence regarding the possible relationship between oral microbiome composition and clinical features of autism. Ragusa et al. (2020) reported significant associations between oral microbiome composition and neurodevelopmental abnormalities, verbal intelligence, social interaction, communication, and repetitive/restrictive behaviors in young children with ASD [151]. Hicks et al. (2018), on the other hand, described associations between oral microbiome composition and severity of autistic symptoms evaluated using the Autism Diagnostic Observation Schedule (ADOS) [152].

MaAsLin2 and LEfSE analyses further confirmed the presence of specific microbiological signatures capable of discriminating ASD subjects from neurotypical controls. According to the authors, these findings suggest that, even in the absence of profound global alterations in microbial diversity, specific taxonomic modifications may characterize the oral microbiome in ASD subjects [153]. Variations in oral microbiological composition were also associated with different clinical and behavioral features of autism, including deficits in social interaction, repetitive behaviors, and anxiety symptoms.

5.2 Artificial Intelligence and the oral microbiota

Alongside the development of metagenomic technologies, artificial intelligence (AI) and machine learning algorithms are emerging as promising tools for analysis of complex

microbiological datasets. Metagenomic datasets generate multidimensional information that is often difficult to interpret using traditional statistical approaches. Machine learning algorithms may identify complex microbiological patterns associated with ASD and develop predictive models based on oral microbiome composition [154].

Several studies have used algorithms such as random forest, support vector machine, and deep neural networks to discriminate ASD subjects from neurotypical controls based on microbiological data. In some investigations, these models achieved high diagnostic accuracy, suggesting that the oral microbiome could represent a future source of non-invasive biomarkers for ASD screening [155]. More recent approaches use multimodal deep learning models integrating metagenomic data with neuroimaging, physiological parameters, clinical variables, and behavioral data [156].

Integration of different types of biological data could significantly improve diagnostic accuracy, clinical stratification, and early identification of subjects at risk. However, authors emphasize that clinical application of these models will require methodological standardization, multicenter validation, control of confounding factors, and reproducibility of results [157]. Variables such as diet, oral hygiene, antibiotic use, geographic variability, age, and environmental conditions may significantly influence the oral microbiome and compromise generalizability of predictive models.

5.3 Limitations of current literature and future perspectives

Despite the progress achieved so far, the precise role of the oral microbiota in ASD pathophysiology has not yet been completely clarified. Currently available findings remain heterogeneous and not fully consistent, probably because of methodological differences among studies and the high interindividual variability of the oral microbiome. Furthermore, the oral microbiome may be strongly influenced by diet, oral hygiene, medications, family environment, and socioeconomic conditions, all of which represent important confounding factors in microbiological studies investigating autism.

Although definitive causal relationships cannot yet be demonstrated, current findings support the hypothesis that the oral microbiome may represent a biological component associated with ASD. Future research should clarify the causal role of the oral microbiome, the interactions among oral microbiome, gut microbiome, and brain, as well as the possible

diagnostic and therapeutic use of microbiological alterations observed in ASD. Additional longitudinal and multicenter studies will be necessary to better understand the biological mechanisms involved and the potential clinical application of oral microbial biomarkers in ASD.

6. Saliva and salivary diagnostics

Saliva is a biological fluid secreted by the major salivary glands – parotid, submandibular, and sublingual glands – as well as by thousands of minor salivary glands distributed throughout the oral mucosa. Among its principal roles, saliva contributes to maintenance of oral health and oral hygiene and performs numerous biological functions; indeed, it is involved in swallowing, lubrication of oral tissues, and maturation of dental structures. Furthermore, thanks to its antimicrobial properties, saliva contributes to maintenance of oral microbial balance and prevention of infections [158].

Saliva is secreted in quantities ranging from 0.75 to 1.5 liters per day; it is odorless, colorless, and generally presents a pH ranging between 6.5 and 7.5. It consists predominantly of water but also contains organic and inorganic substances together with numerous microorganisms performing different biological and pathological functions [159]. Organic substances include urea, uric acid, glucose, fatty acids, glycerides, creatinine, amylase, peroxidase, and hormones, whereas inorganic components include ions such as Na^+ , K^+ , Ca^{2+} , Cl^- , Mg^{2+} , F^- , I^- , HCO_3^- , and H_2PO_4^- [160].

These substances perform different physiological functions. Amylase and lipase participate in digestion of carbohydrates and fats, whereas thiocyanate ions possess bactericidal activity. Calcium contributes to remineralization processes of dental tissues, while antioxidant enzymes present in saliva protect the oral cavity from the harmful effects of free radicals, reactive oxygen species, and different nitrogen-derived compounds. Saliva also contributes to regulation of oral pH and maintenance of oral environmental homeostasis through buffering capacity and antimicrobial activity [161].

Oral health can be preserved through primary prevention measures, early diagnosis, and prompt treatment of initial oral infections, namely dental caries and periodontitis, conditions closely related to imbalance of microbial biofilms within the oral cavity [162]. Prevention of these pathological conditions through home oral hygiene and fluoride application is undoubtedly fundamental; however, it is equally important to monitor and identify early alterations in oral health status to prevent disease onset and progression.

Although clinical examination and radiographs performed during dental visits still represent the current diagnostic gold standard, these methods may fail to detect very early cellular or molecular alterations. For this reason, increasing importance has progressively been

attributed to additional diagnostic tools and modern technological approaches. In this regard, different biological fluids, including peripheral blood, gingival crevicular fluid (GCF), and saliva, have been investigated for their diagnostic and prognostic potential in disease identification.

In recent years, interest in saliva as a diagnostic tool has progressively increased, mainly because of the possibility of obtaining biological information in a simple, rapid, and non-invasive manner. Saliva is indeed an easily accessible biological fluid whose collection can be performed without causing discomfort to the patient and without the need for invasive procedures, a feature particularly advantageous in children, fragile individuals, and patients with limited cooperation, as frequently observed in subjects with ASD. Moreover, salivary sampling can easily be repeated over time, allowing longitudinal monitoring of both oral and systemic health status.

Saliva contains many biologically active molecules, including proteins, enzymes, hormones, immunoglobulins, metabolites, nucleic acids, and microbial components, which may reflect different physiological and pathological processes occurring within the organism [163]. For this reason, numerous salivary biomarkers associated with oral and systemic diseases have been investigated in recent years, with the aim of identifying increasingly early, sensitive, and specific diagnostic tools [162].

A particularly interesting aspect is that saliva continuously bathes all surfaces of the oral cavity and may therefore provide a relatively faithful representation of the overall oral microbiome. This makes it possible to analyze microbiological variations associated with different pathological conditions, including dental caries, periodontitis, and oral microbiome alterations observed in individuals with ASD. Furthermore, saliva also allows evaluation of the host local immune response toward microorganisms present within the oral cavity.

Advances in molecular biology, genomics, and proteomics have led in recent years to the development of the concept of “salivaomics”, a term encompassing the genomic, proteomic, metabolomic, and microbiological information obtainable from salivary fluid. Modern sequencing technologies and highly sensitive methodologies now allow identification of biomarkers even at very low concentrations, considerably expanding the diagnostic potential of saliva [164].

Research on salivary diagnostics currently includes many clinical studies using saliva as a target vehicle for different biomarkers associated with oral diseases, measurable even at low

concentrations. Single salivary measurements generally represent a condition related to a specific time point; such evaluations may be useful for identifying specific patient groups or profiles associated with different diseases, as well as detecting outlier subjects. However, repeated salivary collections over time also demonstrate high utility since they provide information regarding disease progression and response to different therapeutic treatments. This approach also allows monitoring of the patient during maintenance phases and offers the possibility of timely intervention whenever necessary. According to several authors, these findings may contribute to identification of personalized salivary patterns in response to different stimuli and, in the future, may support development of personalized therapeutic approaches within precision dentistry [162].

Within the field of precision dentistry, the possibility of identifying specific salivary profiles associated with different pathological conditions could eventually allow increasingly personalized approaches in prevention, diagnosis, and therapeutic monitoring. Several authors hypothesize that integration of salivary biomarkers, microbiological data, clinical parameters, and artificial intelligence technologies may contribute to development of predictive models useful for early identification of subjects at risk of disease or monitoring pathological progression over time.

Some agents present in salivary fluid cross transcellular and paracellular pathways from the bloodstream, and their concentrations vary according to physiological and pathological processes; this makes saliva a potential non-invasive diagnostic tool. Developments in molecular biology, genomics, and proteomics have also led to the introduction of the term “salivaomics”, emphasizing the importance of salivary biomarkers in diagnosis of oral and systemic diseases and in evaluation of their different stages of progression [164].

The most important step in treatment nevertheless remains formulation of an accurate diagnosis. Salivary biomarkers investigated in longitudinal studies have shown potential usefulness in early diagnosis, evaluation of disease progression, monitoring of therapeutic response, and identification of specific pathological profiles. Among the most extensively studied biomarkers are inflammatory proteins, cytokines, enzymes, immunoglobulins, microbial metabolites, oxidative biomarkers, and components of the oral microbiome [166].

In clinical practice, numerous studies demonstrated the usefulness of saliva for monitoring periodontitis, dental caries, oral mucosal lesions, and peri-implant conditions. Some investigations also highlighted associations between salivary biomarkers and systemic

conditions, supporting the potential role of saliva as a non-invasive diagnostic tool also for extraoral diseases such as diabetes mellitus, cardiovascular diseases, and other infections [162]. For this reason, saliva is currently considered a promising source of biomarkers within personalized medicine.

In addition to oral diseases, saliva may also be used for diagnosis of systemic diseases. Furthermore, it finds application in drug detection and forensic identification [166]. In recent years, saliva has also proven useful in sports dentistry, where it represents an important diagnostic tool for evaluation of athletes' oral health status through analysis of microbiological, immunological, and ecological parameters of the oral cavity.

Several studies conducted by Tripodi et al. highlighted how physical activity may influence salivary parameters such as pH, salivary flow, buffering capacity, bacterial load, and secretory IgA levels, leading to alterations in oral microbiome balance and increased risk of oral diseases including dental caries, dental erosion, and gingival-periodontal diseases. Variations in oral microbiota composition have been observed in athletes according to the type of sport practiced, dietary habits, hydration status, and exposure to specific environments, such as aquatic sports settings [167].

Modern molecular technologies have further expanded the diagnostic potential of saliva. Advanced techniques currently allow simultaneous identification of proteins, nucleic acids, metabolites, and microbial components even at very low concentrations. Combined use of different biomarkers may improve diagnostic accuracy, prognostic capacity, and therapeutic monitoring. However, salivary composition and clinical interpretation of biomarkers may be influenced by different biological and environmental factors, including age, sex, circadian rhythm, salivary flow, diet, medications, systemic conditions, and oral hygiene.

Despite promising findings, several methodological limitations still make direct comparison of available literature difficult. Salivary composition may indeed be influenced by numerous factors, including age, sex, diet, circadian rhythm, hormonal status, medications, oral hygiene, salivary flow, and systemic conditions. Collection, storage, transport, and processing procedures may also significantly modify the microbiological and molecular results obtained.

For this reason, many studies in recent years have attempted to standardize salivary collection and analysis protocols. Nevertheless, complete methodological uniformity has not yet been achieved, and this represents one of the main limitations affecting comparability of

currently available data. Differences in DNA extraction techniques, sequencing platforms, and bioinformatic pipelines may indeed introduce important biases and contribute to heterogeneity of findings observed across different studies.

In addition, many studies present small sample sizes, methodological differences, variability in collection protocols, and different analytical approaches. For this reason, standardization of collection, storage, transport, processing, analysis, and interpretation of salivary samples appears essential. Although exploration of the oral microbiome has increased because of its association with different systemic diseases, standardization of saliva collection for microbiological analysis has not yet been fully achieved, and this contributes to the lack of truly comparable data.

Comparability of data is essential when evaluating different studies and longitudinal results since lack of comparability frequently leads to inconsistent findings and misleading conclusions. While standardization may be achieved by ensuring traceability to the International System of Units, harmonization is obtained by ensuring traceability to an agreed reference system together with verification of measurement comparability, which strongly depends on the analytical techniques used [169]. However, achieving comparability of microbiological data remains extremely difficult because sample collection methods, storage, DNA extraction, sequencing, and bioinformatic analysis may all introduce significant bias. This explains why apparently similar studies may reach different conclusions and why microbiome studies frequently present high methodological bias.

According to several authors, future technological developments may facilitate integration of salivary diagnostics into routine clinical practice, especially through point-of-care devices and precision dentistry approaches, with potential application both in early diagnosis and longitudinal monitoring of different oral and systemic diseases.

7. Aim of the study

Autism Spectrum Disorder is a neurodevelopmental condition characterized by marked heterogeneity in clinical presentation, associated comorbidities, sensory alterations, and the presence of numerous environmental and genetic factors potentially related to its pathogenesis.

In recent years, research has increasingly focused on the possible involvement of the oral microbiota in inflammatory, immunological, and metabolic processes associated with ASD, particularly regarding alterations of the oral microbial ecosystem observed in these patients. The present study was therefore designed to explore the relationship between selected oral microbiota parameters and Autism Spectrum Disorder through salivary sampling of ASD subjects and their cohabiting parents/caregivers. The analysis focused on descriptive patterns and possible associations with oral health status, eating habits, clinical variables, and behavioral characteristics. In addition, the study explored the relationship between ASD support level and oral clinical conditions observed in ASD subjects.

A further objective of the study was to assess the feasibility of dental management of ASD patients in an outpatient setting through personalized behavioral approaches, with the aim of promoting collaboration between patient and clinician and gradually reducing, when possible, the need for general anesthesia.

The purpose of this work is therefore to contribute to expanding current knowledge regarding oral and microbiological issues associated with ASD and to promote a more precise multidisciplinary and personalized approach to dental management in this patient population.

8 - Materials and methods

8.1 Study design and population

This observational cross-sectional pilot study was conducted on children and adolescents diagnosed with Autism Spectrum Disorder (ASD) and their cohabiting parents/caregivers. The study explored the distribution of selected oral microbiota parameters, oral health status, behavioral characteristics, and clinical variables through salivary analysis and clinical oral examination.

Participants were recruited at the Dental Clinic of the Department of Medical, Oral and Biotechnological Sciences, University “G. d’Annunzio” of Chieti–Pescara. ASD diagnosis had been previously established by specialized neuropsychiatric services according to DSM-5 diagnostic criteria.

The study population included children and adolescents with ASD undergoing dental evaluation together with their parents/caregivers living in the same household environment. Cohabiting family members were included in order to evaluate possible intra-familial similarities and environmental influences on oral microbiota composition.

Dear Parent/Caregiver,

Thank you for your willingness to participate in our research project "Patients with Autism Spectrum Disorder: dental examination, salivary sampling and microbiological analysis". To provide your child with the best possible assistance during the dental visit and to collect valuable data for our study, we kindly ask you to complete the following questionnaire. The information provided will help us better understand the patient's needs and personalize the clinical and behavioral approach during the dental examination, salivary sampling, and any necessary treatment.

All collected information will be treated confidentially and used exclusively for research and clinical purposes.

Question List

- 1. Patient's full name*
- 2. Date and place of birth*
- 3. Address and province of residence*

4. *Parent/Caregiver full name*
5. *Date and place of birth*
6. *Address and province of residence*
7. *Telephone number*
8. *E-mail address*
9. *When was the patient's last medical examination?*
10. *Does the patient currently have or has the patient ever had any of the following conditions?*
11. *Does the patient have any oral habits (tongue thrusting, thumb sucking, mouth breathing, others)?*
12. *Does the patient currently take or has the patient ever taken medications? If yes, which ones?*
13. *Has the patient ever undergone blood tests? If yes, when was the last time?*
14. *Has the patient ever been hospitalized? If yes, when and for what reasons?*
15. *Is the patient followed by a pediatrician or general practitioner?*
16. *If yes, please provide name, surname, and contact details.*
17. *Is the patient followed by other specialists?*
18. *Is this the patient's first dental visit?*
19. *If no, when was the last dental visit?*
20. *If no, at what age was the first dental visit performed?*
21. *Has the patient previously undergone dental treatment?*
22. *If yes, was the patient conscious or was the treatment performed under general anesthesia?*
23. *Has the patient ever undergone dental anesthesia?*
24. *Is or has the patient ever been treated with orthodontic appliances?*
25. *At what age did the first primary teeth erupt?*
26. *At what age did the replacement of primary teeth begin?*
27. *Does the patient report oral pain?*
28. *If yes, is the pain constant or does it occur under specific circumstances?*
29. *Does the patient present bruxism or clenching?*
30. *Does the patient report headaches?*
31. *Has the patient been influenced by previous dental experiences?*

32. *If yes, in what way?*
33. *How many members are there in the patient's household?*
34. *Who does the patient live with?*
35. *What occupations/jobs do the other family members have?*
36. *What are the patient's weekly activities?*
37. *Did the patient experience significant events or traumas during childhood?*
38. *If yes, which ones?*
39. *At what age did the patient receive the diagnosis of Autism Spectrum Disorder?*
40. *What events or situations led you to seek further evaluations?*
41. *Could you briefly describe the patient's main behavioral characteristics?*
42. *Is the patient followed by behavioral specialists?*
43. *Which behavioral management techniques are most effective with the patient?*
44. *The delivery was:*
45. *Was labor induction performed?*
46. *The childbirth was:*
47. *Were there any complications during pregnancy, labor or delivery?*
48. *If yes, which ones?*
49. *What were the baby's birth weight and length?*
50. *Were any problems identified at birth?*
51. *If yes, which ones?*
52. *What type of feeding was used for the patient? For how long?*
53. *When did weaning begin? With which foods?*
54. *Has the patient ever received oral hygiene instructions? If yes, from whom?*
55. *When were oral hygiene procedures started? Which devices/products were used?*
56. *Does the patient brush independently? If yes, from what age?*
57. *How many times per day does the patient brush their teeth and for how long?*
58. *What type of toothbrush does the patient use?*
59. *Does the patient use additional oral hygiene devices (dental floss, mouthwash, etc.)?*
60. *Does the patient follow a specific diet?*
61. *If yes, which one?*
62. *How many meals per day does the patient consume?*
63. *How much water does the patient drink per day?*

64. *Does the patient eat or drink before going to bed? If yes, what?*
65. *How many times per week does the patient consume milk and dairy products?*
66. *How many times per week does the patient consume sugary and/or carbonated drinks?*
67. *How many times per week does the patient consume sweets, packaged snacks, Chocolate, or candies?*
68. *How many times per week does the patient consume pasta, bread, or rice?*
69. *How many times per week does the patient consume meat?*
70. *How many times per week does the patient eat fish?*
71. *How many times per week does the patient eat legumes?*
72. *How many times per week does the patient eat fruit?*
73. *How many times per week does the patient eat vegetables*

8.2 Study protocol

Dental examinations and salivary sample collection were carried out at the Pediatric Dentistry Unit of the Dental Clinic of the University “G. d’Annunzio” of Chieti–Pescara and at the Dental and Oral Medicine Unit of the “A. Cardarelli” Hospital in Campobasso.

Upon arrival at the dental facility, parents/caregivers were asked to read and sign an informed consent form authorizing both the initial dental examination and salivary sample collection.

An initial dental visit was offered to all interested patients; however, inclusion in the research project required fulfillment of specific eligibility criteria.

The study sample was recruited according to the following criteria:

Inclusion Criteria

- Age > 4 years,
- Diagnosis of Autism Spectrum Disorder (ASD),
- Signed informed consent provided by a parent and/or caregiver (legal guardian).

Exclusion Criteria

- Suspected but unconfirmed ASD diagnosis,
- Absence of signed informed consent from a parent and/or caregiver (legal guardian),

- Presence of systemic diseases.

Based on completeness of the collected clinical data and compliance with the inclusion criteria, fifty subjects were ultimately included in the final study sample.

Clinical documentation regarding ASD diagnosis was collected for each participant, and the corresponding support level/severity grade was recorded.

In addition, one parent and/or caregiver for each patient was included in the study and underwent salivary sampling in order to obtain comparative information regarding the oral microbiome.

8.3 Clinical procedures

During the first visit, the following procedures were performed:

- Collection of anamnestic information, including patient demographic data, parent/caregiver demographic data, family history, physiological and pathological medical history, and dental history.
- Collection of clinical documentation related to ASD diagnosis.
- Application of behavioral management techniques.

According to the severity of the autism spectrum condition, different evidence-based behavioral approaches supported by scientific literature were used to improve the interaction between the patient with ASD, the dentist, and the dental examination. Specifically, the following techniques were adopted: visual pedagogy, video modeling, Applied Behavior Analysis (ABA), Treatment and Education of Autistic and related Communication Handicapped Children (TEACCH), desensitization and fading techniques, use of touch-pad devices, and music therapy.

This phase represented an integral component of both the first dental visit and any subsequent appointments when required.

The standard duration of the complete clinical session, including behavioral approach techniques, medical history collection, and clinical examination, ranged from 30 to 60 minutes per patient, depending on the patient's level of compliance and severity of the disorder.

In order to make the first dental visit a positive experience, a dedicated clinical area specifically designed for patients with ASD was used, characterized by reduced sensory

stimulation (dimmed lighting, noise reduction, and use of specific visual/audio materials) and absence of a chaotic environment.

- Clinical oral examination:

Assessment of clinical and diagnostic indices:

- Plaque Index (PI)

- Bleeding on Probing (BOP)

- DMFT/dmft index (Decayed, Missing, Filled Teeth)

- Soft tissue examination:

- Evaluation of lingual and labial frenula

- Occlusal assessment

- Evaluation of dental eruption and mixed dentition stage

- Radiographic examinations, when necessary.

8.4 Salivary sample collection

Salivary sampling was performed using the SaliveFe® device in both ASD patients and their caregivers. Before sample collection, participants were instructed to fast for at least 3 hours, avoid oral hygiene procedures during the 3 hours preceding collection, and refrain from taking occasional medications within the 7 days prior to the visit.

Saliva samples were collected in sterile containers and subsequently stored at low temperature (−80°C) until DNA extraction.

Professional oral hygiene procedures were performed in one or more dental sessions depending on patient cooperation, while fully respecting the patient's individual needs.

Whenever dental treatment was required, appointments were scheduled at appropriate time intervals to progressively carry out the necessary procedures.

8.5 Microbiological analysis

Salivary samples were anonymously processed at the Medical Genetics Service of the CAST (Center for Advanced Studies and Technology).

Microbial DNA was extracted from salivary samples and subjected to 16S rRNA gene sequencing.

Starting from the collected swab samples, bacterial DNA concentration was quantified using the Qubit 3.0 fluorometer (Thermo Fisher Scientific).

Next Generation Sequencing (NGS) was performed using the Ion 16S™ Metagenomics Kit, designed for analysis of mixed microbial populations through the Ion Torrent™ semiconductor sequencing workflow. The kit enables PCR amplification of hypervariable regions of the bacterial 16S rDNA gene.

Following library quantification using the Real-Time Step One PCR System (Thermo Fisher Scientific, Waltham, MA, USA), amplified fragments were sequenced using the Ion S5™ platform (Thermo Fisher Scientific), loaded onto an Ion 520™ chip, and analyzed using the Ion 16S™ Metagenomics analysis module for complex microbial samples.

Microbiological parameters were additionally categorized according to exploratory reference thresholds (F/B ≥ 3 ; Proteobacteria $\geq 30\%$) adopted based on available literature regarding healthy oral microbiota composition [170]. Considering the absence of universally validated pathological cut-offs for oral Firmicutes/Bacteroidetes ratio and Proteobacteria relative abundance, these thresholds should be interpreted as exploratory reference values.

8.6 Statistical analysis

Descriptive statistics were used to summarize the study sample. Continuous variables were reported as mean and standard deviation or median and interquartile range, according to their distribution and the presence of extreme values. Categorical variables were reported as absolute and relative frequencies. Comparisons among groups were performed using chi-square or Fisher's exact test for categorical variables and non-parametric tests for continuous variables when appropriate. Given the exploratory nature of the study and the limited sample size, analyses were interpreted as descriptive and hypothesis-generating. No predictive machine-learning model was retained in the final analysis because the available sample was not adequate for robust training, internal validation, or reliable estimation of classification performance.

8.6.1 Sample size considerations

The final sample included 50 ASD subjects and one cohabiting parent/caregiver for each subject. The sample size was therefore determined by feasibility and availability of eligible participants within the recruitment period. For this reason, the study should be interpreted as

a pilot exploratory investigation. The available sample allows preliminary description of clinical and microbiological patterns, but it is not adequate to support stable multivariable prediction models or classification analyses stratified across three ASD.

9. Results

Clinical and microbiological variables were categorized according to the cut-off values reported in Table 2. Subjects included in the study were identified as “ASD” when referring to patients with Autism Spectrum Disorder and as “Caregiver” when referring to the corresponding parent/caregiver.

Table 2. Cut-Off values used for variable categorization

Variable	0	1	2	3
F/B ratio	<3	≥3	—	—
Proteobacteria	<30%	≥30%	—	—
Oral hygiene	Poor	Fair	Good	—
Toothbrush type	Manual	Electric	—	—
Calculus	Absent	Mild	Moderate	Severe
PI	<0.2	0.2–0.4	>0.4	—
BOP	<10%	10–30%	>30%	—
DMFT	Absent	Present	—	—
Malocclusion	Absent	Present	—	—
Crowding	Absent	Present	—	—
Pharmacological therapy	Absent	Present	—	—
Type of dental treatment	Outpatient	General anesthesia	—	—
Previous dental visit	No	Yes	—	—
Last dental visit	>12 months/never	6–12 months	<6 months	—
Cooperation	Poor	Fair	Good	—
Sugar consumption	Low	Moderate	High	—
Pasta/bread consumption	Low	Moderate	High	—
Dairy product consumption	Low	Moderate	High	—
Meat consumption	Low	Moderate	High	—
Fish consumption	Low	Moderate	High	—
Legume consumption	Low	Moderate	High	—
Fruit consumption	Low	Moderate	High	—
Vegetable consumption	Low	Moderate	High	—
Hydration	<1 L/day	1–1.5 L/day	>1.5 L/day	—
Delivery mode	Vaginal	Cesarean section	—	—
Gestational age at birth	Preterm	Full-term	Post-term	—
Pregnancy/delivery complications	Absent	Present	—	—
Neonatal complications	Absent	Present	—	—
Feeding type	Breastfeeding	Formula feeding	—	—

Weaning	Early	Normal	Delayed	—
Behavioral therapy	No	Yes	—	—

9.1 Descriptive analysis of the sample

As shown in Table 3, from a demographic perspective the sample consisted predominantly of male subjects (70%), in agreement with the higher prevalence of ASD reported in the literature.

Regarding ASD support level, more than half of the patients presented level 3 ASD (54%), indicating a population characterized by significant clinical impairment and high support needs.

Mixed dentition was the most frequent condition (44%), followed by permanent dentition (38%) and deciduous dentition (18%).

From a microbiological perspective, 50% of the sample presented a Firmicutes/Bacteroidetes ratio ≥ 3 , while Proteobacteria values $\geq 30\%$ were observed in 58% of subjects, suggesting a frequent alteration of oral microbiota balance.

The age difference between ASD subjects and caregivers, shown in Table 4, averaged 35.9 ± 4.6 years, ranging from 27 to 45 years. Median age difference was 35 years (IQR: 33–40).

Table 3. Descriptive analysis of the sample

Variable	Category	n (%)
Gender	Male	35 (70)
	Female	15 (30)
ASD Level	Level 1	9 (18)
	Level 2	14 (28)
	Level 3	27 (54)
Dentition	Deciduous	9 (18)
	Mixed	22 (44)
	Permanent	19 (38)
F/B Ratio	<3	25 (50)
	≥ 3	25 (50)
Proteobacteria	<30%	21 (42)
	$\geq 30\%$	29 (58)

Table 4. Age difference between ASD subjects and caregivers

Variable	Mean $\pm$ SD	Median (IQR)	Min–Max
ASD–Caregiver age difference	35.9 ± 4.6	35 (33–40)	27–45

9.2 Clinical and dental variables of the sample

The sample predominantly showed fair or poor oral hygiene levels, whereas only 20% of subjects presented good home oral hygiene.

Manual toothbrushes represented the most used oral hygiene device (62%). Moderate or severe calculus accumulation was observed in 62% of subjects, while moderate/high PI and BOP values were frequently detected, suggesting widespread impairment of oral hygiene and a relatively common gingival inflammatory condition within the studied sample.

Malocclusion and crowding were observed in 58% and 32% of the sample, respectively. DMFT values were also elevated: 56% of patients presented a DMFT ≥ 4 , a finding consistent with high exposure to cariogenic factors and possible preventive and behavioral difficulties associated with ASD.

Regarding therapeutic and behavioral aspects, 42% of subjects were receiving chronic pharmacological therapy.

Previous dental treatments had been performed under general anesthesia in 36% of patients, highlighting the difficulties associated with the autism spectrum condition.

A total of 82% of subjects had already undergone at least one previous dental examination; however, 56% of patients had not attended regular dental check-ups for more than 12 months or had never undergone recent dental visits.

Cooperation during dental procedures was heterogeneous, with a relatively balanced distribution among poor, fair, and good cooperation levels.

Table 5. Clinical and dental variables of the sample

Variable	Category	n (%)
Oral hygiene	0 = poor	17 (34%)
	1 = fair	23 (46%)
	2 = good	10 (20%)
Toothbrush type	0 = manual	31 (62%)
	1 = electric	19 (38%)
Calculus	0 = absent	7 (14%)
	1 = mild	12 (24%)
	2 = moderate	17 (34%)
	3 = severe	14 (28%)
PI	0 = absent/mild	16 (32%)
	1 = moderate	14 (28%)

	2 = severe	20 (40%)
BOP	0 = absent/mild	16 (32%)
	1 = moderate	16 (32%)
	2 = severe	18 (36%)
Malocclusion	0 = absent	21 (42%)
	1 = present	29 (58%)
Crowding	0 = absent	34 (68%)
	1 = present	16 (32%)
DMFT	0 = <4	22 (44%)
	1 = ≥4	28 (56%)
Pharmacological therapy	0 = absent	29 (58%)
	1 = present	21 (42%)
Type of dental treatment	0 = outpatient	32 (64%)
	1 = general anesthesia	18 (36%)
Previous dental visit	0 = no	9 (18%)
	1 = yes	41 (82%)
Last dental visit	0 = >12 months/never	28 (56%)
	1 = 6–12 months	8 (16%)
	2 = <6 months	14 (28%)
Cooperation	0 = poor	16 (32%)
	1 = fair	16 (32%)
	2 = good	18 (36%)

9.3 Dietary variables of the sample

From a dietary perspective, the sample showed a high prevalence of frequent carbohydrate and sugar consumption. 54% of subjects presented high sugar intake, while 86% consumed pasta and bread frequently.

Dairy product consumption was also high in most of the sample (60%).

Regarding protein sources, meat consumption appeared predominantly moderate or high, whereas fish and legume intake was generally low, respectively in 68% and 66% of subjects. Fruit and vegetable consumption was more heterogeneous, with a relatively balanced distribution between low and high intake levels. Nevertheless, a substantial proportion of the sample showed poor fruit (38%) and vegetable (36%) consumption.

Regarding hydration, 44% of subjects consumed between 1 and 1.5 liters of water per day, whereas 38% reported intake below 1 liter/day. Only a minority of the sample showed high water intake (>1.5 L/day).

Overall, the dietary pattern was characterized by high carbohydrate intake together with limited nutritional variety in part of the sample, an aspect potentially relevant in modulation of the oral microbiota.

Table 6. Dietary variables of the sample

Variable	Category	n (%)
Sugar consumption	0 = low	8 (16%)
	1 = moderate	15 (30%)
	2 = high	27 (54%)
Pasta/bread	0 = low	2 (4%)
	1 = moderate	5 (10%)
	2 = high	43 (86%)
Dairy products	0 = low	11 (22%)
	1 = moderate	9 (18%)
	2 = high	30 (60%)
Meat	0 = low	9 (18%)
	1 = moderate	19 (38%)
	2 = high	22 (44%)
Fish	0 = low	34 (68%)
	1 = moderate	13 (26%)
	2 = high	3 (6%)
Legumes	0 = low	33 (66%)
	1 = moderate	14 (28%)
	2 = high	3 (6%)
Fruit	0 = low	19 (38%)
	1 = moderate	13 (26%)
	2 = high	18 (36%)
Vegetables	0 = low	18 (36%)
	1 = moderate	9 (18%)
	2 = high	23 (46%)
Hydration	0 = <1 L	19 (38%)
	1 = 1–1.5 L	22 (44%)
	2 = >1.5 L	9 (18%)

9.4 Perinatal and medical history variables of the sample

Within the perinatal variables, cesarean delivery, observed in 54% of subjects, was more frequent than vaginal delivery.

Most patients were born full-term (56%), whereas 36% were preterm; post-term births were less frequent (8%).

Pregnancy or delivery complications were reported in 36% of the sample, while neonatal complications at birth were reported in 28% of subjects.

Regarding infant feeding during the first months of life, formula feeding was more frequent than breastfeeding (56% vs 44%).

Weaning occurred predominantly within the normal time frame (52%), whereas equal proportions of subjects presented early or delayed weaning (24% each).

Finally, almost the entire sample (92%) was receiving behavioral therapy specific for Autism Spectrum Disorder.

Table 7. Perinatal variables of the sample

Variable	Category	n (%)
Delivery mode	0 = vaginal	23 (46%)
	1 = cesarean section	27 (54%)
Gestational age at birth	0 = preterm	18 (36%)
	1 = full-term	28 (56%)
	2 = post-term	4 (8%)
Pregnancy/delivery complications	0 = absent	32 (64%)
	1 = present	18 (36%)
Neonatal complications	0 = absent	36 (72%)
	1 = present	14 (28%)
Feeding type	0 = breastfeeding	22 (44%)
	1 = formula feeding	28 (56%)
Weaning	0 = early	12 (24%)
	1 = normal	26 (52%)
	2 = delayed	12 (24%)
ASD behavioral therapy	0 = no	4 (8%)
	1 = yes	46 (92%)

9.5 Distribution of F/B ratio and Proteobacteria in ASD and Caregiver samples

The categorical distribution analysis of the Firmicutes/Bacteroidetes (F/B) ratio showed a predominance of values ≥ 3 in both the ASD and Caregiver groups. Specifically, 52% of ASD subjects and 58% of caregivers presented values above the selected cut-off.

Regarding Proteobacteria, values $\geq 30\%$ were observed in 58% of ASD samples and 60% of Caregiver samples, suggesting a frequent high relative abundance of this phylum within the oral microbiota of the studied subjects.

Overall, these findings indicate that microbiological patterns above the selected exploratory thresholds were frequent both in ASD subjects and in caregivers. Therefore, these results should be interpreted cautiously and cannot be considered specific to ASD without comparison with an independent control group.

Table 8. Distribution of F/B ratio and Proteobacteria in ASD and Caregiver groups

Variable	Category	ASD n (%)	Caregiver n (%)
F/B Ratio	0 = <3	24 (48%)	21 (42%)
	1 = ≥3	26 (52%)	29 (58%)
Proteobacteria	0 = <30%	21 (42%)	20 (40%)
	1 = ≥30%	29 (58%)	30 (60%)

9.6 Distribution of clinical, microbiological, dental, Oral hygiene, and dietary variables according to dentition

9.6.1 Stratified analysis according to dentition of clinical, microbiological, and dental variables

Stratified analysis according to dentition revealed a heterogeneous distribution of clinical, microbiological, and behavioral variables among subjects with deciduous, mixed, and permanent dentition.

Male sex was predominant across all dentition groups.

At the same time, subjects with permanent dentition showed a higher frequency of more severe ASD levels, likely related to the higher mean age of this subgroup.

From a microbiological perspective, the distribution of the Firmicutes/Bacteroidetes ratio and Proteobacteria showed some variability among the different dentition groups. In particular, subjects with mixed and permanent dentition showed a tendency toward a higher prevalence of values compatible with dysbiotic patterns, although differences did not reach statistical significance.

Clinical dental indices also showed descriptive differences among the groups: moderate and high PI and BOP values tended to be more frequently represented in subjects with mixed and permanent dentition — as did DMFT values — suggesting a possible progressive worsening of oral hygiene and oral health conditions with increasing dental complexity and longer exposure time of permanent teeth to cariogenic factors.

Regarding behavioral and dietary variables, differences in dietary habit distribution were observed among dentition groups, although without statistically significant associations. These differences may reflect physiological age-related modifications in food preferences and dietary habits.

Categorical variables were compared using the chi-square test or Fisher's exact test when appropriate. A p-value <0.05 was considered statistically significant.

Overall, although statistically significant associations were not observed for most analyzed variables, the results describe potentially relevant clinical and microbiological trends.

Table 9. Stratified analysis according to dentition

Variable	Category	Permanent Dentition (2)	Mixed Dentition (1)	Deciduous Dentition (0)	p-value
GENDER	F	5 (26.3%)	8 (36.4%)	2 (22.2%)	0.668
	M	14 (73.7%)	14 (63.6%)	7 (77.8%)	
ASD LEVEL	1	1 (5.3%)	5 (22.7%)	3 (33.3%)	0.333
	2	5 (26.3%)	7 (31.8%)	2 (22.2%)	
	3	13 (68.4%)	10 (45.5%)	4 (44.4%)	
F/B (ASD)	0	7 (36.8%)	15 (68.2%)	3 (33.3%)	0.073
	1	12 (63.2%)	7 (31.8%)	6 (66.7%)	
Proteobacteria (ASD)	0	11 (57.9%)	7 (31.8%)	3 (33.3%)	0.203
	1	8 (42.1%)	15 (68.2%)	6 (66.7%)	
Calculus	0	0 (0.0%)	4 (18.2%)	2 (22.2%)	0.042
	1	2 (10.5%)	7 (31.8%)	3 (33.3%)	
	2	6 (31.6%)	8 (36.4%)	2 (22.2%)	
	3	11 (57.9%)	3 (13.6%)	2 (22.2%)	
PI	0	3 (15.8%)	8 (36.4%)	5 (55.6%)	0.231
	1	6 (31.6%)	7 (31.8%)	1 (11.1%)	
	2	10 (52.6%)	7 (31.8%)	3 (33.3%)	
BOP	0	1 (5.3%)	8 (36.4%)	6 (66.7%)	<0.001
	1	4 (21.1%)	12 (54.5%)	1 (11.1%)	
	2	14 (73.7%)	2 (9.1%)	2 (22.2%)	
DMFT	0	6 (31.6%)	11 (50.0%)	7 (77.8%)	0.071
	1	13 (68.4%)	11 (50.0%)	2 (22.2%)	
Malocclusion	0.0	7 (36.8%)	10 (45.5%)	4 (44.4%)	0.780
	0.01	1 (5.3%)	0 (0.0%)	0 (0.0%)	
	1.0	11 (57.9%)	11 (50.0%)	5 (55.6%)	
	2.0	0 (0.0%)	1 (4.5%)	0 (0.0%)	
Crowding	0	13 (68.4%)	13 (59.1%)	5 (55.6%)	0.539
	1	6 (31.6%)	7 (31.8%)	4 (44.4%)	

	2	0 (0.0%)	2 (9.1%)	0 (0.0%)	
Sugar Consumption	0	5 (26.3%)	4 (18.2%)	1 (11.1%)	0.059
	1	6 (31.6%)	2 (9.1%)	5 (55.6%)	
	2	8 (42.1%)	16 (72.7%)	3 (33.3%)	
Pasta/Bread	0	1 (5.3%)	1 (4.5%)	0 (0.0%)	0.674
	1	1 (5.3%)	2 (9.1%)	2 (22.2%)	
	2	17 (89.5%)	19 (86.4%)	7 (77.8%)	
Dairy Products	0	4 (21.1%)	6 (27.3%)	3 (33.3%)	0.579
	1	3 (15.8%)	3 (13.6%)	3 (33.3%)	
	2	12 (63.2%)	13 (59.1%)	3 (33.3%)	
Meat	0	5 (26.3%)	3 (13.6%)	1 (11.1%)	0.487
	1	7 (36.8%)	10 (45.5%)	2 (22.2%)	
	2	7 (36.8%)	9 (40.9%)	6 (66.7%)	
Fish	0	9 (47.4%)	19 (86.4%)	5 (55.6%)	0.008
	1	10 (52.6%)	1 (4.5%)	4 (44.4%)	
	2	0 (0.0%)	2 (9.1%)	0 (0.0%)	
Legumes	0	12 (63.2%)	17 (77.3%)	6 (66.7%)	0.427
	1	5 (26.3%)	5 (22.7%)	3 (33.3%)	
	2	2 (10.5%)	0 (0.0%)	0 (0.0%)	
Fruit	0	6 (31.6%)	9 (40.9%)	2 (22.2%)	0.761
	1	6 (31.6%)	6 (27.3%)	2 (22.2%)	
	2	7 (36.8%)	7 (31.8%)	5 (55.6%)	
Vegetables	0	6 (31.6%)	11 (50.0%)	3 (33.3%)	0.525
	1	2 (10.5%)	4 (18.2%)	2 (22.2%)	
	2	11 (57.9%)	7 (31.8%)	4 (44.4%)	
Hydration	0	7 (36.8%)	9 (40.9%)	4 (44.4%)	0.496
	1	7 (36.8%)	10 (45.5%)	5 (55.6%)	
	2	5 (26.3%)	3 (13.6%)	0 (0.0%)	
Oral Hygiene	0	6 (31.6%)	9 (40.9%)	2 (22.2%)	0.866
	1	10 (52.6%)	9 (40.9%)	5 (55.6%)	
	2	3 (15.8%)	4 (18.2%)	2 (22.2%)	

9.6.2 Stratified analysis according to sex of clinical, microbiological, and dental variables

Stratified analysis according to sex showed a predominance of male subjects within the studied sample.

Distribution of ASD severity levels showed a higher frequency of more severe forms among males compared with females, although differences were not statistically significant.

From a microbiological perspective, the Firmicutes/Bacteroidetes ratio and Proteobacteria abundance showed a relatively heterogeneous distribution in both sexes. Male subjects showed a slight tendency toward a higher prevalence of microbiological patterns compatible with oral dysbiosis, although observed differences did not reach statistical significance.

Clinical dental indices also showed variability between males and females. Moderate and high PI and BOP values were more frequently represented in male subjects, suggesting possible greater impairment of oral hygiene conditions in this subgroup, whereas DMFT values appeared relatively homogeneous between sexes without statistically significant differences.

Analysis of dietary habits showed an overall similar distribution between males and females, despite some descriptive differences regarding frequency of consumption of specific food groups.

Sex-stratified analyses did not reveal statistically significant associations for most of the considered variables ($p>0.05$).

Table 10. Stratified analysis according to sex

Variable	Category	M	F	p-value
ASD LEVEL	1	5 (14.3%)	4 (26.7%)	0.571
	2	10 (28.6%)	4 (26.7%)	
	3	20 (57.1%)	7 (46.7%)	
F/B (ASD)	0	19 (54.3%)	6 (40.0%)	0.537
	1	16 (45.7%)	9 (60.0%)	
Proteobacteria (ASD)	0	13 (37.1%)	8 (53.3%)	0.453
	1	22 (62.9%)	7 (46.7%)	
Calculus	0	4 (11.4%)	2 (13.3%)	0.071
	1	6 (17.1%)	6 (40.0%)	
	2	15 (42.9%)	1 (6.7%)	
	3	10 (28.6%)	6 (40.0%)	
PI	0	10 (28.6%)	6 (40.0%)	0.632
	1	11 (31.4%)	3 (20.0%)	
	2	14 (40.0%)	6 (40.0%)	
BOP	0	9 (25.7%)	6 (40.0%)	0.360
	1	14 (40.0%)	3 (20.0%)	
	2	12 (34.3%)	6 (40.0%)	
DMFT	0	18 (51.4%)	6 (40.0%)	0.665
	1	17 (48.6%)	9 (60.0%)	
Malocclusion	0.0	15 (42.9%)	6 (40.0%)	0.796
	0.01	1 (2.9%)	0 (0.0%)	
	1.0	18 (51.4%)	9 (60.0%)	
	2.0	1 (2.9%)	0 (0.0%)	
Crowding	0	22 (62.9%)	9 (60.0%)	0.578
	1	11 (31.4%)	6 (40.0%)	
	2	2 (5.7%)	0 (0.0%)	
Sugar Consumption	0	7 (20.0%)	3 (20.0%)	0.375

	1	11 (31.4%)	2 (13.3%)	
	2	17 (48.6%)	10 (66.7%)	
Pasta/Bread	0	1 (2.9%)	1 (6.7%)	0.733
	1	4 (11.4%)	1 (6.7%)	
	2	30 (85.7%)	13 (86.7%)	
Dairy Products	0	9 (25.7%)	4 (26.7%)	0.540
	1	5 (14.3%)	4 (26.7%)	
	2	21 (60.0%)	7 (46.7%)	
Meat	0	8 (22.9%)	1 (6.7%)	0.022
	1	9 (25.7%)	10 (66.7%)	
	2	18 (51.4%)	4 (26.7%)	
Fish	0	24 (68.6%)	9 (60.0%)	0.749
	1	10 (28.6%)	5 (33.3%)	
	2	1 (2.9%)	1 (6.7%)	
Legumes	0	26 (74.3%)	9 (60.0%)	0.568
	1	8 (22.9%)	5 (33.3%)	
	2	1 (2.9%)	1 (6.7%)	
Fruit	0	12 (34.3%)	5 (33.3%)	0.981
	1	10 (28.6%)	4 (26.7%)	
	2	13 (37.1%)	6 (40.0%)	
Vegetables	0	15 (42.9%)	5 (33.3%)	0.685
	1	6 (17.1%)	2 (13.3%)	
	2	14 (40.0%)	8 (53.3%)	
Hydration	0	14 (40.0%)	6 (40.0%)	0.451
	1	14 (40.0%)	8 (53.3%)	
	2	7 (20.0%)	1 (6.7%)	
Oral Hygiene	0	9 (25.7%)	8 (53.3%)	0.034
	1	21 (60.0%)	3 (20.0%)	
	2	5 (14.3%)	4 (26.7%)	

9.6.3 Stratified analysis according to ASD support level of clinical, microbiological and dental variables

Stratified analysis according to ASD support level showed descriptive differences in the distribution of clinical, microbiological, and behavioral variables among subjects with ASD levels 1, 2, and 3.

Male sex was predominant across all levels of ASD severity, with a higher frequency among subjects classified as ASD level 3.

Regarding dentition, subjects with more severe ASD more frequently presented permanent dentition, probably in relation to the higher mean age of this subgroup.

Microbiological analysis showed a tendency toward a higher prevalence of dysbiotic patterns in subjects with ASD level 3. Elevated Firmicutes/Bacteroidetes ratio and Proteobacteria

values were more frequently represented in subjects with more severe clinical forms, although without statistically significant differences.

Clinical dental indices also showed a progressive tendency toward worsening with increasing ASD severity. Moderate and high PI and BOP values were more frequently observed in subjects with ASD level 3.

Similarly, presence of caries experience and calculus tended to be more frequent among subjects with higher ASD levels.

Analysis of dietary habits showed some heterogeneity among the different ASD levels, with possible differences related to food selectivity and behavioral characteristics typically associated with the disorder.

Overall, although statistically significant associations were not observed for most of the considered variables ($p>0.05$), the observed trends suggest a possible relationship between ASD clinical severity, oral health conditions, and oral microbiota alterations.

Table 11. Stratified analysis according to ASD support level

Variable	Category	3	2	1	p-value
GENDER	F	7 (25.9%)	4 (28.6%)	4 (44.4%)	0.571
	M	20 (74.1%)	10 (71.4%)	5 (55.6%)	
F/B (ASD)	0	10 (37.0%)	10 (71.4%)	5 (55.6%)	0.106
	1	17 (63.0%)	4 (28.6%)	4 (44.4%)	
Proteobacteria (ASD)	0	11 (40.7%)	3 (21.4%)	7 (77.8%)	0.028
	1	16 (59.3%)	11 (78.6%)	2 (22.2%)	
Calculus	0	0 (0.0%)	1 (7.1%)	5 (55.6%)	0.001
	1	7 (25.9%)	5 (35.7%)	0 (0.0%)	
	2	8 (29.6%)	5 (35.7%)	3 (33.3%)	
	3	12 (44.4%)	3 (21.4%)	1 (11.1%)	
PI	0	6 (22.2%)	5 (35.7%)	5 (55.6%)	0.244
	1	7 (25.9%)	4 (28.6%)	3 (33.3%)	
	2	14 (51.9%)	5 (35.7%)	1 (11.1%)	
BOP	0	4 (14.8%)	6 (42.9%)	5 (55.6%)	0.095
	1	11 (40.7%)	3 (21.4%)	3 (33.3%)	
	2	12 (44.4%)	5 (35.7%)	1 (11.1%)	
DMFT	0	11 (40.7%)	8 (57.1%)	5 (55.6%)	0.537
	1	16 (59.3%)	6 (42.9%)	4 (44.4%)	
Malocclusion	0.0	10 (40.0%)	7 (50.0%)	4 (44.4%)	0.906
	1.0	15 (55.6%)	7 (50.0%)	5 (55.6%)	
	2.0	1 (3.7%)	0 (0.0%)	0 (0.0%)	
Crowding	0	15 (55.6%)	11 (78.6%)	5 (55.6%)	0.466
	1	10 (37.0%)	3 (21.4%)	4 (44.4%)	

	2	2 (7.4%)	0 (0.0%)	0 (0.0%)	
Sugar Consumption	0	6 (22.2%)	1 (7.1%)	3 (33.3%)	0.488
	1	8 (29.6%)	3 (21.4%)	2 (22.2%)	
	2	13 (48.1%)	10 (71.4%)	4 (44.4%)	
Pasta/Bread	0	1 (3.7%)	1 (7.1%)	0 (0.0%)	0.120
	1	1 (3.7%)	1 (7.1%)	3 (33.3%)	
	2	25 (92.6%)	12 (85.7%)	6 (66.7%)	
Dairy Products	0	9 (33.3%)	2 (14.3%)	2 (22.2%)	0.748
	1	4 (14.8%)	3 (21.4%)	2 (22.2%)	
	2	14 (51.9%)	9 (64.3%)	5 (55.6%)	
Meat	0	3 (11.1%)	5 (35.7%)	1 (11.1%)	0.103
	1	10 (37.0%)	3 (21.4%)	6 (66.7%)	
	2	14 (51.9%)	6 (42.9%)	2 (22.2%)	
Fish	0	15 (55.6%)	14 (100.0%)	4 (44.4%)	0.025
	1	11 (40.7%)	0 (0.0%)	4 (44.4%)	
	2	1 (3.7%)	0 (0.0%)	1 (11.1%)	
Legumes	0	20 (74.1%)	9 (64.3%)	6 (66.7%)	0.537
	1	5 (18.5%)	5 (35.7%)	3 (33.3%)	
	2	2 (7.4%)	0 (0.0%)	0 (0.0%)	
Fruit	0	8 (29.6%)	6 (42.9%)	3 (33.3%)	0.864
	1	7 (25.9%)	4 (28.6%)	3 (33.3%)	
	2	12 (44.4%)	4 (28.6%)	3 (33.3%)	
Vegetables	0	9 (33.3%)	10 (71.4%)	1 (11.1%)	0.036
	1	4 (14.8%)	1 (7.1%)	3 (33.3%)	
	2	14 (51.9%)	3 (21.4%)	5 (55.6%)	
Hydration	0	12 (44.4%)	3 (21.4%)	5 (55.6%)	0.338
	1	10 (37.0%)	8 (57.1%)	4 (44.4%)	
	2	5 (18.5%)	3 (21.4%)	0 (0.0%)	
Oral Hygiene	0	10 (37.0%)	5 (35.7%)	2 (22.2%)	0.933
	1	12 (44.4%)	7 (50.0%)	5 (55.6%)	
	2	5 (18.5%)	2 (14.3%)	2 (22.2%)	

Overall, the performed stratified analyses mainly highlighted descriptive trends rather than statistically significant associations, likely because of the relatively limited sample size and the pilot nature of the study.

Nevertheless, some observed patterns suggest a possible multifactorial interaction among ASD clinical severity, oral health, dietary habits, and oral microbiota composition.

9.7 Analysis of microbiological parameters

Graphical analysis of microbiological parameters revealed marked interindividual heterogeneity in both ASD subjects and caregivers, suggesting considerable variability in oral microbiota composition within the studied sample.

Regarding the Firmicutes/Bacteroidetes (F/B) ratio, ASD subjects showed a distribution characterized by the presence of several samples with elevated values, in some cases exceeding the dysbiosis cut-off considered in the study (Graph 1).

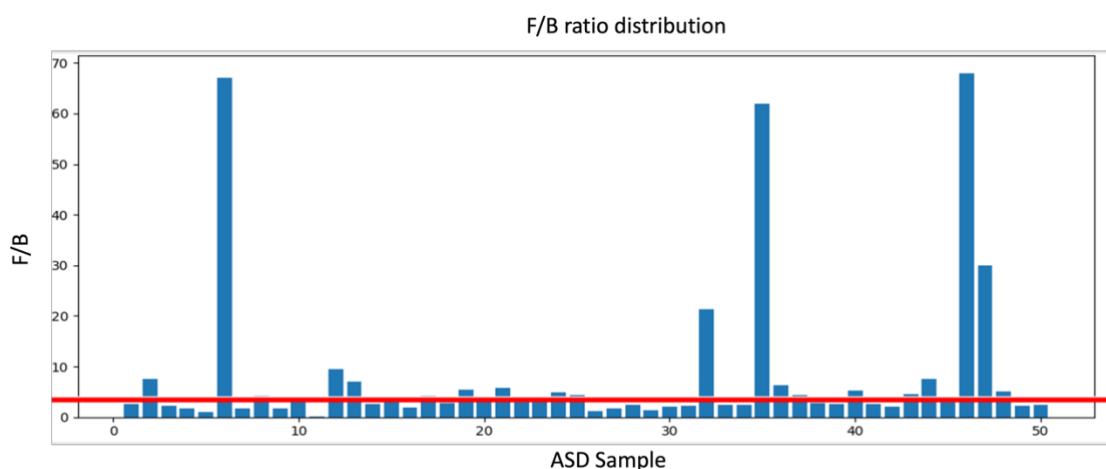
In the Caregiver group (Graph 2), the F/B ratio showed a relatively more homogeneous distribution, despite some degree of interindividual variability.

Analysis of Proteobacteria also revealed interesting differences between the two groups: ASD subjects (Graph 3) showed greater dispersion of values, with several samples characterized by elevated percentages compared with the 30% cut-off.

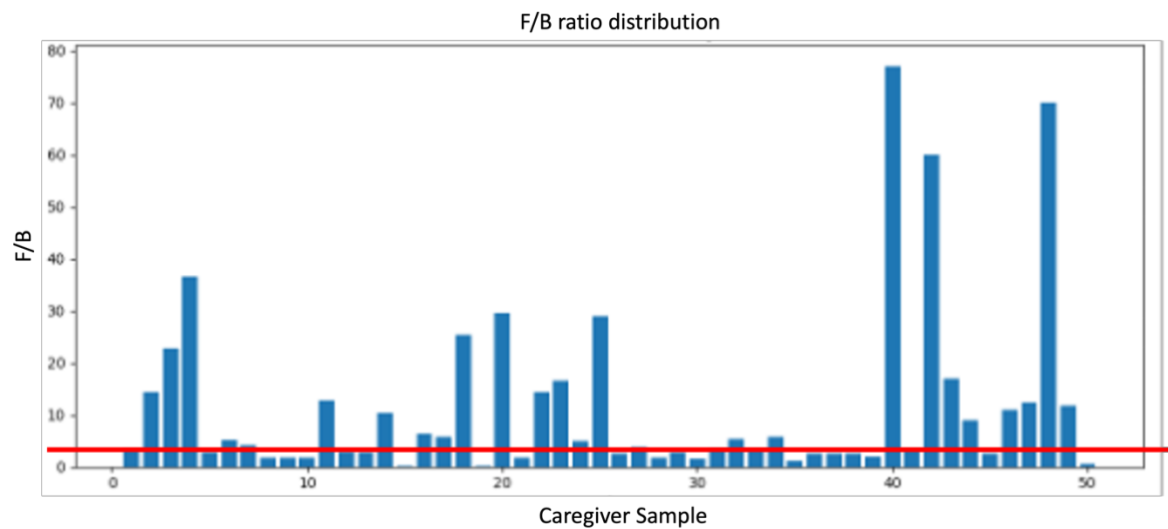
In the Caregiver group (Graph 4), Proteobacteria levels appeared more stable overall, although some individuals still presented elevated values.

Overall, the graphs suggest that the oral microbiota of ASD subjects tends to present greater variability and a more frequent presence of potentially dysbiotic patterns compared with caregivers.

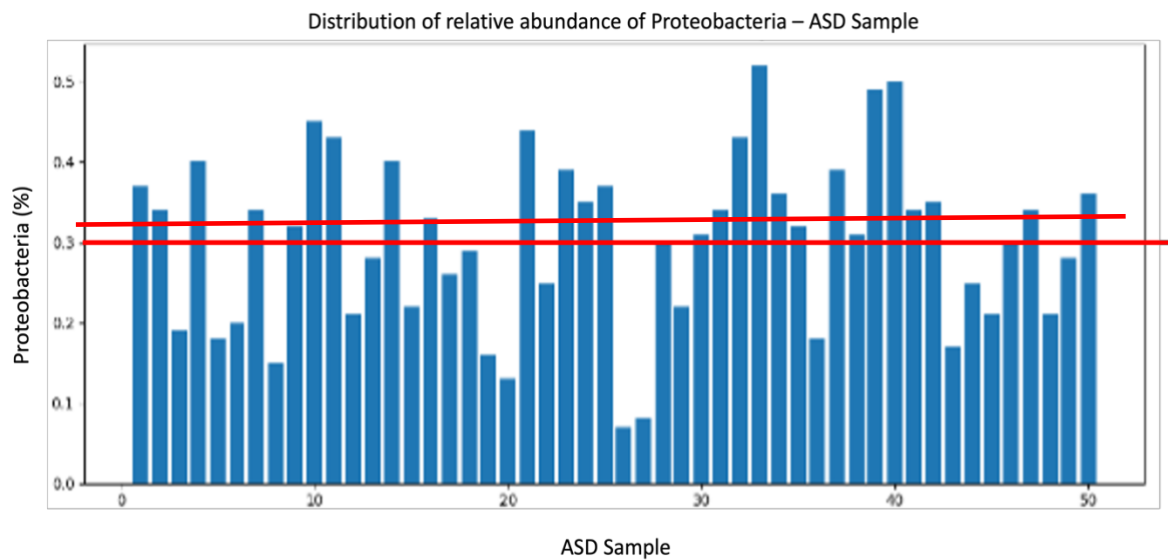
Graph 1. Distribution of F/B values in the ASD sample



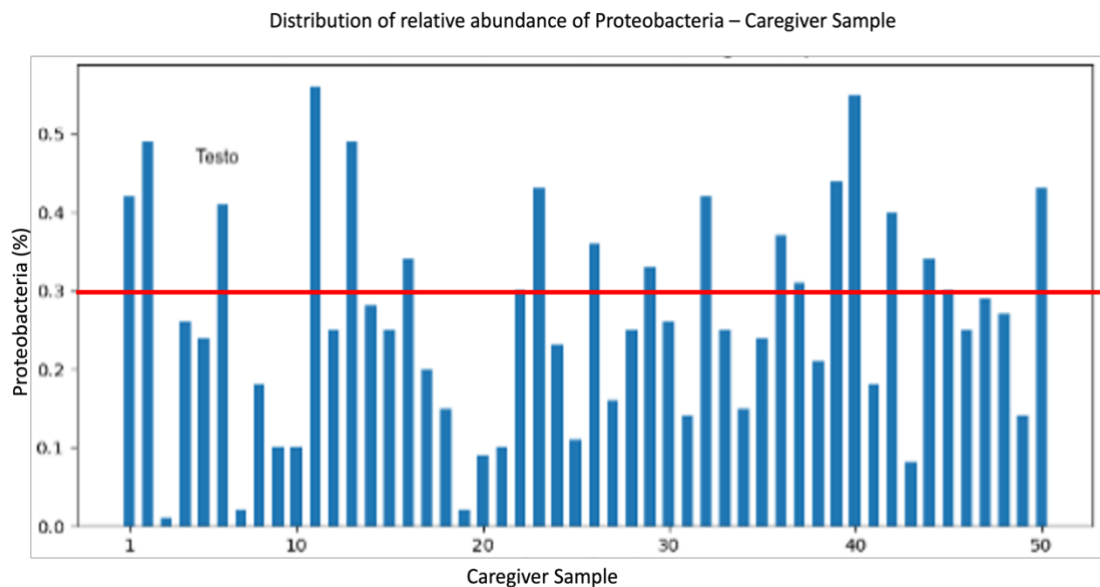
Graph 2. Distribution of F/B values in the Caregiver Sample



Graph 3. Distribution of Proteobacteria values in the ASD sample



Graph 4. Distribution of Proteobacteria values in the Caregiver sample



9.8 Descriptive statistics of microbiological variables

Descriptive analysis of quantitative microbiological variables — including mean $\pm$ standard deviation (SD), median, minimum–maximum range, and interquartile range (IQR) — showed wide interindividual variability in both ASD subjects and caregivers.

Regarding the Firmicutes/Bacteroidetes (F/B) ratio, values observed in the ASD group showed a heterogeneous distribution, with the presence of samples characterized by elevated values compared with the sample median. Similarly, variability of the F/B ratio was also observed in the Caregiver group, although with a more homogeneous distribution overall.

Analysis of Proteobacteria showed marked dispersion of values in the ASD group, with the presence of subjects characterized by elevated percentages; variable values were also observed in the Caregiver group, although with a generally more stable distribution compared with ASD subjects.

Differences between mean and median values observed for some variables suggest the possible presence of asymmetric distributions and extreme values.

Table 12. Descriptive statistics of microbiological parameters

Variable	Mean $\pm$ SD	Median	Min–Max	IQR
F/B (ASD)	8.41 $\pm$ 15.52	3.08	0.97–68.00	2.33–5.35
Proteobacteria (ASD)	0.30 $\pm$ 0.11	0.32	0.07–0.52	0.21–0.37
F/B (Caregiver)	11.54 $\pm$ 16.95	4.56	0.14–77.00	2.58–12.70
Proteobacteria (Caregiver)	0.26 $\pm$ 0.14	0.25	0.01–0.56	0.15–0.35

9.9 Descriptive analysis of quantitative microbiological parameters stratified according to dentist

Stratified analysis according to dentition showed descriptive differences in quantitative microbiological values among subjects with deciduous, mixed, and permanent dentition.

Subjects with permanent dentition tended to present higher mean values for some microbiological parameters, including the F/B ratio and Proteobacteria abundance, although not at statistically significant levels.

The presence of wide ranges and extended interquartile intervals nevertheless highlights significant intra-group heterogeneity, suggesting the possible involvement of additional clinical, environmental, and behavioral factors in modulation of the oral microbiota.

Table 13. Descriptive statistics of microbiological variables according to dentition

Variable	Group	Mean $\pm$ SD	Median	Min–Max	IQR	p-value
F/B (ASD)	2	9.40 $\pm$ 15.57	4.40	1.25–67.00	2.41–6.9	0.256
	1	6.72 $\pm$ 14.31	2.67	0.97–68.00	2.08–4.1	
	0	10.43 $\pm$ 19.42	4.25	1.72–62.00	2.38–5.71	
Proteobacteria (ASD)	2	0.26 $\pm$ 0.12	0.22	0.07–0.52	0.18–0.36	0.219
	1	0.33 $\pm$ 0.09	0.34	0.15–0.50	0.26–0.39	
	0	0.32 $\pm$ 0.09	0.31	0.16–0.45	0.28–0.32	
F/B (Caregiver)	2	13.61 $\pm$ 16.19	5.72	2.47–70.00	3.51–15.65	0.001
	1	13.64 $\pm$ 19.93	4.71	0.52–77.00	2.63–13.44	

	0	2.04 ± 1.54	1.68	0.14–5.71	1.6–1.78	
Proteobacteria (Caregiver)	2	0.26 ± 0.15	0.25	0.01–0.56	0.15–0.36	0.115
	1	0.30 ± 0.12	0.30	0.02–0.55	0.22–0.38	
	0	0.20 ± 0.14	0.20	0.02–0.49	0.1–0.25	

9.10 Descriptive analysis of quantitative microbiological parameters stratified according to sex

Stratified analysis according to sex did not reveal marked differences in the distribution of quantitative microbiological parameters between males and females.

Mean values of the F/B ratio and Proteobacteria were relatively comparable between the two groups, despite some interindividual variability. Observed ranges and interquartile intervals suggest a heterogeneous distribution of microbiological values regardless of biological sex.

Overall, these results appear to indicate that sex may not represent the principal determinant of the microbiological alterations observed within the studied sample.

Table 14. Descriptive statistics of microbiological variables according to sex

Variable	Group	Mean ± SD	Median	Min–Max	IQR	p-value
F/B (ASD)	M	8.00 ± 15.31	2.93	1.25–68.00	2.3–4.75	0.257
	F	9.36 ± 16.50	4.40	0.97–67.00	2.49–7.25	
Proteobacteria (ASD)	M	0.30 ± 0.11	0.32	0.07–0.52	0.22–0.37	0.641
	F	0.30 ± 0.10	0.29	0.15–0.50	0.23–0.34	
F/B (Caregiver)	M	11.62 ± 16.20	4.13	0.14–70.00	2.51–14.28	0.849
	F	11.35 ± 19.19	5.00	1.65–77.00	2.81–10.35	
Proteobacteria (Caregiver)	M	0.24 ± 0.13	0.25	0.01–0.49	0.12–0.34	0.261
	F	0.31 ± 0.14	0.25	0.14–0.56	0.2–0.38	

9.11 Descriptive analysis of quantitative microbiological parameters according to ASD support level

Stratified analysis according to ASD severity level showed a tendency toward increased values of some microbiological parameters in subjects with more severe clinical levels; however, statistical significance was not reached.

Subjects with ASD level 3 tended to show higher mean values of the Firmicutes/Bacteroidetes ratio and Proteobacteria abundance compared with lower ASD levels. This trend may suggest a possible association between clinical severity of the disorder and alterations of the oral microbiota.

Also in this case, the wide dispersion of the data and the relatively extended interquartile intervals indicate considerable biological heterogeneity within the sample.

Table 15. Descriptive statistics of microbiological variables according to ASD support level

Variable	Group	Mean $\pm$ SD	Median	Min–Max	IQR	p-value
F/B (ASD)	3	10.56 $\pm$ 16.90	4.40	1.71–67.00	2.55–6.65	0.081
	2	7.51 $\pm$ 17.49	2.41	1.25–68.00	1.87–3.99	
	1	3.35 $\pm$ 1.88	2.94	0.97–7.50	2.31–4.13	
Proteobacteria (ASD)	3	0.31 $\pm$ 0.12	0.34	0.08–0.52	0.2–0.39	0.261
	2	0.32 $\pm$ 0.09	0.34	0.07–0.49	0.3–0.36	
	1	0.26 $\pm$ 0.09	0.25	0.15–0.45	0.21–0.28	
F/B (Caregiver)	3	11.93 $\pm$ 19.17	3.76	0.14–77.00	2.71–12.6	0.516
	2	14.54 $\pm$ 17.32	5.98	1.60–60.00	2.63–22.55	
	1	5.71 $\pm$ 4.80	2.81	1.78–14.25	1.83–9.0	
Proteobacteria (Caregiver)	3	0.27 $\pm$ 0.16	0.25	0.01–0.56	0.15–0.42	0.379
	2	0.29 $\pm$ 0.11	0.26	0.11–0.49	0.24–0.36	

	1	0.21 ± 0.09	0.20	0.10– 0.34	0.14–0.3	
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Considering the relatively limited sample size and the high biological variability of microbiological parameters, the obtained results should be interpreted with caution. Nevertheless, the observed trends suggest the presence of potentially relevant microbiological patterns deserving further investigation in larger cohorts.

9.12 Association Between F/B Ratio in ASD Subjects and Caregivers

Analysis of the association between the Firmicutes/Bacteroidetes (F/B) ratio in ASD subjects and caregivers showed a tendency toward concordance of microbiological patterns within the same family unit.

In particular, ASD subjects presenting elevated F/B values were more frequently associated with caregivers also characterized by high F/B ratios, suggesting the possible influence of shared environmental, dietary, behavioral, and familial factors on oral microbiota composition.

Although the association did not reach strong statistical significance, the observed distribution supports the hypothesis of partial intra-familial sharing of oral microbiological characteristics.

Table 16. Association between F/B ratio in ASD subjects and Caregiver

F/B (ASD)	F/B (Caregiver) <3	F/B (Caregiver) ≥3	Total
<3	13	11	24
≥3	8	18	26
Total	21	29	50

9.13 Association Between Proteobacteria in ASD Subjects and Caregivers

Analysis of Proteobacteria abundance also showed a tendency toward intra-familial concordance between ASD subjects and caregivers.

Specifically, ASD subjects with Proteobacteria values ≥30% were more frequently associated with caregivers presenting similarly elevated values.

These findings may reflect the influence of shared lifestyle, dietary habits, environmental exposure, and microbiological transmission within the household environment.

Overall, the results suggest the possible existence of shared oral microbiological patterns between ASD subjects and cohabiting caregivers, although further investigations on larger cohorts are required to confirm these preliminary observations.

Table 17. Association between proteobacteria in ASD subjects and Caregivers

Proteobacteria (ASD)	Proteobacteria (Caregiver) <30%	Proteobacteria (Caregiver) ≥30%	Total
<30%	11	10	21
≥30%	9	20	29
Total	20	30	50

10 - Discussion

The rationale behind the present study was not only to analyze the clinical and microbiological characteristics of subjects with Autism Spectrum Disorder, but also — and above all — to promote a more inclusive and personalized dental approach for these patients. Individuals with ASD commonly present behavioral, sensory, and communication difficulties that may compromise oral health management and oral hygiene practices, with all the related consequences. For this reason, dental treatment is frequently performed in hospital settings under general anesthesia. However, general anesthesia is not without organizational difficulties — including long waiting times, during which oral conditions may progressively and irreversibly worsen — as well as risks and potential complications for the patient. Furthermore, delayed access to dental care may often result in more invasive and less conservative treatments for dental elements. One of the principals aims of the present study was therefore to highlight how, through appropriate clinical and behavioral knowledge and multidisciplinary approaches, it is possible to establish gradual and personalized care pathways for ASD patients.

Application of behavioral management techniques, together with adequate familiarization time and repeated appointments over time, may indeed allow progressive improvement in dental compliance and achievement of levels of cooperation often exceeding initial expectations [171]. While the study, many patients progressively managed to undergo professional oral hygiene procedures and calculus removal, although requiring a variable number of appointments and a gradual approach. In some cases, it was also possible to perform conservative treatments and dental extractions atraumatically and in an outpatient setting, thus avoiding general anesthesia. Even when this goal was not achieved, it is important to emphasize that a hospital–university collaborative network was established with the common objective of ensuring the best possible solution for each individual patient. These findings underline the importance of building a trusting relationship with both the patient and the family, as well as transforming dental appointments into a predictable and familiar routine. The possibility of reducing the use of general anesthesia through preventive programs and dedicated behavioral approaches therefore represents a clinically relevant objective, not only for improving the quality of dental care but also for promoting the overall well-being and quality of life of individuals with Autism Spectrum Disorder.

Nevertheless, much work remains to be done in this field. Many patients had never undergone a dental examination, and an even larger proportion had not attended a dental visit for more than one year, often much longer. This finding suggests that primary prevention is still not considered sufficiently important. Indeed, many parents/caregivers reported that they had never taken their child to the dentist to avoid causing psychological trauma, or because they believed that absence of pain corresponded to good oral health. The literature shows that attending dental visits before the onset of pain represents the best way to experience dental care in the least traumatic manner possible, allowing routine monitoring and preventing progression of pathological conditions [172].

The present research also investigated the clinical, dental, and microbiological characteristics of ASD subjects, comparing their microbiological parameters with those of one cohabiting parent/caregiver with whom they spent most of their time. The aim was to evaluate the distribution of the Firmicutes/Bacteroidetes (F/B) ratio and Proteobacteria within the oral microbiota, analyzing possible associations with clinical, behavioral, dietary, and dental variables.

The obtained results demonstrated marked heterogeneity within the sample, both from clinical and microbiological perspectives. This observation appears consistent with the multifactorial nature of ASD and the complexity of the oral microbial ecosystem, which is influenced by multiple biological, environmental, dietary, and behavioral factors [146].

From an epidemiological perspective, the sample showed a clear predominance of male subjects, in agreement with international literature reporting a male-to-female ratio in ASD generally ranging between 3:1 and 4:1 [24].

Microbiological analysis represented one of the central aspects of the study. In particular, the Firmicutes/Bacteroidetes ratio exceeded the pathological cut-off in 52% of ASD subjects and 58% of caregivers. Similarly, elevated Proteobacteria values (>30%) were observed in 58% of ASD subjects and 60% of caregivers. These findings suggest the frequent presence of microbiological patterns compatible with oral dysbiosis. Although the F/B ratio has traditionally been used mainly in gut microbiota studies, increasing evidence suggests that alterations of this ratio may also reflect microbial imbalance at the oral level. Increased relative abundance of Firmicutes compared with Bacteroidetes has indeed been associated with chronic inflammatory processes, metabolic alterations, and modifications of bacterial ecosystem homeostasis [173].

The relative increase in Proteobacteria is also of particular interest, since this phylum includes numerous Gram-negative bacteria potentially associated with inflammatory processes and dysbiotic conditions. In the literature, elevated concentrations of these bacteria have frequently been correlated with ecological instability of both gut and oral microbiota [174].

In the present study, microbiological analysis revealed marked interindividual heterogeneity, especially within the ASD group. Graphical distribution of F/B and Proteobacteria values showed the presence of subjects characterized by particularly elevated values, suggesting the existence of microbiologically distinct subgroups within the ASD population. Such heterogeneity may be explained by the considerable clinical variability typical of autism; oral microbiota composition may therefore be influenced by dietary behavior, food selectivity, cooperation during oral hygiene procedures, chronic pharmacological therapy, and daily habits.

When comparing ASD subjects and caregivers, although association analyses between F/B ratio and Proteobacteria in the two groups did not reveal statistically significant results, the presence of relatively similar microbiological patterns between patients and caregivers may suggest a possible influence of the shared family environment, as previously reported in the literature. Caregivers share numerous environmental and behavioral factors with ASD subjects, including dietary habits, domestic environment, daily routines, and interpersonal contact. It is well established that the oral microbiota may be influenced by intra-familial transmission mechanisms and shared environmental exposures. Therefore, the presence of elevated F/B and Proteobacteria values also among caregivers may at least partially reflect these shared dynamics [175].

One of the main limitations of the present study is that caregivers cannot be considered fully independent healthy controls, since cohabiting individuals may share environmental exposures, dietary habits, lifestyle factors, and microbiological exchanges that could influence oral microbiota composition. Nevertheless, this intra-familial design may also represent a relevant element of interest, supporting the hypothesis that shared environmental and interpersonal factors contribute to shaping the oral microbiota.

The lack of statistical significance in association analyses may mainly be attributed to the relatively limited sample size. Although suitable for a pilot study, the sample of 50 subjects

may not have been sufficient to demonstrate robust statistical associations between highly heterogeneous microbiological variables.

Stratified analyses provided additional interesting findings. Stratification according to dentition showed a tendency toward more altered microbiological values in subjects with mixed and permanent dentition, thus in older subjects. Individuals with permanent dentition tended to present higher mean values of both F/B ratio and Proteobacteria, likely because of maturation of the oral ecosystem during growth. With tooth eruption and transition to permanent dentition, the anatomical complexity of dental surfaces increases, favoring formation of more mature and structured biofilms. In addition, longer exposure of permanent teeth to environmental and dietary factors may contribute to selection of different bacterial communities.

At the same time, subjects with permanent dentition also showed higher PI, BOP, and DMFT values. These results suggest a possible progressive worsening of oral health conditions with increasing age and dental complexity.

Among the variables stratified according to dentition, BOP showed a statistically significant difference ($p < 0.001$). Subjects with permanent dentition more frequently presented elevated gingival bleeding values compared with subjects with deciduous dentition, likely reflecting greater susceptibility to gingival inflammatory processes in older subjects, promoted by plaque accumulation, difficulties with home oral hygiene, and modifications of oral biofilm composition [176]. Similarly, calculus accumulation also showed a statistically significant association with permanent dentition ($p = 0.042$), a finding consistent with the pathophysiology of calculus formation, which is closely related to prolonged biofilm retention on dental surfaces.

From a dietary perspective, sugar consumption showed a trend approaching statistical significance ($p = 0.059$). These differences may reflect physiological age-related changes in dietary habits and food preferences.

Sex-stratified analysis did not reveal significant microbiological differences between males and females. Mean values of F/B ratio and Proteobacteria were relatively comparable between the two groups, suggesting that biological sex may not represent the principal determinant of the observed microbiological alterations.

Nevertheless, some clinical differences deserve attention. Oral hygiene showed a statistically significant difference between sexes ($p = 0.034$), with poorer oral hygiene more frequently

observed in male subjects, possibly reflecting behavioral differences in cooperation with home oral hygiene procedures.

Stratification according to ASD level represented one of the most relevant aspects of the study. Subjects with ASD level 3 indeed tended to present more altered microbiological and clinical patterns compared with lower support levels. Elevated F/B ratio and Proteobacteria values were more frequently represented in subjects with severe ASD. Although the F/B ratio did not reach statistical significance, Proteobacteria percentages showed a statistically significant difference among ASD levels ($p=0.028$). This finding suggests a possible relationship between clinical severity of the disorder and oral microbiota alterations. It is plausible that subjects with more severe ASD experience greater difficulties in oral hygiene management, more pronounced food selectivity, reduced cooperation, and more frequent chronic pharmacological therapy, all factors potentially involved in modulation of the oral microbial ecosystem.

Calculus accumulation also showed a statistically significant association with ASD level ($p=0.001$), with moderate and severe accumulation more frequently observed in subjects with ASD level 3. Similarly, BOP tended to show higher values in subjects with more severe ASD.

Food selectivity represents one of the behavioral aspects most frequently described in autism literature. Many ASD subjects exhibit restricted food preferences, rejection of specific textures, colors, or tastes, and a tendency toward monotonous diets rich in simple carbohydrates [177]. In the present study, sugar and refined carbohydrate consumption were particularly elevated. Eighty-six percent of the sample reported high pasta and bread consumption, whereas 54% showed elevated sugar intake; conversely, fish and legume consumption were generally low. These dietary patterns may contribute both to oral microbiota alterations and worsening of clinical dental indices. Diets rich in fermentable carbohydrates indeed promote proliferation of acidogenic and aciduric bacteria, increasing the risk of oral dysbiosis and dental caries.

Clinical dental indices of the sample also deserve particular attention. Fifty-six percent of subjects presented $DMFT \geq 4$, while moderate/high PI and BOP values were highly prevalent. Overall, these findings suggest generally compromised oral health conditions among ASD subjects included in the study, likely related to difficulties in home oral hygiene management, reduced cooperation, sensory sensitivities, and food selectivity.

Perinatal variables also appear interesting. The relatively high prevalence of cesarean delivery (54%), formula feeding (56%), and preterm birth (36%) may have potential microbiological implications. Several studies suggest that delivery mode, infant feeding, and early environmental exposure significantly influence early microbial colonization of the newborn. Consequently, alterations during initial microbiota development could contribute to persistent modifications of the bacterial ecosystem [178]. However, the present study does not allow establishment of causal relationships between perinatal variables and the observed microbiological alterations.

Another important aspect to underline is that more than half of the sample had not undergone regular dental check-ups for over 12 months or had never attended dental visits at all, highlighting the difficulties in access to dental care among ASD subjects and the lack of a reciprocal trust-based relationship with dental professionals.

Dental management of ASD patients represents a particularly relevant challenge in clinical practice. Anxiety, sensory alterations, communication difficulties, and behavioral problems may significantly interfere both with home prevention and with execution of dental treatment. In the present study, 36% of subjects had previously undergone dental treatment under general anesthesia. This finding emphasizes the clinical difficulties frequently associated with dental management of ASD patients, especially in cases characterized by poor cooperation. Interestingly, even subjects with ASD support levels 1 and 2 — who responded positively during this project — had previously undergone general anesthesia, sometimes even for professional hygiene procedures, because of their diagnosis rather than real clinical necessity.

In this regard, one particularly interesting aspect emerging from the clinical experience was the unexpectedly positive cooperation shown by many patients and their families. Many subjects progressively managed to undergo calculus removal sessions, although requiring multiple appointments and a gradual personalized approach. In some cases, it was also possible to perform conservative dental procedures and dental extractions atraumatically and in an outpatient setting, avoiding general anesthesia. These findings demonstrate how progressive development of trust, behavioral adaptation, and familiarization with the dental environment may significantly improve clinical management of ASD patients.

Another positive element was that many patients subsequently returned for periodic follow-up visits. This aspect is particularly relevant because it contributes to making the dentist a

familiar figure and the dental visit a predictable and less stressful routine for the patient. Organization of continuous and personalized preventive pathways may therefore represent a fundamental strategy for improving both dental compliance and oral health conditions over time in subjects with Autism Spectrum Disorder.

The present study presents several limitations. From a methodological perspective, the main limitations are the relatively limited sample size and the cross-sectional design, which does not allow establishment of causal relationships between oral microbiota and ASD clinical characteristics.

Another limitation concerns the absence of a healthy control group external to the family unit. Comparison with caregivers provides interesting information regarding possible shared environmental influences, but it does not allow determination of whether the observed microbiological patterns are specifically associated with ASD.

Furthermore, it should be considered that the oral microbiota represents a highly dynamic ecosystem influenced by multiple factors, including diet, oral hygiene habits, chronic and non-chronic pharmacological therapy, and local inflammatory conditions.

Despite these limitations, the study also presents several strengths. In particular, the integrated clinical–microbiological approach allowed simultaneous analysis of dental, behavioral, dietary, and microbiological parameters. Moreover, inclusion of the ASD–Caregiver comparison represents an original and potentially useful element for understanding the role of the family environment in modulation of the oral microbiota.

Overall, the obtained findings suggest that ASD subjects may present a higher frequency of microbiological patterns compatible with oral dysbiosis, associated with poorer oral health conditions and specific behavioral and dietary characteristics. Although not always statistically significant, the observed trends support the hypothesis of a possible multifactorial interaction among ASD clinical severity, dietary behavior, dental conditions, and oral microbiota.

Further studies on larger samples and with longitudinal design will be necessary to clarify the real role of the oral microbiota in subjects with Autism Spectrum Disorder and to determine whether these alterations represent simple behavioral consequences or potential biological biomarkers of the disorder. Future research perspectives may include more in-depth metagenomic analyses, investigation of correlations between oral and gut microbiota,

evaluation of microbial metabolites, and monitoring of the effects of dietary and preventive dental interventions.

Finally, the obtained results underline the importance of a multidisciplinary approach in management of ASD subjects, integrating dental, microbiological, nutritional, and neuropsychiatric expertise to improve both oral health and quality of life of these patients.

11 - Conclusion

The present study described the clinical, dental, and microbiological characteristics of a pilot sample of subjects with Autism Spectrum Disorder and explored selected oral microbiota parameters in relation to behavioral, dietary, and clinical variables.

The results showed marked interindividual microbiological heterogeneity and frequent values above the exploratory thresholds used for the Firmicutes/Bacteroidetes ratio and Proteobacteria relative abundance. At the same time, the sample showed frequent impairment of oral hygiene, plaque accumulation, gingival bleeding, calculus, and caries experience.

Stratified analyses suggested possible descriptive trends involving ASD support level, dental indices, dietary habits, and selected microbiological parameters. However, these findings require cautious interpretation because of the limited sample size, cross-sectional design, absence of an independent healthy control group, and lack of multivariable adjustment.

The comparison with parents/caregivers suggested that shared familial, environmental, behavioral, and dietary factors may contribute to shaping the oral bacterial ecosystem. This comparison is informative but does not allow the identification of ASD-specific microbiological signatures. This aspect should be carefully considered when interpreting the microbiological similarities observed between ASD subjects and caregivers, since cohabiting individuals cannot be considered fully independent controls.

Beyond the microbiological findings, the present study also highlighted a clinically relevant aspect: many ASD patients, when included within gradual and personalized treatment pathways, showed unexpectedly good levels of cooperation. In numerous cases, it was possible to perform professional oral hygiene sessions, conservative treatments, and dental extractions in an outpatient setting and in an atraumatic manner, thus avoiding general anesthesia.

These findings emphasize the importance of a multidisciplinary and behavioral approach in dental management of subjects with Autism Spectrum Disorder. Therapeutic success does not depend exclusively on the dental technique used. Establishment of a trusting relationship with both the patient and the family, combined with continuous preventive programs and periodic follow-up visits, may help transform the dental appointment into a predictable, less

stressful, and sustainable routine over time, thus preventing or limiting development of oral health problems.

Despite the limitations related to sample size and the cross-sectional design of the study, the obtained data represent an interesting preliminary contribution to research on the oral microbiota in ASD subjects. Future longitudinal studies including independent healthy controls and larger cohorts are needed to better clarify the specific contribution of ASD-related factors and shared environmental influences on oral microbiota composition.

In conclusion, the present work supports the need for dedicated, preventive, and personalized dental care pathways for individuals with Autism Spectrum Disorder. The microbiological findings should be considered preliminary and hypothesis-generating, and require confirmation in larger, controlled, and preferably longitudinal studies.

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