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AUTISM SPECTRUM DISORDER AND THE GUT MICROBIOTA – A SYSTEMATIC REVIEW

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Autism spectrum disorders (ASD) are a varying group of disorders characterized by deficiency in social interaction and restrictive patterns of behavior and interests. While there are a number of studies focusing on the neuropsychiatric pathogenesis of ASD, its etiology remains unclear. The role of gut-brain-axis in ASD has been studied increasingly and a connection between symptoms and the composition of gut microbiota has been documented in various works. Despite this, the significance of individual microbes and their function is still widely unknown. This work aims to elucidate the current knowledge of the connection between ASD and the gut microbiota in children based on scientific evidence.

This is a systematic review done by a literature search from PubMed database focusing on the main findings concerning the gut microbiota composition, interventions targeting the gut microbiota, and possible mechanisms explaining the results in children aged between 2 and 18 years of age. 31 articles that met the selection criteria were included in this systematic review.

Most studies in this review found significant differences when comparing the microbial communities of ASD children to healthy controls, while there was notable variation in results when looking at diversity indices or taxonomic level abundance. It seems that there are certain microbial taxa, such as Proteobacteria and *Sutterella*, and functional markers, such as lower levels of butyrate, that are characteristic for ASD. More research is needed to discover whether some of these features could be used as potential biomarkers for ASD and how the gut microbiota could be targeted in therapeutical interventions.

Asiasanat: Autisminkirjon häiriöt, suolistomikrobisto

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Autism Spectrum Disorder and the Gut Microbiota – a Systematic Review

1. Introduction

Autism spectrum disorders (ASD) are a varying group of disorders characterized by deficiency in social interaction and restrictive patterns of behavior and interests. Although varying severity of symptoms and clinical heterogeneity is a hallmark of ASD, most people with ASD face a number of challenges in everyday life. The neuropsychiatric pathogenesis of ASD has been studied extensively. However, its etiology remains unclear (Masi et al. 2017). No reliable biomarkers for ASD have been established, and the diagnosis is done on the basis of behavioral symptoms and neurocognitive performance (Lord et al. 2018). ASD is primarily treated with psychosocial methods, while pharmacological treatments can be used to treat the behavioral symptoms (Vanhala 2018).

A significant number of children with ASD have gastrointestinal symptoms alongside with restricted diets (Bauman 2010, Bresnahan et al. 2015, Cermak et al. 2010). The gut microbiota consists of trillions of microbes inhabiting our gastrointestinal tract. It is commonly suggested that the gut microbiota has a role in the development of not only somatic, but also psychiatric disorders. The role of gut-brain-axis in ASD has been studied increasingly and a connection between symptoms and the composition of gut microbiota has been documented in various works. Despite this, the significance of individual microbes and their function is still widely unknown. (Cryan et al. 2019)

This work aims to elucidate the current knowledge of the connection between ASD and the gut microbiota in children based on scientific evidence. This is a systematic review done by a literature search from PubMed database focusing on the main findings concerning the gut microbiota composition, interventions targeting the gut microbiota, and possible mechanisms explaining the results in children aged between 2 and 18 years of age.

2. Methods

2.1 Search strategy

A systematic search was conducted in June 2020 using PubMed, using a search filter for the studies written in English. The search terms were: ("autism spectrum disorder" OR ASD OR autistic* OR autism*) AND (microbiota* OR microbiome* OR microflora*) AND (child* OR children*). In all, 237 articles from the years 1998-2020 were found.

2.2 Selection criteria

Studies about non-human subjects as well as reviews, case reports and duplicate works were excluded. Additionally, studies that failed to provide data for the subjects' microbiota or age and studies with less than 20 participants with ASD were excluded.

The inclusion criteria for the review were: observational prospective and retrospective studies, case-control studies, or cohort studies investigating gut bacteria in children aged 2-18 years diagnosed with autism or ASD. The included articles had to include information about the sample size and gut microbiota. The articles had to be written in English. The detection of gut microbiota was to be done by fecal samples and/or gut biopsy. Finally, the analysis of the microbiota was to be done with metagenomic sequencing, 16S rRNA sequencing, quantitative real-time PCR techniques (qPCR), polymerase chain reaction (PCR) or fluorescence in situ hybridization (FISH).

One person went through the material without blinding. However, consensus meetings on article inclusion and exclusion were performed in a group formed additionally by one post doctoral and one senior researcher. Information about the articles was collected in a spreadsheet, including the publishing year, study design, number of ASD participants and controls, age, sex distribution, ASD diagnosis criteria, the method of collecting gut microbiota samples, sequencing method, and if GI symptoms, diet and antibiotics were acknowledged. In another spreadsheet, the results were collected. This was done by recording all the significant differences in microbe taxa between study groups and if there was an intervention included.

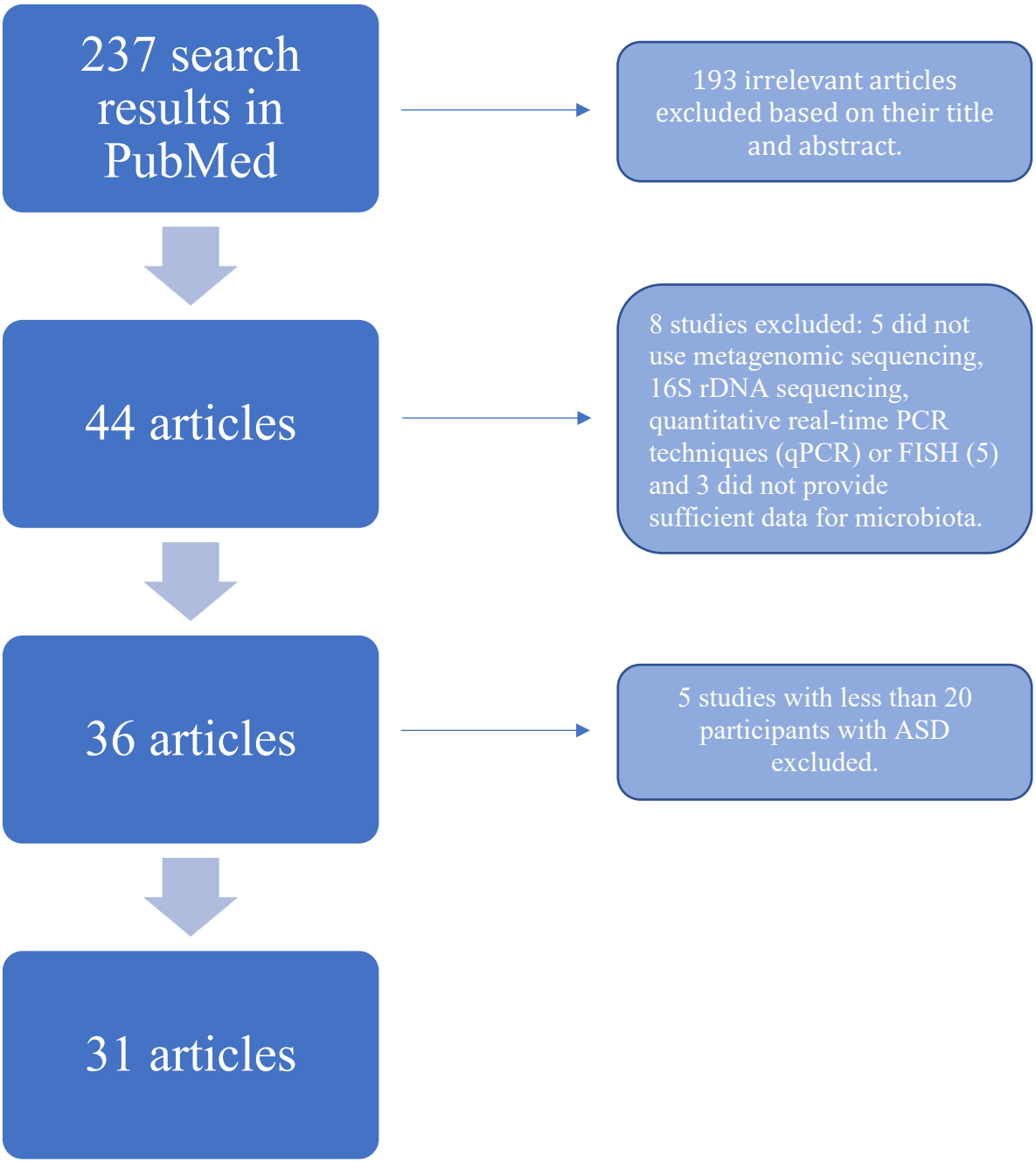

Figure 1 *Flow chart of study selection*.

3. Results

3.1 Study Selection

Out of the 237 search results, 44 articles were chosen. Studies unrelated to the topic, studies written in other language than English, reviews, case reports and duplicate publication were excluded after

title and abstract evaluation. Out of those 44 articles further 8 were excluded, because they did not use the specified sequencing methods or did not provide sufficient data for the microbiota. Lastly, 5 more studies were excluded due to having less than 20 participants. Finally, there were 31 eligible articles included in this review. (Figure 1)

3.2 Study characteristics

Out of the 31 chosen articles the total of 27 were case-control studies, three were randomized controlled trials (RCTs) and one was a non-randomized controlled trial. All had a control group, and four studies included an intervention. Articles were published between the years 2013 and 2020. The study subject number ranged from 30 to 154 of which number of subjects with ASD ranged from 20 to 114. The age of the participants with ASD ranged from 2 to 17 years. Three of the 31 studies included only boys, 24 both girls and boys and 4 did not report the sex distribution of subjects. For ASD diagnosis, DSM-5, ADI-R, DSM-4, ICD-10, ATEC, PDD-BI, CARS, GMDS, ADOS-2 and PDDBI-SV were used. In nearly every study ASD evaluation was done by a clinician. Four studies did not provide information about the diagnosis criteria for ASD.

For the gut microbiota characterization, 29 studies used fecal samples, one study duodenal biopsy and one both ileal and cecal biopsies. For microbiota sequencing, 22 of the studies used 16S rRNA sequencing, two quantitative real-time PCR, two *Clostridium Perfringens* targeted PCR, three metagenomic sequencing and two FISH.

For confounders, 28 of the 31 studies took study subjects' GI-symptoms into account, of which five used a GI severity index (GSI or GSI-6) and one used a GIF (GI failure) score to evaluate the severity of the symptoms. In three studies subjects with any GI symptoms were not included at all. 25 studies acknowledged subjects' diets, and in five of those studies subjects with restrictive diets were excluded. In 21 studies possible use of antibiotics was taken into account, of which the total of 18 studies reported that the subjects had not had antibiotics in the last one to three months.

	Result	Number of studies	References
α-diversity	ASD > control group	5	Zhai et al., Li et al., De Angelis et al.,Wang et al., Tomova et al. (2020b)
	ASD < control group	6	Kang, Ilhan et al., Ma et al., Carissimi et al., Kang, Park et al., Liu et al., Wang et al.
	No significant group difference	12	Plaza-Días et al., Kong et al., Grimaldi et al, Zhang et al., Pulikkan et al., Kushak et al., Son et al., Berding and Donovan 2018, Strati et al., Berding and Donovan (2020), Ahmed et al., Tomova et al. (2020a)
β-diversity	GM composition significantly different between ASD and control groups	20	Kong et al., Grimaldi et al., Zhai et al., Li et al., De Angelis et al., Kang et al., Zhang et al., Ma et al., Pulikkan et al., Kang et al., Kushak et al., Son et al., Niu et al., Berding and Donovan (2018), Wang et al., Liu et al., Strati et al., Berding and Donovan (2020), Wang et al., Tomova et al. (2020b)

Table 1 *Alpha and beta diversities in autism spectrum disorder (ASD) participants compared to controls.*

3.3 Alpha and beta diversity

24 studies of all 31 studies analyzed the alpha diversity, i.e. intra-individual diversity, of subjects' gut microbiota. 12 studies found no significant difference between study groups. In five studies there was higher gut microbiota diversity in the ASD group, while another six studies found opposite results indicating that children with ASD had in fact lower diversity. Niu et al. found that

children with ASD had a higher intestinal microbiota Shannon index, which accounts for richness and evenness of taxa, and a lower Simpson index, which accounts for taxa dominance.

21 studies analyzed the gut microbiota for beta diversity, i.e. inter-idividual diversity, of which 20 confirmed that ASD children have a significantly different gut microbiota composition compared to a control group. Tomova et al. (2020a) found that dietary habits accounted for the difference in the beta diversity of ASD children. (Table 1)

	Abundance in ASD participants compared to controls	Number of studies	References
Firmicutes/ Bacteroidetes ratio	↑	3	Strati et al., Kong et al., Niu et al.
	↓	2	Ahmed et al., Zhang et al.
Bacteroide*tes*	↑	1	De Angelis et al.
	↓	2	Strati et al., Niu et al.
Proteobacteria	↑	3	Plaza-Días et al., Kong et al., Li et al.
Bacteroides	↑	2	Ahmed et al., Zhai et al.
	↓	2	Niu et al., Kushak et al.
Bacilli	↑	2	Plaza-Días et al., Grimaldi et al.
Bifidobacteria	↑	2	Pulikkan et al., Plaza-Días et al.
	↓	3	Ahmed et al., Niu et al., De Angelis et al.
	↑ after intervention	2	Shaabam et al., Grimaldi et al.
Ruminococcus	↑	1	Ahmed et al.
	↓	3	Liu et al., Niu et al., De Angelis et al.
	↑ after intervention	1	Grimaldi et al.

	↓ after intervention	1	Wang et al. (2020)
Veillonella	↑	1	Pulikkan et al.
	↓	2	Strati et al., Zhang et al.
Lactobacillus	↑	3	Tomova et al. (2020b), Strati et al., Pulikkan et al.
	↑ after intervention	1	Shaabam et al.
Clostridium	↑	2	Kandeel et al., De Angelis et al.
	↓ after intervention	1	Wang et al. (2020)
Nitriliruptor	↑	2	Tomova et al. (2020a), Tomova et al. (2020b)
Sutterella	↑	3	Williams et al., Zhang et al., Zhai et al.
E. Coli	↓	2	Kushak et al., Carissimi et al.
Prevotella	↑	1	De Angelis et al.
	↓	2	Pulikkan et al., Kang et al.
Bilophila	↑	1	Zhai et al.
	↓	1	Strati et al.
Alistipes	↑	1	De Angelis et al.
	↓	1	Strati et al.
Parabacteroides	↑	1	Zhai et al.
	↓	1	Strati et al.
Barnesiella	↑	1	Liu et al.
	↓	1	Averina et al.

Streptococcus	↑	1	Li et al.
	↓	1	Zhang et al.
Lachnospiraceae	↓	1	Liu et al.
	↑ after intervention	1	Grimaldi et al.

Table 2 *Phyla and taxa level abundances in participants with autism spectrum disorder (ASD) compared to controls when more than one study had significant results concerning the taxa.*

3.4 Specific phyla and genus level differences

In three studies (Strati et al. 2017, Kong et al. 2019, Niu et al. 2019), Firmicutes/Bacteroidetes ratio was increased in autistic children's samples compared to controls. However, Zhang et al. had results indicating the opposite and Ahmed et al. found that the same ratio was in fact decreased in both autistic children and their healthy siblings. Furthermore, Proteobacteria was found to be increased in autistic samples in three studies (Plaza-Díaz et al. 2019, Kong et al. 2019, Li et al. 2019).

On genus level, two studies found that *Bifidobacteria* were decreased in subjects with ASD (De Angelis et al. 2013, Niu et al. 2019) and one study reported lower levels of *Bifidobacteria* in ASD children compared to their healthy siblings (Ahmed et al. 2020). Whereas, Pulikkan et al. and Plaza-Días et al. found opposite results. Three studies (Pulikkan et al. 2018, Strati et al. 2017, Tomova et al. 2020b) found that *Lactobacillus* was more abundant in the samples of autistic subjects, along with *Sutterella* (Zhai et al. 2019, Zhang et al. 2018, Williams et al. 2012). Meanwhile *Ruminococcus* was reported less abundant compared to controls in several studies (De Angelis et al. 2013, Niu et al. 2019, Liu et al. 2019) although one study (Ahmed et al. 2020) found that *Ruminococcus* was more abundant in both autistic children and their siblings compared to controls.

Liu et al. (2019) noted that while the gut microbiota profiles of children with ASD and their mothers had a clear correlation, *Clostridium* was one of the unique biomarkers found mainly in the ASD children's samples. Consistently, two studies (De Angelis et al. 2013, Kandeel et al. 2020) found that *Clostridium* genus was more increased in autistic subjects' samples, while especially *C. perfringens* was increased in two (Finegold et al. 2017, Alshammari et al. 2020) *Clostridium cluster*

XVIII was found to be specifically high in constipated autistic individuals (Strati et al. 2017). (Table 2)

3.5 Intervention outcomes

Grimaldi et al. conducted a randomized placebo-controlled trial with 26 participants with ASD. The participants were divided into two groups based on their dietary habits. The intervention was a 6-week treatment with B-GOS prebiotics. Children with restricted diets and B-GOS intervention resulted with significantly higher *B. longum* abundance together with improvements in anti-social behavior. In the same study, the effects of exclusion diets on gut microbiota composition and metabolism in ASD children were assessed. In baseline, *Bifidobacterium* and *Veillonellaceae* family were less abundant in children on exclusion diets while *Faecalibacterium prausnitzii* and *Bacteroides* were found in higher abundance compared to children with an unrestricted diet. Children with restricted diets also were reported with lower scores in abdominal pain and bowel movement.

Another randomized controlled trial by Niu et al. studied the intestinal microbiota and probiotics treatment in children with ASD in China. 37 children with ASD received a 4-week probiotic treatment combined to an applied behavior analysis (ABA) training while other 28 ASD children were treated with ABA training only. After the intervention the group with combination treatment including both probiotics and ABA training had a greater decrease in ATEC and GI scores compared to the group who didn't receive probiotics.

In a placebo-randomized controlled trial by Wang et al. (2020) a probiotics and fructo-oligosaccharide intervention was conducted in order to modulate the microbiota-gut-brain axis and to improve ASD symptoms. In this study 26 children with ASD were divided into two groups where the first group received both probiotics and fructo-oligosaccharide (FOS) treatment while the second group received placebo supplementation. All subjects were placed in the same hospital ward where they had received identical diet for the preceding 12 months. The duration of the intervention was 30 days, 60 days and 108 days. After the intervention there was an increase in *Bifidobacteriales* and *B. longum* in the probiotics + FOS group, while *Clostridium* and *Ruminococcus* abundances were decreased. ATEC scored were decreased after probiotics and FOS treatment of 30 to 60 days. Also 6-GSI scores assessing gastrointestinal symptoms were reduced after intervention. These differenced did not occur in the placebo group.

A prospective, open-label study assessing the role of probiotics in children with ASD was done by Shaabam et al. 30 children with ASD were given probiotics supplementation including *Lactobacillus acidophilus*, *Lactobacillus rhamnosus* and *Bifidobacteria longum* for 3 months. This resulted in an increase in *Bifidobacteria* and *Lactobacilli* levels as well as improved ATEC scores indicating decreased manifestation of autistic behavior. There was also a decrease in body weight and gastrointestinal symptoms in patients compared to the baseline. However, it should be noted that there was no placebo group in this study. (Table 2)

3.6 Functional characteristics

Few studies also made interesting remarks on the functional markers of the gut microbiota of ASD children. In four studies, lower levels butyrate was associated with ASD. Wang et al. (2020) and Liu et al. found lower levels of butyrate in the ASD group, while interestingly in another study there were decreases in the abundance of genes linked to production of butyrate in the ASD metagenomes. Furthermore, Zhang et al. found that butyrate and lactate producers were less abundant in the ASD group compared to controls. However, Kang et al. found no significant difference in fecal butyrate levels. Children with ASD also had lower levels of fecal acetic acid (Wang et al. 2020, Liu et al. 2019).

After the Probiotics + FOS intervention study by Wang et al. (2020) they reported elevated levels of SCFAs, approaching those in the control group. ASD children had altered glutamate metabolites in addition to an abundance with microbiota associated with glutamate metabolism. Gut 2-keto-glutaramic acid was validated as a potential biomarker for ASD as there was a significant decline of it in the ASD group (Wang et al. 2019). Interestingly calprotectin, a marker for gut inflammation, was found to correlate with behavioral and GI symptoms in ASD (Tomova et al. 2020b). It was also noted that increased Clostridium botulinum correlated with altered glutamate metabolites (Wang et al. 2019) and in another study, disaccharidase activity (Kushak et al. 2017).

Averina et al. found that in the ASD metagenomes there were decreases in the abundance of genes linked to production of not only butyrate, but also GABA and melatonin. Consistently, GABA levels in feces were found to be decreased in ASD group in a study by Kang et al. (2018). However, Wang et al. (2020) found no significant difference in plasma GABA levels when comparing ASD group to controls. De Angelis et al. found that ASD children had dysregulated metabolism of

Flavone-8-acetic acid (FAA). In the study conducted by Kang et al. (2018), there were higher isopropanol concentrations in the feces on ASD children and in addition, increased serotonin and decreased homovallinic acid, indicating a dopamine metabolism disruption.

4. Discussion

The aim of this study was to determine the current knowledge on ASD and the gut microbiota in children. The results of this systematic review show that the gut microbiota of children with ASD is altered compared to one of neurotypically developed children. Most studies in this review found significant differences between microbial communities, while there was notable variation in results regarding diversity indices or taxonomic level abundance. However, results regarding functional markers were generally more consistent.

Beta diversity describes whether there is a dissimilarity between two microbial communities. 20 studies in this review confirmed that that gut microbiome of ASD children was significantly different compared to the control samples. However, results regarding alpha diversity were more contradictory. Alpha diversity describes the diversity of a single sample. Most studies did not specify the alpha diversity index; therefore, it remains unclear whether the difference in the diversity is explained by richness of different species or a dominance in a specific species. Liu et al. (2019) made similar remarks indicating that while it seems that the gut microbiota is indeed altered in ASD, the significance of diversity in a single sample remains unclear.

All studies that found no significant difference in the alpha diversity had acknowledged the diet of participants. In many of those studies participants with restricted diets were excluded or all participants were given a similar diet. Diet is known to directly alter the composition of gut microbiota (Sandhu et al. 2017), therefore homogenous diets could result in less variety in gut microbiota as well. Three out of five studies that had results indicating that participants with ASD had higher diversity did not report the diets of participants, while most studies with results indicating that that ASD children had lower alpha diversity had reported the diets. Berding and Donovan (2018) found no difference in alpha diversity when comparing the gut microbiota of ASD children to healthy controls but found that dietary patterns were associated with specific microbial profiles. Children who consumed more vegetables, legumes, nuts and seeds, fruit alongside with refined carbohydrates and starchy vegetables had microbiota profiles characterized by lower abundance if *Enterobacteriacae*, *Lactococcus, Roseburia*, *Leuconostoc* and *Ruminococcus*, while

the gut microbiota of children who consumed less of those foods had higher levels of multiple species, including *Barnesiellacaea* and *Alistipes*. More restricted diet could therefore result in increased abundance of certain species. However, in the same study, diet and microbiota did not predict social deficit scores (PDDBI-SV). Many children with ASD have restricted diets (Sharp et al. 2013) which can result in varying gut microbiota (Sandhu et al. 2017). In a recent large autism stool metagenomics study by Yap et al. (2021) restricted dietary preferences seemed to result in less diverse gut microbiota and additionally, looser stool consistency. They did not find evidence for gut microbiota directly contributing to the etiopathogenesis of ASD. These results show that instead of solely focusing on the microbial differences, more research is needed to understand the connection between diet, gut microbiota and ASD in children, and furthermore, the factors affecting microbial diversity.

The most coherent results regarding species level differences in ASD children's gut microbiota compared to controls were in Firmicute/Bacteroidetes ratio, and Proteobacteria and *Sutterella* levels. In the study conducted by Zhang et al. the Firmicute/Bacteroidetes level was noticed to be decreased, and the result was speculated to be explained by cultural factors. In this review there were similar results indicating an increased Firmicute/Bacteroidetes ratio in both European and Chinese cohorts, so while the geographical and cultural differences are likely to affect the gut microbiota's composition, they do not fully explain the differing results in this review.

Williams et al. (2012), Zhang et al. (2018) and Zhai et al. (2019) had aligned results indicating that *Sutterella* is more abundant in children with ASD. Williams et al. discovered that *Sutterella* could be found in the gut microbiota of more than half of children with ASD, while none of the controls had it. In the same study both ASD and control group included participants with GI symptoms. Meanwhile in the study by Zhang et al. subjects with the most severe GI symptoms were excluded and in the study by Zhai et al. none of the subject had GI symptoms. These findings could indicate that *Sutterella* may be involved in the pathogenesis of ASD regardless of GI symptoms.

Li, Yang et al. (2019) found that also the mothers of children with ASD had a higher abundance of Proteobacteria, which could be explained by dietary habits and other environmental factors but also with congenital factors that alter the gut microbiota of children. Plaza-Diaz et al. (2019) found that Proteobacteria were more abundant in ASD children with mental regression compared to those with ASD and no mental regression. There is evidence for Proteobacteria being associated with inflammatory processes in the GI tract (Matsuoka et al. 2015, Shin et al. 2015). These results could

indicate that Proteobacteria, that include many pathogens such as *Escheria*, *Vibrio*, *Helicobacter*, *Salmonella* and *Yersinia* could affect not only the gastrointestinal tract but also neuropsychiatric function of children.

Three trials that included an intervention had interesting results regarding autistic behavior symptoms. In the study conducted by Grimaldi et al. (2018) children with both restricted diet and B-GOS intervention had higher levels of *B. longum* alongside with improved ATEC scores indicating improvements in anti-social behavior. Consistently, Wang et al. (2020) discovered that *Bifidobacteriales* and *B. longum* were significantly increased after probiotics and fructo-oligosaccharide supplementation. Both ATEC and 6-GSI score were decreased indicating improvements in autistic and GI symptoms. Shaabam et al. (2018) had similar results: after probiotic supplementation, children with ASD had an increase in *Bifidobacteria* abundance as well as improvements in ATEC scores. Meanwhile, Ahmed et al. (2020) discovered that the only difference in the gut microbiota of ASD children compared to their healthy siblings was that the siblings had higher levels of *Bifidobacteria*, which could act as a protecting factor. *B. longum* has been previously connected to reduced stress levels (Allen et al. 2016), which indicates that it has a significant role in the gut-brain-axis function. In a randomized controlled trial by Pärtty et al. (2015) infants were given a probiotic supplementation including *Lactobacillus rhamnsosus* and followed for 13 years. They discovered that specific probiotics may reduce risk for ASD and ADHD, but interestingly, there was no significant effect on microbiota composition. They suggested that the mechanism explaining the reduced risk for these conditions was not directly associated with the gut microbiota itself, but instead the probiotics could affect the central nervous system via altered vagal signaling or by systematic metabolic changes. *Bifidobacteria* and especially *B. longum* could be targeted more specifically in probiotic treatment of ASD children in the future, although as concluded in a systematic review by Ng et al. (2019), more research with standardized intervention regimen is needed to study the potential benefits of supplementation treatments.

Some of the studies had also studied different microbial metabolites. These results give more mechanistic insight to understanding the gut-brain-axis. There are multiple pathways for information to go between gut and brain. These pathways include autonomic nervous system, enteric nervous system, (entero) endocrine signaling, neurotransmitters, immune system, spinal mechanisms, HPA-axis, and different molecules including neurotransmitters, BCAA, SCFAs and peptidoglycans. (Cryan et al. 2019) In the current review, multiple studies discovered that SCFAs were associated with ASD. Studies that had looked at SCFA fecal concentrations, SCFA producing

bacteria or genes coding SCFA producing enzymes all confirmed differences in children with ASD compared to controls. Differences in SCFA levels can be explained by abundance of certain SCFA producing bacterial taxa or the amount of carbohydrates in diet. Studies that had reported SCFA levels had excluded participants with major dietary differences, indicating that there were differences in certain SCFA producing bacterial abundances. Two studies confirmed that there was indeed a connection between SCFA concentrations and levels of certain taxa. Four studies found that lower level of butyrate was associated with ASD. Only one study found no significant difference in butyrate levels. Butyrate is a SCFA that has been mostly associated with maintaining gut health but there has been growing interest of the neuromodulative effects of butyrate as well (Hamer et al. 2018). Butyrate has been associated with attenuating ASD behavior in animal models before (Kratsman et al. 2016) and in a study by Rose et al. (2018) it was discovered that it could affect genes linked to behavior and cognition via energy metabolism. Also, acetic acid levels seemed to be lower in ASD. The results of this review further support the idea of butyrate and other SCFAs playing a role in developing both gastrointestinal and behavioral symptoms in ASD, although the number of studies with fecal metabolomics data in child ASD research is still scarce.

It has been speculated whether specific microbial differences could be used as biomarkers for ASD. While this review confirms that there seems to be multiple differences in the gut microbiota of ASD children compared to healthy controls, the specific species that could be used as biomarkers remain controversial. There is significant variety in how the differences are classified, for example whether the study has looked at phyla or genus level. Most of the studies included in this review used 16s rRNA sequencing to analyze the composition of the gut microbiota, but there were studies using *Clostridium Perfringens* targeted PCR, quantitative real-time PCR, shotgun metagenomics and FISH methods as well. Thereby, results that have been achieved using different methods cannot be fully compared. Studies than included an intervention all investigated the effects of probiotic supplementation, but the composition of the supplementation varied. Almost all studies had used stool samples to analyze the gut microbiota. Using gut biopsies could provide more information on species that cannot be detected in stool samples. Many studies in this review had a relatively small sample size, only six of all studies including more than 100 participants. Additionally, in several studies used data was collected in one clinic or hospital. This review includes studies that have been conducted in different continents, including Asia, Europe, Africa, and Northern America, which can explain contradictory results, as the gut microbiota is likely to be affected by environmental and host genetic factors as well as cultural dietary characteristics. All these factors partly explain the complex results in this review. It could be beneficial to study larger cohorts including participants

from different locations and utilize replication data sets. This could bring more epidemiological or population level insight on the subject as well.

All studies in this review had included more male than female participants, three of the studies including only male participants. This is explained by well-known differences in prevalence of ASD among boys and girls, prevalence in boys being one in 53 and in girls one in 252 (Prevalence of autism spectrum disorders--Autism and Developmental Disabilities Monitoring Network, 14 sites, United States, 2008; 2012). Thereby, it should be noted that the sex-ratio of children with diagnosed ASD affects participant recruitment and furthermore, results of a study, and the results might not be as representative when looking at girls with ASD. Also, the selection of controls varied in different studies. Some of the studies had used typically developed siblings as controls, while in other studies, controls were unrelated, which can influence the interpretation of results. In a study by Ahmed et al., there were three study groups: children with ASD, their neurotypical siblings and unrelated neurotypical children. They discovered the gut microbiota of healthy siblings was more similar to ASD group than to unrelated controls. Parracho et al. found that healthy siblings had intermediate levels of certain bacteria when compared to ASD children and unrelated controls. This can be explained by shared environmental and host genetic factors that inevitably affect the gut microbiota. Nevertheless, most of the studies using siblings as controls did find significant differences between ASD children and their healthy siblings. To fully understand the key differences in gut microbiota it could be beneficial to have more studies with large populations with both related and unrelated controls. These characteristics reflect the complexity of gut microbiota research. More homogenous research and especially randomized controlled clinical trials focusing on specific microbial species is needed to understand the role of microbiota in ASD.

This review highlights what is already known best about the gut microbiota and ASD in children. The inclusion of a wider range of databases than what was used in this review could provide more knowledge on the subject. This review focused only on bacterial taxa as this domain has been researched in the context of ASD sufficiently to conduct systematic literature review, but it must be acknowledged that the gut microbiota consists of viral and fungal communities as well (Hallen-Adams et al. 2019, Dalmasso et al. 2014). As a conclusion, gut microbiota appears to be a contributive factor in especially gastrointestinal, but also behavioral symptoms that are typical for ASD. It also seems that there are certain microbial taxa, such as Proteobacteria and *Sutterella*, and functional markers, such as lower levels of butyrate, that are robust microbial-related characteristic for ASD. Probiotic interventions improve the comorbid GI symptoms, and thus it seems that the

ASD-related gut microbiota features are interrelated with GI symptoms. More research is needed to discover whether some of these features could be used as potential biomarkers for ASD and how the gut microbiota could be targeted in therapeutical interventions.

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