UNIVERSIDADE BEIRA INTERIOR

# Microbiome and Autism

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# Abstract

Background: Autism Spectrum Disorders (ASD) are a group of neurologic conditions that affect behavior, communication, and social interaction in children and adults all over the world. Several recent studies correlate these disorders with alterations of the gut microbiome due to the possible imbalance of the gut-brain axis. This possible connection opens new avenues to explore the unknown areas of ASD pathogenesis and the new opportunities for managing this disorder. The goal of this systematic review is to analyze the existing knowledge on microbiome changes in ASD and to understand its importance in the biological and behavioral context of ASD patients.

Methods: A systematic search covering the topics of ASD and microbiome was performed on PubMed and completed on October 7, 2019. Twenty-eight articles were included and their quality was assessed. The data extracted for analysis was related to the participants characteristics, the study type and method of analysis, the instrument used to diagnose ASD and the main outcomes of said investigation.

Results: Most of the reviewed studies found microbiome changes in ASD patients in comparison with neurotypical subjects. However, there was no specific pattern of bacterial changes found. The studies focused mostly on *Firmicutes*, *Bacteroidetes, Actinobacteria* and *Proteobacteria*. Out of all these phyla, the only one that exhibited a clear trend in ASD subjects was *Firmicutes*, mainly the order *Clostridiales* and *Clostridium* species, with a documented increase in ASD subjects in ten studies.

Conclusion: This review suggests that there is an altered microbiome in ASD. However, the current analysis was not able to establish a set of bacterial changes characteristic to this pathology. Nevertheless, the gut-brain axis relationship seems to be one worth pursuing in hopes to establish a clear pathophysiological path to this disorder.

# Keywords

Autism; microbiome; immunomodulation

# Resumo alargado

As Perturbações do Espetro Autista (PEA) são um conjunto de condições neurológicas que afetam a comunicação e a interação social, com padrões repetitivos e restritivos de comportamento e hipo ou hiper-reactividade a estímulos sensoriais e ambientais. Estas condições são definidas pela sua clínica, visto que a sua patogénese não se encontra ainda esclarecida. Existem fatores genéticos e ambientais implicados na génese das PEA, e também múltiplas comorbilidades com destaque para os distúrbios gastrointestinais.

A elevada prevalência destes distúrbios em crianças com PEA leva à hipótese de que, para além de meras comorbilidades, estes possam ser parte do mecanismo causal desta patologia. Assim, através da teoria do "*gut-brain axis*" ou eixo intestino-cérebro, é possível estabelecer uma ligação entre estas duas componentes da PEA.

O eixo intestino-cérebro define-se como o conjunto de interações nervosa, endócrina e imunológica que se estabelece entre o SNC e o trato GI. Um elemento fundamental desta comunicação é a microbiota, o conjunto de bactérias e outros microrganismos que residem num particular nicho biológico, neste caso o trato intestinal humano. No meio intestinal, estas bactérias produzem metabolitos essenciais para a sinalização endócrina e imunológica, comunicando também com o SNC através de recetores do nervo vago.

O microbioma intestinal é também promotor da motilidade, produtor de vitaminas e tem um efeito protetor contra organismos patogénicos entéricos. No entanto, quando em desequilíbrio ou disbiose, pode produzir toxinas que atingem o SNC.

Esta revisão sistemática procurou explorar a relação entre as alterações no microbioma humano e a patogénese da PEA.

Foi realizada uma pesquisa na base de dados PubMed usando a expressão: "(("Autistic Disorder"[Mesh]) OR ("Autism Spectrum Disorder"[Mesh])) AND (("Microbiota"[Mesh]) OR ("Gastrointestinal Microbiome"[Mesh]))". Os critérios de inclusão foram: estudos observacionais ou de intervenção, realizados em indivíduos com PEA e com referência à sua relação com a microbiota intestinal, redigidos em inglês. Foram também incluídos estudos referidos nas referências das revisões sistemáticas e meta-analises englobadas na pesquisa inicial. A qualidade dos estudos foi avaliada segundo os critérios STROBE e TREND e os principais dados extraídos foram: o número de participantes do estudo, o tipo de estudo e a metodologia usada, o/os instrumento/os usados para diagnosticar PEA e os principais resultados obtidos.

Usando as recomendações do PRISMA (*Preferred Reporting for Systematic Reviews and Meta-Analysis*) foram incluídos 28 estudos nesta revisão. Foram estudadas 1169375 crianças com idades compreendidas entre 1 e 18 anos. Os estudos foram divididos em 3 grupos para facilitar a sua análise e discussão: "*Standard comparison*", "*Comparison by exposure variables*" e "*Comparison after intervention*". O primeiro grupo comparava linearmente o microbioma de indivíduos com PEA com o de sujeitos neurotípicos. O segundo reunia os estudos de coorte que

procuravam verificar o impacto de variáveis que alterariam o microbioma, segundo os autores, para concluir se essa exposição teria influência num posterior diagnóstico de PEA. O último grupo reunia os estudos de intervenção com suplementos ou probióticos em crianças com PEA.

A maioria dos estudos revelou uma diferença significativa entre o microbioma dos indivíduos com PEA e o dos controlos, mas as diferenças registadas não foram constantes entre estudos, com a notável exceção da ordem *Clostridiales* e da espécie *Clostridium*, que demostrou um notável aumento nos indivíduos com PEA. No primeiro grupo de estudos, apenas 2 em 18 consideraram que não havia uma divergência entre os microbiomas. No entanto, os próprios estudos foram realizados em condições bastante diferentes: 9 comparavam as crianças com PEA com os seus irmãos neurotípicos, enquanto os restantes 11 usaram controlos da comunidade; apenas 2 estudos abordaram a micobiota; um estudo analisou crianças e mães como uma unidade em termos de distribuição destes microrganismos e outro estudo recolheu os seus dados usando biopsias retais, ao invés de amostras fecais, por exemplo.

Em relação aos estudos de coorte, não foi encontrada nenhuma relação causal entre os fatores testados (parto por cesariana, uso de antibióticos nos primeiros anos de vida) e a incidência de PEA. Os estudos de intervenção demostraram um efeito positivo da suplementação e probióticos na alteração da composição do microbioma, mas estes efeitos nem sempre se revelaram a nível sintomático.

Assim, foi verificada uma diferença não negligenciável entre o microbioma de um indivíduo com PEA e o microbioma neurotípico. Esta conclusão pode ser uma base para futura pesquisa nesta aérea, através de um estudo que procure uniformizar os fatores que influenciam a microbiota e as suas condições de desenvolvimento.

# Palavras chave

Autismo; microbioma; imunomodulção

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# List of acronyms

- 6-GSI - Gastrointestinal Severity Index (modified)
- ABC - Autism Behavior Checklist
- ADI-R - Autism Diagnostic Interview-Revised
- ADOS - Autism Diagnostic Observation Schedule
- ASD - Autism Spectrum Disorder
- ATEC - Autism Treatment Evaluation Checklist
- CARS - Childhood Autism Rating Scale
- CBCL - Child Behavior Check List
- DBC-P - Development Behavior Checklist
- DSM-4/5 - Diagnostic and Statistical Manual of Mental Disorders $4^{th}$ or $5^{th}$ Edition
- DSR - Daily Stool Records
- FGID - Functional Gastrointestinal Disorders
- GMDS-ER - Griffiths Mental Development Scale-Extended Revised
- GSRS - Gastrointestinal Symptoms Rating Scale
- IBS - Irritable Bowel Syndrome
- ICD-9/10 - International Classification Disease 9 or 10th Edition
- INDT-ASD - DSM-5 approved AIIMS-modified INCLEN Diagnostic Tool for Autism Spectrum Disorder
- ISAA - Indian Scale for Assessment of Autism
- Mc Arthur-CDI - MacArthur-Bates Communicative Development Inventories
- MoviPrep - laxative
- PDD-BI - Pervasive Developmental Disorders Behavior Inventory
- PGI-III - Parent Global Impressions-III
- Prilosec - acid pump inhibitor
- PSI - Parenting Stress Index
- QPGS - Questionnaire on Pediatric Gastrointestinal Symptoms
- RBS-R - Repetitive Behavior Scale-Revised
- SCFA – Short-Chain Fatty Acid
- SCQ - Social Communication Questionnaire
- SHGM - Standardized Human Gut Microbiome
- SRS - Social Responsiveness Scale
- TBPS - Total Behavior Problem Score
- VABS-II - Vineland Adaptive Behavior Scale

# 1. Introduction

Autism spectrum disorders (ASD) are a group of conditions[1], usually detected in childhood, described by difficulties in social behavior and communication, very specific interests and repetitive activities that are typical to each patient. These children and adults can experience socioemotional difficulties, struggling with verbal communication and relationship construction. ASD subjects are also prone to be very attached to their routine and easily disturbed by stimuli that do not have any effect on neurotypical individuals like some specific sounds or textures. These symptoms usually manifest early in life, but they can have a later onset when social demands start being too difficult to handle and they can have a severe impact in day-to-day life for these children and adults. (1–3)

In the past few years, there has been a substantial rise in ASD prevalence, with 1 in 160 children in the world experiencing ASD. (3–6) In Portugal, the estimated prevalence is 1 in 1000 children from ages 6 to 9. (7) But, as Finegold et al have pointed out, this increase could just be the product of changes in diagnostic criteria and increased awareness. (5) Nevertheless, there are several possible explanations, since ASD diagnosis remains quite unexplored from a pathophysiological standpoint, no particular conclusion can be assumed.

Autism is usually defined by its symptoms, due to its unknown and varied pathophysiology. Compart (8) selects several biomedical factors that might be associated with autism: nutritional deficiencies, food sensitivities, altered intestine permeability, brain, and gut inflammation due to excess of cytokines and mitochondrial dysfunction. Nevertheless, ASD causes can be easily divided into genetic and non-genetic, including here all the gastrointestinal (GI) and immune changes that can occur in these subjects. So far, we can ascertain that ASD children's behavior defines the syndrome and that genetic and environmental factors can be of influence in the pathogenesis on a variable basis. As Silver et al (9) put it ASD "is not a disease as it does not have a unique biologic cause".

Some of the aforementioned comorbidities that complicate ASD pathology are gut disorders like constipation, diarrhea, and abdominal pain and discomfort. (10–13) The GI problems that children with ASD experience can be related to some behavior problems and aggressive demeanor they experience. (14) Considering that gut microbiota is considered vital for GI wellbeing, there is a clear connection between the gut disorders associated with ASD and dysbiosis, which attests to the importance of exploring this section of ASD pathology. (15)

As stated by Finegold et al, there are several ways microbiota could influence the pathology of ASD: a) by producing toxins, b) creating autoantibodies or c) allowing toxin production by invading bacteria when it is altered or weakened. (16) This way, studying the microbiota can be a good first step to better understand autism.

[1] ASD includes Autism Disorder (AD), Asperger's Syndrome and Pervasive Developmental Disorder Not Otherwise Specified (PDD-NOS).

The human microbiota is, in essence, the complete assembly of microbes that reside in a particular biological niche including bacteria, fungi, viruses, and others. (12) Particularly in the gut, this colonization begins as a newborn but it can be continuously altered by individual factors like genetics, external factors, and microorganism interactions. (15,17) Genetic factors are essentially related to hyper immunity due to overexpression of factors like IL-6, IL-12 and TNF or immunodeficiency caused by mutations in IL-10 or NOD2. Lifestyle, diet, hygiene, antibiotics, metabolic dysfunction and chronic inflammation can also influence the microbiome in composition and distribution. (18)

In a neurotypical healthy gut, the most important phyla are *Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria, Fusobacteria,* and *Verrucomicrobia,* with the first two amounting to the majority of all bacteria present. *Firmicutes* are mostly represented in the form of *Clostridium* but *Lactobacillus* is also an important part of this phylum. *Bifidobacterium*, on the other hand, is a part of *Actinobacteria*. (19)

Gut microbiota is a known immunomodulator and it contributes to the human metabolism as well. (4) It can help breakdown some nutrients, promote motility, produce vitamins and compete against pathogens. (14,20–22)

In the epithelium, the microbiota facilitates the production of SCFAs, metabolites of carbohydrates, that affect the level of some gut hormones, participating in glucose homeostasis. The microbiome also has a beneficial effect on "leaky gut" promoting GLP-2 production by L cells and restoring the balance that affects, for example, obese subjects. (23)

However, the microbiome can have some pathogenic effects, producing toxins that can reach the central nervous system, showing a real connection between the gut and the central nervous system (CNS) through the "gut-brain" axis.

The "gut-brain" axis can be defined as the biochemical interaction between the GI tract and the SCN. (17) This communication is bidirectional and not only nervous but also endocrine and immune, affecting the afferent neural pathways that connect the gut to the brain. (4,24) Still, the microbial participants of this connection have not yet been identified (4).

This bond is complex and multilayered, involving several communication pathways between bacteria and their metabolites with both gut cells and neurons. As for endocrine signaling, the above mentioned SCFAs are an example of a bacterial fermentation metabolite that triggers the production of gut peptides inducing satiety. Tryptophan, another metabolite, is also a precursor of serotonin, which is mostly stored in gut enterochromaffin cells.

Concerning the SNC interaction with the GI system, vagal receptors can be stimulated as well by gut peptides and bacterial metabolites alike. Immune responses are part of this system too, with Gram-negative bacteria promoting cytokine production (mainly IL-6) through activation of B cells and microglia. (25,26)

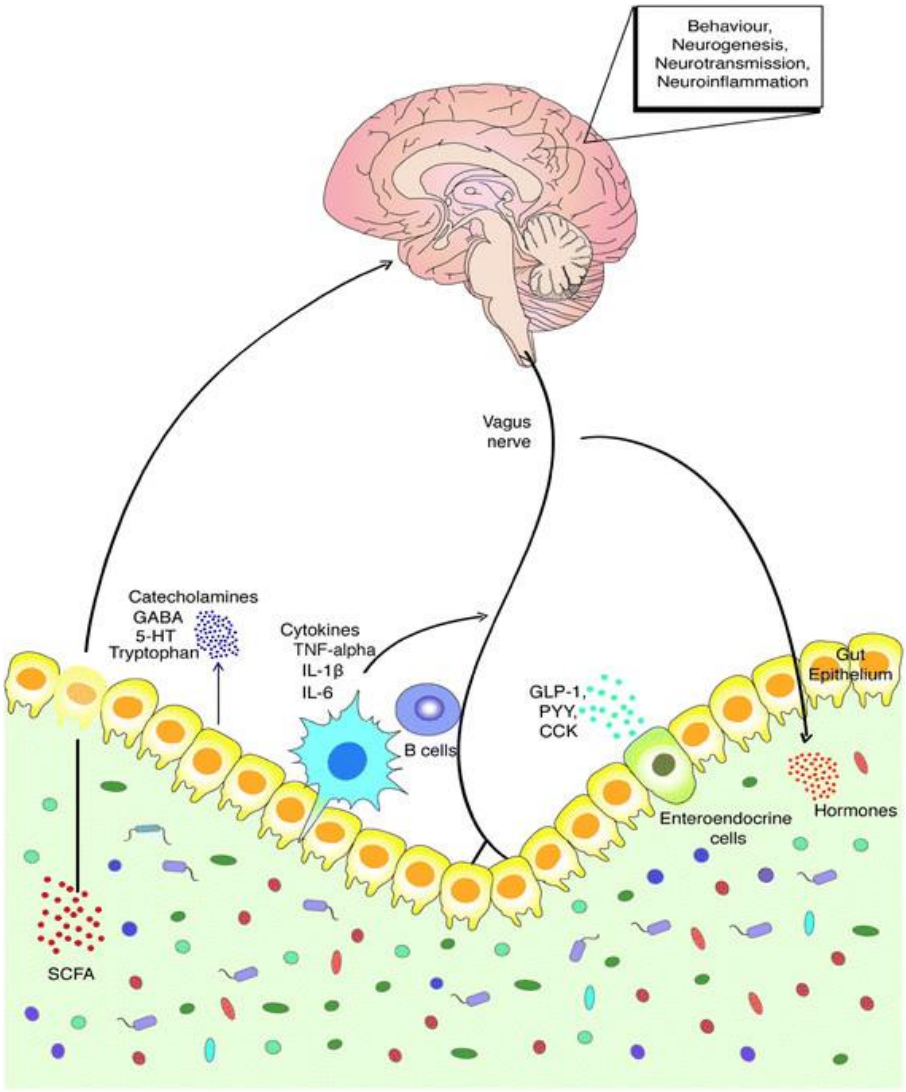


Figure 1- Representation of the gut-brain axis. (adapted from 26)

The "gut-brain" axis, as explained, impacts various organs and systems so it comes as no surprise that it is proposed to be involved in the pathogenic mechanism of several conditions like schizophrenia, IBS, Parkinson's Disease and more. (25,26)

As for ASD, its connection with the axis seems to be intuitive, since it affects behavior, neural and GI functions. The studies analyzed in this review sought to approach the axis focusing on microbiome changes or dysbiosis in ASD patients, studying its prevalence and how it influences behavior and GI comorbidities.

# 2. Methods

In this systematic review, the question at hand was: “Is there a relationship between microbiome changes in ASD subjects and the physiopathology of this condition?”. To explore this issue, a thorough search on the PubMed database was conducted for the chosen keywords: “autism”, “ASD”, “microbiota” and “intestinal microbiome”. The search expression was the following: (("Autistic Disorder"[Mesh]) OR ("Autism Spectrum Disorder"[Mesh])) AND (("Microbiota"[Mesh]) OR ("Gastrointestinal Microbiome"[Mesh])). This search included all the articles found from the inception of the database until October 7, 2019.

All of the resulting articles were screened based on their title and abstract and selected, based on pre-agreed criteria. The inclusion criteria were: a scientific study (whether it was a randomized controlled trial, a cohort study or a cross-sectional study), with human patients with ASD, that referred to its relationship with large intestinal microbiota and that was written in English. The exclusion criteria were: systematic reviews and meta-analyses and studies conducted on animal models, tissues or cells.

After this initial selection, the references of existing systematic reviews and meta-analyses were examined to find other articles that fitted the inclusion criteria and that were not reached by the initial research.

The evaluation of literature was conducted by two independent researchers and the scientific quality of the studies was evaluated using the STROBE scale for case-control and cross-sectional studies and the TREND statement for non-randomized controlled trials (Table S1, S2 and S3). (27,28) Studies complying with ≥ 75 % of the statements were considered to have good quality, 75 % - 50 % intermediate quality and ≤ 50 % bad quality.

The data extracted from the studies were summarized in tables identifying: the participants of the study, the study type and method of analysis, the instrument used to diagnose ASD and the main outcomes to facilitate comparison between studies.

This systematic review was performed according to the recommendations established by the Preferred Reporting Items for Systematic reviews and Meta-Analysis (PRISMA) statement guidelines. (29)

# 3. Results

A total of 109 articles were found in the initial PubMed search and 13 more studies were identified in the references of systematic reviews. All 122 articles were screened by abstract reading and 92 were excluded: 3 were not written in English, 17 didn't address ASD in a significant way, 31 were performed in animal models or cell lines, 17 didn't mention microbiome as a key part of their study, 1 focused on duodenal microbiome (30) and 24 were literature reviews. After the application of the inclusion and exclusion criteria, the remaining 29 articles were read in full and 1 was excluded: it described a study that was yet to take place, so it showed no results to interpret. (31) This way, 28 studies were included in the qualitative synthesis (Figure 1). Most of the reports considered had good quality, with only 6 showing intermediate quality according to the used criteria (Table S1-3).

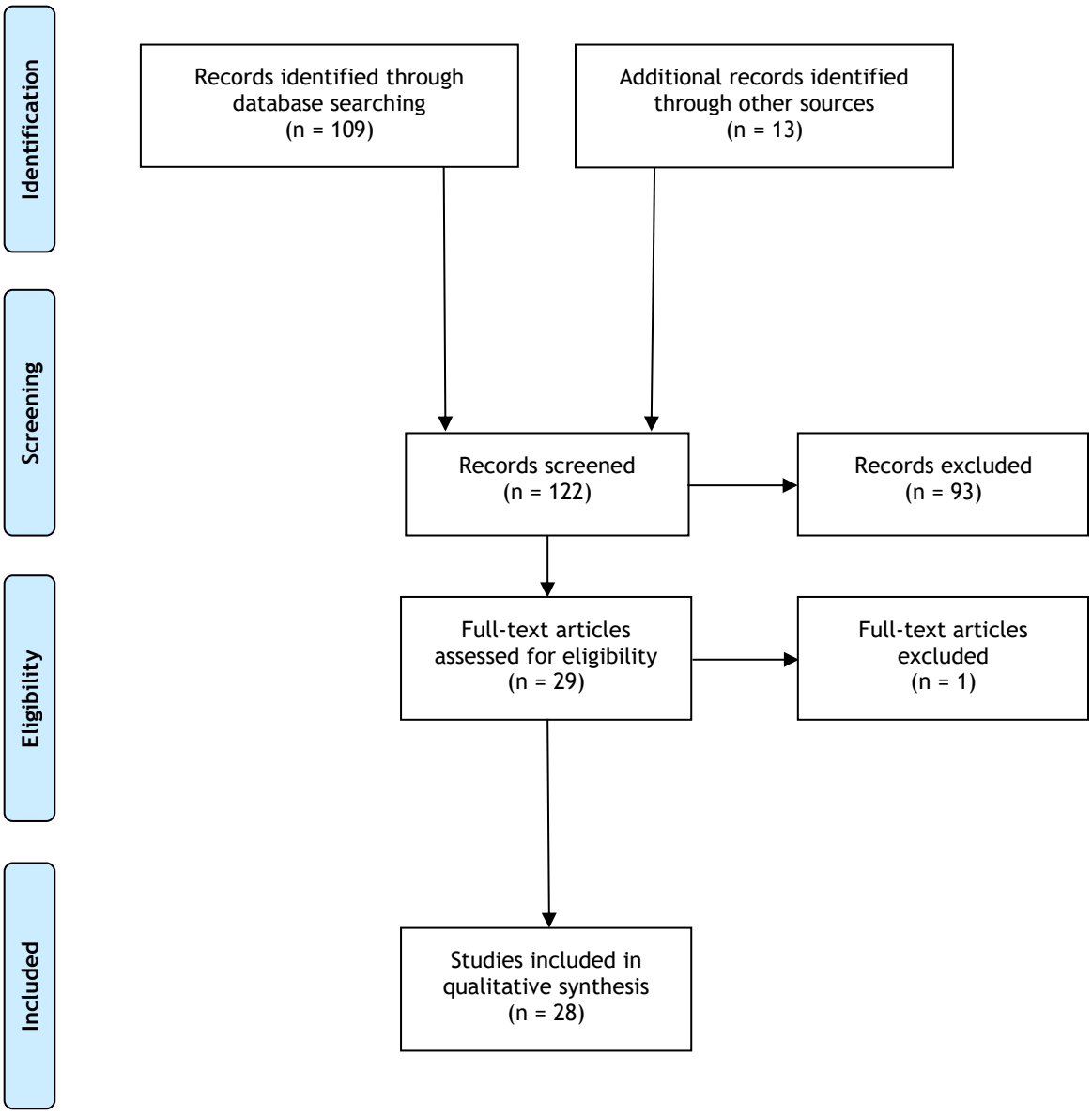


Figure 2 - PRISMA flowchart of the studies selection process

The 28 included studies were divided into 3 types of scientific research: “Standard Comparison” (Table 1), “Comparison by exposure variables” (Table 2) and “Comparison after intervention” (Table 3). Age was mentioned in 23 articles, including children between 1 and 18 years old. It was not possible to determine the median age due to lack of exact values for each participant and the central tendency measurements were variable between the studies as well. In total, 359142 children were a part of control groups, 765 were in the ASD groups and, overall, including the population-based studies described in Table 2, 1169375 children were studied.

Specific gender distribution was mentioned in 20 articles, with almost all reports showing a male predominance of ASD. Of the articles in which gender is mentioned, 504 subjects were male, in a total of 604 ASD subjects. Kang et al (32), Plaza-Díaz et al (12) and Finegold et al (33) did not refer to the gender distribution of the groups, only attesting it was similar between the ASD and control group. As for Axelsson et al (34), there was no information about the gender composition of the studied cohort, but using the between-within model, females were found to have a reduced risk of autism. Song et al (10) and Finegold et al (16) showed no reference to the studied group’s age or gender distribution and De Angelis et al (35) studied more females than males but did not specify if the ASD group had that same distribution. Mcartney et al (36) mentioned gender and age distribution of the initial group but it did not share that information in regards to the subset that completed the full trial.

The research summarized in Table 1 dealt with studies that simply compared the microbiome of subjects with ASD or AD with microbiota from neurotypical volunteers. Four studies used siblings or family members of the ASD patients as controls, 12 studies used unrelated controls, with 5 using both.

In Table 2, the mentioned reports are cohort studies. By examining some exposure variables that are said to impact the constitution of the microbiome, the authors sought to conclude if that contact influenced ASD diagnosis later in life.

Table 3 displays the intervention studies included in this research: each clinical trial compared the difference in microbiome composition after an antibiotic, supplement or probiotic intervention.

## 3.1 Standard comparison of ASD and NT microbiome

Twenty studies conducted a standard comparison between the microbiome of ASD and NT controls. Nine studies were based on comparison with sibling controls (4,5,11,17,22,35,38–40) with the remaining eleven using community controls. Of the latter, two focused on the mycobiota (20,37), one used mother-child pairings, adding the hereditary factor to the microbiome comparison (24), one studied mental regression in ASD (12), four elected specific bacterial groups to guide their comparisons (10,15,33,41), one used rectal biopsies instead of standard fecal analysis (42) and the remaining two (14,16) executed a standard comparison. A summary of all the data is reported in Table 1.

Son et al (38) sought to compare microbiota using a sibling study format, finding almost no significant changes in the microbiome in ASD siblings. Also, ASD children's behavior (measured by CBCL) did not seem to be influenced by the presence of FGID. Gondalia et al (22) similarly concluded there were no significant differences between ASD microflora and that of their siblings ($P>0.05$), suggesting that stress and anxiety could be the causes for the high prevalence of GI disorders in the ASD population. However, Tomova et al (4) demonstrated that ASD dysbiosis also affects neurotypical siblings as well, maybe due to their GI dysfunction. Besides, there were some specific changes found only in ASD children, suggesting a possible involvement of *Clostridia* and *Desulfovibrio* species. This finding can be supported due to the parallel increase of these bacterial counts with ASD severity and their decrease with the severity of GI problems.

Finegold et al (5) positioned the sibling control group is between community controls and ASD children in terms of bacterial abundance, with *Firmicutes* amounting to less than 50% of the bacteria. A significantly altered genus in ASD was *Desulfovibrio*, with notable increases in all 3 species, noting a possible role in autism pathogenesis.

Older studies like De Angelis et al (35) separated ASD in AD and PDD-NOS, using the DSM-IV classification, but their findings can still be useful. Contrary to what was verified in other works the *Firmicutes*/*Bacteroidetes* ratio was decreased in AD subjects. However *Enterobacteriaceae* and *Clostridiaceae* were increased, as was *Akkermansia muciniphila*.

The latter bacteria was also a focal point of study in Wang et al (39), but their results showed a significative decrease of its abundance relative to controls ($P=0.029$), less so in siblings. This trend in bacterial changes repeated itself concerning FGID, which were more prevalent in ASD children, as expected but was also slightly increased in their siblings, compared to community controls.

The aforementioned report based on the same population of Wang et al (11) evidenced an increase of *Sutterella spp.* and *Ruminococcus torques* in ASD children (the latter in those with FGID) and, less so, in their siblings.

As for Parracho et al (40), GI problems were significantly more frequent in ASD patients than controls, even with ASD subjects experiencing a variety of diets and supplementation (66% ASD subjects and 8% of siblings were following a restricted diet and 53.4% of ASD children and 41.7% of siblings were taking probiotics). As for bacterial changes, one *Clostridium* species showed a significant increase in ASD, with intermediate values shown by the sibling group, paralleling the incidence of GI problems.

On the contrary, Pulikkan et al (17) portrayed keeping the children on their native diet as a strength. This study found *Prevotellaceae* were decreased and *Lactobacillus* and *Bifidobacterium* were increased in ASD.

As evidenced by Strati et al (20) the fungal mycobiota can also be altered in ASD, which can negatively impact the experienced GI symptoms. In this case, *Candida* counts were double in ASD subjects ($P<0.001$). This work also concluded that constipated ASD subjects showed high levels of *Clostridium cluster XVII*.

Iovene et al (37) also focused on mycobiota changes with a particular interest in increased *Candida* counts in ASD subjects. A decrease in *Lactobacillus spp.* and *Clostridium spp.* counts seemed to contribute to the ASD dysbiosis while the former showed a significant correlation with CARS score severity ($P=0.0322$). There is no correlation demonstrated between the high prevalence of GI symptoms in the ASD group with the elevated counts of *Candida* detected, suggesting that this fungus can only thrive due to preexisting dysbiosis in ASD subjects.

Li et al (24) compared microbiomes using mother-child pairings in a very interesting and uncommon way, looking to associate maternal gut microbiota with the changes in ASD children's biological profile. Adding the genetic variable to this complex equation, the results found that both mothers and children in the ASD group showed a similar proliferation of *Proteobacteria* and *Enterobacteriaceae*, while *Clostridium* and *Streptococcus* were more specifically increased in ASD children. Mothers of children with ASD also had an altered microbiome composition compared with parents of NT children, implying a hereditary element in this dysbiosis.

Plaza Díaz et al sought to correlate the degree of mental regression in ASD children with changes in the microbiome, finding different compositions within all the studied groups. The standout bacteria were *Actinobacteria* and *Proteobacteria* increased in ASD children. The latter one was particularly increased in children that evidenced mental regression. (12)

Park et al (15) focused their study on *Prevotella* and other fermenters and they found a decrease of these bacteria among ASD subjects, showing also a correlation between this genus and ASD status ($P<0.05$).

Song et al (10) focused on the specific hypothesis that *Clostridium* had a meaningful role in Autism pathogenesis and found that *C. bolteae* and *Clostridium* clusters I and XI were increased in ASD. Finegold et al (33) also showed that *C. perfringens* was increased in ASD with GI symptoms ($P=0.031$). Similarly, Martirosian et al (41) showed an increase of *Clostridium* species counts in ASD, particularly *C. perfringens*.

Instead of fecal analysis, Luna et al (42) used rectal biopsies to ascertain microbiome changes and compare those to the ones obtained with the previous method. *Clostridiales* were shown to be increased in ASD with FGID and *Sutterella* was decreased.

Lastly, Adams et al (14) established a significant correlation between GI symptoms and ASD severity ($P<0.001$). *Bifidobacterium* was significantly decreased in ASD while *Lactobacillus* was increased, the latter possibly due to low seafood consumption in the ASD group ($P=0.0008$). Finegold et al (16) found high counts of *Clostridium* and *Ruminococcus* in ASD children.

Table 1- Standard Comparison[2]

| Reference | Study type | Study group | Control group | ASD diagnosis | Method of analysis | Changes in microbiome |
|---|---|---|---|---|---|---|
| Plaza-Díaz et al, 2019 (12) | Descriptive observational study | 30 children with ASD and no mental regression (ANMR), 18 with ASD and mental regression (AMR) | 57 matched neurotypical (NT) controls | ADI-R, DSM-5, ICD-10, And ADOS. PDDBI, Battelle developmental test, and CARS for the severity of ASD. | Sequencing | Phylum: *Actinobacteria* was augmented in ANMR and *Proteobacteria* in AMR. Family: *Enterobacteriaceae* were higher in the whole ASD group. *Clostridiales* family XVII were only higher in ANMR. Genus: *Bacillus*, *Bifidobacterium*, and *Prevotella* were higher in ASD. *Enterococcus* was higher just in AMR. |
| Strati et al, 2017 (20) | Not specified | 40 ASD subjects | 40 matched NT controls | DSM-5 and CARS (for ASD severity) | Sequencing | Phylum: Increase of *Firmicutes*/*Bacteroidetes* ratio in ASD due to a reduction of the *Bacteroidetes*. Genus: *Lactobacillus* and *Candida* were significantly increased in ASD. |
| Pulikkan et al, 2018 (17) | Not specified | 30 severe ASD children | 24 family matched (siblings or blood relatives) NT children | CARS, INDT-ASD and ISAA | Sequencing | Phylum: *Firmicutes* were higher in ASD. Family: *Prevotellaceae* were decreased. Genus: *Lactobacillus* and *Bifidobacterium* were increased in ASD. |
| Li et al, 2019 (24) | Cross-sectional study | 59 mother-child pairs | 30 matched mother-child pairs of NT children | DMS-5, ADOS, and ABC | Sequencing | Phylum: Mothers and children with ASD children had more *Proteobacteria*. |

[2] The section "Changes in microbiome" is a simplified version of the collection of bacteria mentioned in each article, including only bacteria that appear in several studies, for the sake of comparison.

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|---|---|---|---|---|---|---|
| | | of ASD children | | | | Family: *Enterobacteriaceae* was increased in mothers of ASD children. *Alcaligenaceae* was increased in ASD children. Genus: *Acinetobacter* and *Streptococcus* were increased in mothers and ASD children. *Clostridium* was increased in ASD children. |
| Iovene et al, 2016 (37) | Not specified | 47 children with ASD | 33 matched NT children | DSM-5, ADI-R, CARS, and ADOS | Microscopic and cultural examination | *Candida spp.* was present in 57.7% of ASD and no controls. Family: *Enterobacteriaceae* was increased in ASD. Genus: *Lactobacillus spp.* and *Clostridium spp.* was decreased in ASD. |
| Son et al, 2015 (38) | Not specified | 59 ASD children | 44 NT siblings | ADOS and ADI-R for diagnosis. CBCL to assess problem behaviors. | Sequencing | Phylum: there was no significant difference in the 4 major phyla (*Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria*) between ASD and NT siblings. Genus: *Cyanobacteria/Chloroplast* was increased in ASD with FGID. *Sutterella* and *Prevotella* were not associated with ASD or FGID. |
| De Angelis et al, 2013 (35) | Not specified | 10 PPD-NOS children and 10 AD children | 10 NT sibling controls | DSM-4 to group the children into AD or PDD-NOS. ADI-R, ADOS, and CARS for evaluation. | Sequencing and culture | Phylum: *Bacteroidetes* were increased in PDD-NOS and AD. *Firmicutes* was decreased in AD. Family: *Sutterellaceae, Enterobacteriaceae* and Clostridiaceae were increased in AD. Genus: *Ruminococcus* was increased in PDD-NOS and NT children. *Clostridium, Bacteroides* and *Prevotella* were increased in AD and *Bifidobacterium, Lactobacillus* and *Streptococcus* were decreased. *Akkermansia* was increased in PDD-NOS and AD. Species: *Akkermansia muciniphila* was increased in AD. |

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| Tomova et al, 2014 (4) | Not specified | 10 ASD subjects | 9 NT siblings and 10 NT controls | Diagnosis by ICD-10 criteria and severity evaluated by ADI and CARS. | Sequencing and cultures | Phylum: ASD and siblings showed a decreased in the *Bacteroidetes*/*Firmicutes* ratio, due to the drop in *Bacteroidetes* abundance. Genus: *Lactobacillus spp.* and *Desulfovibrio* were increased in ASD. *Bifidobacterium* was lower in siblings than in ASD. |
| Park et al, 2013 (15) | Not specified | 20 ASD children | 20 NT children | ADI-R, ADOS, ATEC, and PDD-BI | Sequencing | Genus: *Prevotella* and S*utterella* were decreased in ASD. *Akkermansia* was increased in ASD. |
| Wang et al, 2011 (39) | Not specified | 23 ASD children (17 with AD and 6 with Asperger's syndrome) | 22 NT siblings and 9 unrelated NT children | Diagnosed with CARS and DSM-4 | Sequencing | Genus: *Bifidobacterium spp.* was decreased in ASD. Species: *Akkermansia muciniphila* was decreased in ASD and, less so, in siblings. |
| Finegold et al, 2010 (5) | Not specified | 33 ASD subjects | 7 NT siblings and 8 NT non-sibling controls | Diagnosed and evaluated by a doctor (unknown criteria) | Sequencing | Phylum: *Bacteroidetes* were increased in the ASD group and *Firmicutes* was increased in the control group. The sibling group is between them in *Firmicutes* presence, but closer to the ASD group. *Actinobacteria* was slightly decreased in ASD and *Proteobacteria* was increased. Genus: *Desulfovibrio, Clostridium* and *Ruminococcus spp.* were increased in ASD and *Bifidobacterium* was decreased. *Streptococcus* was increased in controls. |
| Gondalia et al, 2012 (22) | Not specified | 51 ASD children | 53 NT siblings | Diagnosed by a doctor (unknown criteria) and severity was | Sequencing | There were no significant differences between the groups. |

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| | | | | evaluated by CARS. | | |
| Parracho et al, 2005 (40) | Not specified | 58 ASD children | 12 NT siblings and 10 unrelated NT children | No report of diagnosis method. | Sequencing | Genus: *Bacteroides* were decreased in the sibling group. Species: *Clostridium histolyticum* was increased in ASD and less so in siblings. |
| Adams et al, 2011 (14) | Not specified | 58 ASD children | 39 NT controls | Diagnosis by a professional and ATEC (to assess severity) | Culture | Genus: *Bifidobacterium* and *Enterococcus* were decreased in ASD and *Lactobacillus* was increased. |
| Finegold et al, 2002 (16) | Not specified | 13 late-onset AD children | 8 NT control children | No report of diagnosis method. | Culture and sequencing | Genus: *Clostridium* and *Ruminococcus* were increased in ASD. |
| Martirosian et al, 2010 (41) | Not specified | 41 AD children | 10 NT children | ICD-10 was used for diagnosis and the Psychoeducational Profile – Third Edition – Caregiver Report was used to access severity. | Culture | Species: *C. perfringens* was increased in ASD. |
| Finegold et al, 2017 (33) | Not specified | 33 ASD children | 13 matched NT children | No report of diagnosis method. | Culture and sequencing | Species: *C. perfringens* was increased in ASD with GI symptoms. |
| Luna et al, 2017 (42) | Not specified | 14 ASD children with FGID | 15 NT children with FGID and 6 | ADOS and SRS to access NT children to make | Sequencing | Order: *Clostridiales* were increased in ASD with FGID. Genus: *Sutterella* was decreased in ASD with FGID. |

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| | | | healthy NT children | sure they had no ASD-like behavior. | | |
| Wang et al, 2013 (11)[3] | Not specified | 23 ASD children | 22 NT siblings and 9 NT controls | Diagnosed with CARS and DSM-4 | Sequencing | Genus: *Sutterella spp.* was increased in ASD and, less so, in siblings.<br>Species: *Ruminococcus torques* were increased in ASD with FGID and in siblings. |
| Song et al, 2004 (10) | Not specified | 15 ASD subjects | 8 NT controls | No report of diagnosis method. | Sequencing | Species: *C. bolteae* and *Clostridium* clusters I and XI were increased in ASD. |

[3] This article is a short report conducted based on Wang et al, 2011 (39).

## 3.2 Comparison of cohort exposure variables

Three articles mentioned cohort studies done over a long period, observing the effect of exposure variables that could have a modifying effect on the microbiome and the outcomes of ASD incidence.

Axelsson et al (34) conducted a population-based, prospective cohort study for 13 years, studying autism prevalence through exposure to cesarean delivery and antibiotic use in the first 2 years of life, using a sibling model. This article did not support a causal relationship between exposure and autism.

Hamad et al (43) used only antibiotic exposure as a variable, determined by filling one or more antibiotic prescriptions during the first year of life. This study also did not support any connection between antibiotic treatment and risk of ASD, due to a lack of dose-response correlation and lack of association in the sibling-controlled analysis.

Similarly using antibiotic exposure, Vargason et al (13) performed a retrospective analysis, dividing the studied population into two cohorts (ASD and POP) with a subdivision for GI symptoms. This article concluded that having filled more antibiotic prescriptions increased the risk of having GI symptoms, in children with or without ASD. However, GI disorders were much more commonly found in the ASD population, which indicated that this tendency was fully independent of antibiotic exposure.

Table 2 - Comparison by exposure variables

| Reference | Study type | Study group | Exposure variables | ASD diagnosis | Method of analysis | Other relevant outcomes |
|---|---|---|---|---|---|---|
| Axelsson et al, 2018 (34) | Population-based, prospective cohort study (for 13 years) | 671.606 children who had not been diagnosed with autism at their 2nd birthday | Cesarean delivery and antibiotic use in the first 2 years of life | ICD-10 | Statistical analysis with a standard, stratified and between-within model | This article does not support a causal relationship between antibiotic treatment and cesarean delivery with autism. |
| Hamad et al, 2018 (43) | Population-based cohort study (during approximately 18 years) | 214.834 typically developing children, including an 80.225 sibling's cohort (children with a sibling of discordant antibiotic exposure status) | Filling of one or more antibiotic prescription during the first year of life | DSM-5 and ICD-10 | Statistical analysis (cox proportional hazards regression model) | This article doesn't support any connection between antibiotic treatment and the risk of ASD. |
| Vargason et al, 2018 (13) | Retrospective analysis | 3253 children in the ASD cohort and 278.370 in the POP cohort (general population); during the study they were further classified as +GI (with GI symptoms) or -GI. | Number of oral antibiotic prescriptions filled during the first 3 years of the enrollment period | ICD-9 and DSM-4 | Cox regression model | More antibiotic fills increase the risk of having GI symptoms, in children with or without ASD- |

## 3.3 Comparison after supplement intervention

The last set of studies featured in this review are clinical trials of several supplements that are seen as possibly beneficial for ASD children. Four of them use probiotics in several combinations (32,36,45,46) and one uses a vitamin supplement (44). Probiotics are microorganisms that can improve GI health when taken as a supplement (45), so in this case, they are used to combat ASD dysbiosis.

In contrast, Liu et at (44) studied vitamin A influence on ASD and microbiome due to its relationship to CNS regulation through retinoic acid and potential role in the microbiota layer. Only 20 of the 64 initial participants completed the intervention showing increased levels of *Bacteroidetes* and a decrease of *Firmicutes*, with an increased ratio. *Clostridium* and *Bifidobacterium* both decreased. However, despite these changes, all the ASD diagnosis scores showed no significant difference by the end of the intervention.

As for probiotic interventions, Shaaban et al (45) provided a nutritional supplement formula with *Lactobacillus* and *Bifidobacteria* to ASD children, subsequently comparing their microbiome with NT controls. After supplementation, *Bifidobacteria* and *Lactobacilli* increased in comparison to baseline, when *Bifidobacteria* was decreased in comparison to controls. There was a significant correlation (P= 0.0001) showing a decrease in the severity of ASD and GI symptoms after probiotic supplementation.

Mccartney et al (36) used a probiotic containing only *Lactobacillus*, which increased *Lactobacillus* and *Enterococcus* counts, decreasing *Clostridium cluster XIVa*. Probiotic feeding improved behavior scores, but the placebo also lowered the baseline scores ($P<0.05$). Unfortunately, from the 62 ASD children that started the study, only 17 followed the complete protocol which severely undermined its conclusions, but it still an important inclusion in this review.

Already mentioned above, Tomova et al (4) also have a small intervention section with probiotic supplementation that shows some interesting results, decreasing *Firmicutes* and increasing the *Bacteroidetes*/*Firmicutes* ratio to the level of healthy individuals, and so did *Lactobacillus spp., Desulfovibrio spp.* and *Bifidobacterium*.

Kang et al (32) used a Microbiota Transfer Therapy treatment with Standardized Human Gut Microbiota (SHGM), including a course of vancomycin and a laxative before SHGM application. The results showed that *Bifidobacterium, Desulfovibrio,* and *Prevotella* increased after treatment. This study demonstrates a significant negative correlation ($p < 0.001$) between change in GSRS and PGI-III, suggesting a real connection between gut and behavior in ASD. All the ASD scores evaluated in this clinical trial were better at the end of treatment and showed a lasting effect of at least 8 weeks, strongly implying a link with dysbiosis improvement. This study also illustrates a convergence of host-microbiome towards donor microbiome along with all these developments, which exalts the importance of neurotypical microbiota.

Sandler et al (46) was the oldest study found in the research for this review and, although it did not mention specific microbiome alterations explicitly, it was found to be a valid addition due to its farsighted concern with the connection between microbiota and autism pathogenesis. This project assumed that the microbiome alterations could be treated with an antimicrobial agent like vancomycin, followed by a probiotic containing *Lactobacillus* and *Bifidobacterium*. The ASD manifestations improved during the antibiotic therapy but these gains went back to baseline once it ended.

Table 3 - Comparison after intervention

| Reference | Study type | Study group | Intervention | ASD diagnosis | GI symptoms | Method of analysis | Changes in microbiome | Other relevant outcomes | Monitored confounders |
|---|---|---|---|---|---|---|---|---|---|
| Liu et al, 2017 (44) | Follow-up study | 64 children with ASD, with only 20 of the group finishing the protocol | Vitamin A supplementation | ABC, CARS, and SRS | - | Sequencing | Phylum: *Bacteroidetes* increased after the intervention. *Firmicutes*/*Bacteroidetes* ratio, *Firmicutes, Proteobacteria, and Actinobacteria* decreased. Genus: *Prevotella, Bacteroides* increased. *Clostridium* and *Bifidobacterium* decreased. | All the ASD diagnosis scores showed no significant difference by the end of the intervention. | Food frequency and mealtime behaviors. Additionally, no probiotics or antibiotics were taken. |
| Shaaban et al, 2017 (45) | Prospective open-label study | 30 ASD children and 30 gender and age-matched NT controls from the patient's families | Probiotic nutritional supplement formula with *Lactobacillus* and *Bifidobacteria* | ATEC for ASD severity. DSM-5 and ADOS/ADI-R for diagnosis. | GI symptoms of ASD children were evaluated with 6-GSI. | Sequencing | Genus: After supplementation *Bifidobacteria* and *Lactobacilli* increased in ASD, while before *Bifidobacteria* was lower. | After the intervention both the severity of autism and the GI symptoms improved. | No anti-fungal, antibiotics, special diets, psychiatric medications, vitamins or alternative therapies were allowed. |

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|---|---|---|---|---|---|---|---|---|---|
| Kang et al, 2017 (32) | Open-label clinical trial | 18 ASD children and 20 age and gender-matched NT children as controls | Microbiota Transfer Therapy with SHGM (1. Vancomycin, 2. MoviPrep, 3. SHGM and 4. Prilosec) | ADI-R, PGI-III, CARS, ABC, SRS and VABS-II | All the ASD children had moderate to severe GI problems evaluate with the GSRS and DSR. | Sequencing | Genus: *Bifidobacterium, Desulfovibrio,* and *Prevotella* increased after treatment. | 80% reduction of GI symptoms at the end of treatment and improvement of ASD behavioral symptoms as well. | No antibiotics or probiotics. |
| Sandler et al, 2000 (46) | Not specified | 11 regressive AD[4] children | Vancomycin (for 8 weeks) followed by a probiotic (*Lactobacillus* and *Bifidobacterium)* | DSM-4, Developmental Profile II, and CARS for autism severity. To check for improvement children were videotaped while playing and they were evaluated by a | All children had diarrhea. | Cultures | - | There was a significant improvement of AD symptoms during vancomycin treatment but these gains went back to baseline | One of the inclusion conditions was antibiotic usage 2 months or less before AD onset. |

[4] Regressive autism or late-onset autism is a classification from the now outdated DSM-4 that refers to children with reported NT development for 12-24 months that lose previously acquired skills and start demonstrating AD symptoms. (35)

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| | | | | doctor with behavior and communication scales. | | | | once it ended. | |
| Mcartney et al, 2010 (36) | Double-blind, placebo-controlled, crossover-designed study | 62 ASD children started the study but only 17 followed the complete protocol | Probiotic *Lactobacillus* | DBC-P that resulted in a TBPS | GI symptoms were evaluated by a parent filled questionnaire. | Sequencing | Genus: *Lactobacillus* and *Enterococcus* were increased after probiotic use. Species: *Clostridium cluster XIVa* was decreased after probiotic use. | Probiotic feeding improved behavior scores, but the placebo also lowered the baseline scores. | No other probiotics, prebiotics, antibiotics, medication for GI motility or other experimental medication. |

## 3.4 Microbiome changes – Summarized

The table presented bellow summarizes the microbiome changes in ASD reported in the analyzed studies, mentioning the ones deemed relevant for a useful comparison. Only the observational and intervention studies (Tables 1 and 3) were included in this shortened comparison, since the exposure studies (Table 2) did not mention a specific bacterium. Regarding the observational studies, this table reports increased and decreased numbers of microorganisms, as well as unchanged. As for the intervention studies, it summarizes which bacteria groups were increased or decreased by the intervention.

Table 4 - Microbiome changes – Summarized

| | **Observational studies (Table 1)** | | | **Intervention studies (Table 3)** | |
|---|---|---|---|---|---|
| | **Increased in ASD** | **Decreased in ASD** | **No change** | **Increased after intervention** | **Decreased after intervention** |
| *Firmicutes* | Pulikkan et al (17) | De Angelis et al (35), Finegold et al (5) | Son et al (38), Gondalia et al (22) | - | Liu et al (44) |
| *Bacteroidetes* | De Angelis et al (35), Finegold et al (5) | Strati et al (20), Tomova et al (4), | Son et al (38), Gondalia et al (22) | Liu et al (44) | - |
| *Firmicutes/Bacteroidetes* | Strati et al (20), Pulikkan et al (17) | Tomova et al (4), Finegold et al (5), De Angelis et al (35) | - | - | Liu et al (44) |
| *Actinobacteria* | Plaza-Díaz et al (12) | Finegold et al (5) | Son et al (38) | - | Liu et al (44) |
| *Proteobacteria* | Plaza-Díaz et al (12), Li et al (24), Finegold et al (5) | - | Son et al (38), Gondalia et al (22) | - | Liu et al (44) |
| *Enterobacteriaceae* | Plaza-Díaz et al (12), Li et al (24), De Angelis et al (35), Iovene et al (37) | - | - | - | - |

| | | | | | |
|---|---|---|---|---|---|
| *Clostridiales/ Clostridiaceae/ Clostridium* of any species | Plaza-Díaz et al (12), Luna et al (42), Li et al (24), De Angelis et al (35), Finegold et al (5), Parracho et al (40), Finegold et al (16), Martirosian et al (41), Finegold et al (33), Song et al (10) | Iovene et al (37) | - | - | Liu et al (44), Mccartney et al (36) |
| *Bifidobacterium* | Plaza-Díaz et al (12), Pulikkan et al (17) | De Angelis et al (35), Wang et al (39), Finegold et al (5), Adams et al (14), Shaaban et al (45) | - | Shaaban et al (45), Kang et al (32) | Liu et al (44) |
| *Lactobacillus* | Strati et al (20), Pulikkan et al (17), Tomova et al (4), Adams et al (14) | Iovene et al (37), De Angelis et al (35) | - | Shaaban et al (45), Mccartney et al (36) | - |
| *Prevotellaceae/ Prevotella* | Plaza-Díaz et al (12), De Angelis et al (35) | Pulikkan et al (17), Park et al (15) | - | Liu et al (44), Kang et al (32) | - |
| *Candida* | Strati et al (20), Iovene et al (37) | - | - | - | - |

# 4. Discussion

This review aimed to analyze studies, seeking a significant connection between microbiome changes and ASD pathology. The included studies spanned only some bacteria genus in the thousands that populate the microbiome, making it important to better understand the role of each group of microorganisms in the human gut biology. Each report also looked at this question in a slightly different way, so it was relevant to explore these dissimilarities in confounder management and control selection.

The first studies on the relationship of ASD and microbiota hypothesized that antibiotic use in small children was enough to disrupt the healthy gut biome and create a dysbiosis that would originate the GI problems and allow for neurotoxin producing bacteria to proliferate and, possibly affect the CNS function, as ASD. (16,46) However, children treated with an antibiotic that would suppress said bacteria showed no long-term improvements so there seemed to be no choice but to reject this theory. Hamad et al also suggested that antibiotics cannot achieve the microbiome changes necessary for long term ASD dysbiosis. (43)

Since this first hypothesis could not be proven, the following articles focused more on microbiome composition. The most significant phyla in the gut microbiome are, as mentioned previously, *Firmicutes* and *Bacteroidetes*. A few studies found an increase in the *Firmicutes*/*Bacteroidetes* ratio in ASD (17,20), and others a decrease (4,5,35), with no clear tendency. *Proteobacteria*, however, was found to be increased in ASD in three studies (5,12,24), with a decrease to standard levels after vitamin A supplementation in Liu et al (44).

*Proteobacteria* are Gram-negative lipopolysaccharide producers that induce inflammatory responses on the cell wall. (24) This phylum, although being relatively a small presence in the human gut, has been implicated in dysbiosis related to metabolic disorders and gut inflammation. (47) *Enterobacteriaceae*, one of the families of *Proteobacteria*, also exhibit an increase documented in four articles. (12,24,35,37) This family of Gram-negative bacteria can consume oxygen, making the intestinal environment favorable for anaerobe colonization of genera like *Clostridium* and *Bifidobacterium*. *Enterobacteriaceae* also thrive in an inflamed gut, being associated with several conditions from celiac disease to colorectal cancer. (48)

However, the bacterial group that seems to stand out as the most commonly prevalent in ASD, found in ten studies (5,10,12,16,24,33,35,40–42) is *Clostridiales*. This set of bacteria is composed of Gram-positive anaerobes that colonize the mucosal folds of the intestine, producing SCFAs, catecholamines and promoting the development of T cell receptors. (49) Still, some *Clostridiales* species can have a negative effect, especially when other microbiome changes are already present.

*Clostridium perfringens* is a part of *Clostridium* cluster I, and a common Gram-positive bacterium in a healthy microbiome. (33) It's also a toxin-producing organism and a common pathogen, particularly in immunocompromised organisms. *Clostridium difficile* is also a known

pathogen, particularly in those treated with antibiotics, but it can also be a colonizer other cases of microbiome disruption, like ASD. Liu et al (44) and Mccartney et al (36) found *Clostridia* to be decreased after their respective interventions, with vitamin A and probiotic supplementation.

*Bifidobacterium* is another anaerobe that is present in several studies, either as a member of the microbiome or of a probiotic. Five studies considered it lacking in ASD subjects (5,14,35,39,45), with 2 finding it in abundance (12,17). These bacteria have generally been agreed to have positive health benefits to the human gut in probiotic supplements. (45,46) Some of their abilities include improving GI barrier function, inhibiting harmful bacteria and suppressing inflammation. (50) Still, their role in ASD remains unclear, requiring more research and possibly more probiotic intervention with this particular genus.

Alongside *Bifidobacterium*, *Lactobacillus* is a probiotic genus seen as beneficial for human health. They are usually very present in newborn microbiota due to their high lactate diet and suffer a decline in dominance as the microbiome grows more diverse along with the child's food patterns. (17) This bacteria can also help maintain the integrity of the intestinal epithelial barrier and promote it's reparation when injured. (37) About four studies reported they were increased in ASD subjects (4,14,17,20), with 2 stating they were decreased (35,37) and two others (36,45) showing an increase after supplementation. This way, it seems this genus is also inconclusively distributed in ASD. However, both studies that used *Lactobacillus* as a probiotic (36,45) saw some positive results in behavioral scores and GI symptoms, which could be an indicator of a future role of this bacteria in ASD management.

*Prevotella* is part of the *Bacteroidetes* phylum, also plays a significant role in the gut microbiome. This genus is associated with plant-rich diets but also with chronic inflammatory conditions. The abundance of *Prevotella* seems to be related to the capacity to digest carbohydrates and it can be associated with typical diets from India, China, Morocco, Egypt, and others. (51) In this case, two studies reported an increase (12,35), with two others recording a decrease (15,17). Pulikkan et al were set in India, with children on a typical diet and still showed a reduction in *Prevotella* counts, which, along with the increase after intervention in two studies (32,44), leans towards an association between low *Prevotella* levels and ASD.

As for other organisms, *Candida* was the only fungal species discussed in any of the studies, and it was found to be increased in Strati et al (20) and Iovene et al (37). This fungus could be of great importance in ASD due to its corrupting influence on the microbiome structure. When the bacterial population is altered or fragile it can be a colonizer and subsequently, once it is settled, it disrupts the self-reparation of the community.

Out of all the bacteria and fungi mentioned, two studies proposed ASD biomarkers that are worth mentioning as well: Li et al (24) elected *Alcaligenaceae* and *Acinetobacter* as bacterial biomarkers for ASD and Pulikkan et al (17) proposed that the defining families that represented the difference between ASD and neurotypical subjects were *Prevotellaceae,*

*Lactobacillaceae,* and *Mogibacteraceae*. However, it seems no real consensus can be reached based on the existing studies.

As previously discussed, the microbiome is influenced by many factors, which makes this topic a very complex variable. Diet, for example, is one of the main everyday factors that can have a deep impact on the bacterial abundance in the gut. Six (12,17,20,24,35,45) studies opted to ensure the subjects had the same diet, while four (15,32,38,44) asked only for a report of consumed food. The remaining reports opted to ignore this variable or described a diversity of diets, including special diets like gluten-free or casein-free among their subjects. This can have a noticeable impact on the microbiome and ASD children's behavior, but the results of diet modulation are conflicting. (3) Nevertheless, keeping the tested subjects on the same diet can help control this confounding factor.

Probiotics have also been used to model the GI system of ASD children into one that resembles a neurotypical gut. (36) In this review, three out of five intervention studies used probiotics as the testing factor and about eight (5,15,20,24,32,35,36,38) studies banned or controlled probiotic ingestion so it wouldn't affect sample collection. The three studies mentioned above (32,36,45) showed significant improvement of GI symptoms and children's behavior during the probiotic treatment, with only Mccartney et al (36) reporting the same behavioral improvements on the placebo group. Despite this small sample, with only 65 ASD children, the results obtained with probiotic feeding seem to be worth subsequent studies for confirmation and a better understanding of the pathophysiological mechanism at hand.

Another possible influence on microbiome composition is antibiotic usage. Sixteen (4,5,14–17,20,22,24,32,35,36,38,42,44,45) studies considered these antimicrobials as a confounding factor, banning them from use. However, the three exposure studies included in this review that considered antibiotic influence in the first years of life found no significant correlation of this practice with the incidence of ASD in the same subjects. Despite these conclusions, it seems reasonable to limit antibiotic usage during these studies, due to its short-term effects on microbiome composition.

The studies discussed in this review also show some discrepancies regarding the control groups. Ten (4,5,11,17,22,35,38–40,45) studies used sibling controls, arguing for a better management of confounding variables, like genetics and parental characteristics. However, according to Son et al (38), neurotypical siblings may have a higher prevalence of GI disorders than most children. This similarity between ASD subjects and their siblings raises the possibility of transmission of bacteria that can affect the composition of sibling microbiome. (5) However, a more direct explanation for the resemblance between ASD children and their siblings exist: having the same diet and living conditions and, of course, similar genetic influences. (40) The remaining reports used neurotypical community controls, with seven (12,20,24,32,33,37,45) of them using sex and age-matched controls. To achieve a higher elimination of bias, using both sibling and matched community controls seems like the most effective option.

As mentioned before, the microbiome is a vital link between gut and brain function, related to endocrine and neuroimmune pathways. This way, a dysbiotic microbiome affects human health in a myriad of ways.

Schizophrenia, like ASD, is a neuropsychiatric disorder with complex symptoms, a genetic association that, as of yet, points to a heavier environmental component in the pathogenesis and an association with GI disorders. Therefore, possible microbiome alterations are being studied regarding this condition as well. (26)

IBS pathogenesis has also shown to be related to microbiota changes, through multiple studies of fecal bacteria, suggesting a connection between autonomic nervous system changes in motility and intestinal permeability and these microbiome modifications. Parkinson's Disease is mainly a degenerative neurological disorder of the motor system but it can have "IBS-like symptoms", as mentioned by Martin et al. (25) This way, the microbiome causality that may apply to IBS can be studied as a means for early detection of this pathology.

In summary, there seems to be a clear link between psychiatric and some neurologic disorders and gastrointestinal comorbidities, through the "gut-brain axis". As mentioned by Rogers et al (52), SCFAs can stimulate the sympathetic and autonomic nervous system and reach the brain (with effects on behavior and development) and regulate the production of serotonin in enteroendocrine cells. This is a major link to psychiatric disorders, due to the high levels of serotonin present in ASD and depression. This way, "the microbiota–gut–brain axis is fully bidirectional, functioning in a manner through which changes in microbiota affect behavior, while conversely, changes in behavior brought about by chronic stress, genetic manipulation, or pharmacological intervention, result in alterations in microbiota composition.". (52)

Nevertheless, the two-way causality is not yet fully understood, but it is clear that the relationship between ASD pathophysiology and the microbiome is worth pursuing. (15) Two studies found that ASD severity was correlated with dysbiosis (4,37) and two others with GI symptoms. (4,14) In contrast, two studies found no connection between GI symptoms and ASD severity or dysbiosis (15,38), although one of them also found no changes in the ASD microbiome.

This review is limited mainly due to the heterogeneity of study designs, including the focus on different bacteria. The fact that the gut microbiome is still an ill-defined entity, with multiple expressions and influenced by many factors, adds to the complexity of this comparison. All the articles included in this review were based on the comparison of ASD and NT subjects' microbiome, but there was no consistency as to which bacteria should be studied to understand the possible differences. There are also many possible confounding factors when dealing with microbiota, like diet, probiotic and antibiotic use, as above mentioned. This review only selected articles written in English and, despite including studies from other sources, the only primary search performed was on the PubMed database.

# 5. Conclusions

The majority of studies that performed a standard comparison between neurotypical and ASD subjects' microbiome reported that it was significantly different. Conversely, there seems to be no consensus among these articles as to which bacteria define these differences. The most common bacteria altered bacterial group was *Clostridiales*, which was predominantly increased in ASD.

As for works classified as "Comparison by exposure", all three articles concluded neither early antibiotic exposure nor cesarean delivery had any significant impact in predicting an ASD diagnosis later in life.

Finally, the intervention reports with probiotics and vitamin A had mostly positive results, expect for Sandler et al (46). Their study did not yield the expected result due to the lack of long-term effects of vancomycin, but it was a reference for many posterior reports. Liu et al (44) also had mixed results, showing the effective impact of the probiotic in altering the microbiome but no significant changes in ASD severity.

# 6. Future perspectives

This review included works from many parts of the world (20,53), spanning several ethnicities and populations. In future research, it would be ideal to analyze the most varied population possible, residing in different countries, with diverse ethnicities and food patterns. These subjects could be studied as one total cohort and sub-cohorts organized by common characteristics, to find a biomarker common in all subjects or one for each basal microbiome profile. One of the difficulties of microbiome research is the varied definitions of a basal profile for either all human beings or subsets of people based on their ethnicity, geographical location or diet, for example. (54)

It should be noted that any finds that connect microbiota patterns to a neurological syndrome, like ASD can be used to build on research to other conditions.

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# Supplementary Material

Table S 1 - Quality assessment of case-control studies using the STROBE scale (27)

| | Plaza Díaz et al, 2019 | Strati et al, 2017 | Pulikkan et al, 2018 | Li et al, 2019 | Iovene et al, 2016 | Son et al, 2015 | De Angelis et al, 2013 | Tomova et al, 2014 | Park et al, 2013 | Wang et al, 2011 | Finegold et al, 2010 | Gondalia et al, 2012 | Parracho et al, 2005 | Adams et al, 2011 | Finegold et al, 2002 | Martirosian et al, 2018 | Finegold et al, 2017 | Luna et al, 2017 | Wang et al, 2013 | Song et al,2004 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **1. a)** | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **1. b)** | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| **2** | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| **3** | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| **4** | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| **5** | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| **6. a)** | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 0 |
| **6.b)** | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | na | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| **7** | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 0 |
| **8** | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| **9** | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **10** | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 |
| **11** | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| **12. a)** | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 |
| **12. b)** | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 |
| **12. c)** | na | na | na | na | na | na | na | na | na | na | na | na | na | na | na | na | na | 1 | na | na |

| | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **12. d)** | 0 | 0 | 0 | 0 | 0 | 0 | na | na | na | na | na | na | na | na | na | na | 0 | na | na | na |
| **12. e)** | na | na | na | 1 | na | na | na | na | 1 | na | na | na | na | na | na | na | na | na | na | 1 |
| **13. a)** | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 |
| **13. b)** | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 0 |
| **13. c)** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **14. a)** | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 |
| **14. b)** | na | na | na | na | na | na | na | na | na | na | na | na | na | na | na | na | na | 0 | na | na |
| **15** | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| **16. a)** | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| **16. b)** | na | na | na | na | na | na | na | na | na | na | na | na | na | na | na | na | na | na | na | na |
| **16. c)** | na | na | na | na | na | na | na | na | na | na | na | na | na | na | na | na | na | na | na | na |
| **17** | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| **18** | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| **19** | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 |
| **20** | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| **21** | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| **22** | 1 | 1 | 1 | 1 | na | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| **Total** | 89,3 % | 85,7 % | 82,1 % | 86,2 % | 81,5 % | 82,1 % | 77,8 % | 85,2 % | 85,7 % | 70,3 % | 77,8 % | 80,8 % | 85,2 % | 85,2 % | 70,3 % | 70,3 % | 67,9 % | 75,9 % | 66,7 % | 57,1 % |

1: yes; 0: no; na: not applicable; studies with a compliance percentage of the STROBE scale above 75% were considered to have good quality, studies with a compliance percentage between 50 and 75% were considered to have an average quality, studies with a compliance percentage below 50% were considered to have low quality

Table S 2 - Quality assessment of cohort studies using the STROBE scale (27)

| | **Axelsson et al, 2019** | **Hamad et al, 2018** | **Vargason et al, 2018** |
|---|---|---|---|
| **1. a)** | 1 | 1 | 1 |
| **1. b)** | 1 | 1 | 1 |
| **2** | 1 | 1 | 1 |
| **3** | 1 | 1 | 1 |
| **4** | 1 | 1 | 1 |
| **5** | 1 | 1 | 1 |
| **6. a)** | 1 | 1 | 1 |
| **6.b)** | na | na | 1 |
| **7** | 1 | 1 | 1 |
| **8** | 1 | 1 | 1 |
| **9** | 1 | 1 | 1 |
| **10** | 1 | 1 | 1 |
| **11** | 1 | 1 | 1 |
| **12. a)** | 1 | 1 | 1 |
| **12. b)** | 1 | 1 | 1 |
| **12. c)** | 1 | 1 | 1 |
| **12. d)** | 1 | 0 | na |
| **12. e)** | 1 | 1 | na |
| **13. a)** | 1 | 1 | 1 |
| **13. b)** | 1 | 1 | 1 |
| **13. c)** | 1 | 1 | 0 |
| **14. a)** | 1 | 1 | 1 |
| **14. b)** | 1 | 1 | 1 |
| **14. c)** | 1 | 1 | 1 |

| | | | |
|---|---|---|---|
| **15** | 1 | 1 | 1 |
| **16. a)** | 1 | 1 | 1 |
| **16. b)** | 1 | na | na |
| **16. c)** | 0 | 0 | 0 |
| **17** | 1 | 0 | 0 |
| **18** | 1 | 1 | 1 |
| **19** | 1 | 1 | 1 |
| **20** | 1 | 1 | 1 |
| **21** | 1 | 1 | 1 |
| **22** | 1 | 1 | 1 |
| **Total** | 97,0% | 90,6% | 90,3% |

1: yes; 0: no; na: not applicable; studies with a compliance percentage of the STROBE scale above 75% were considered to have good quality, studies with a compliance percentage between 50 and 75% were considered to have an average quality, studies with a compliance percentage below 50% were considered to have low quality

Table S 3- Quality assessment of non-randomized controlled trials using the TREND statement (28)

| | Liu et al, 2017 | Shaaban et al, 2017 | Kang et al, 2017 | Sandler et al, 2000 | Mccartney et al, 2010 |
|---|---|---|---|---|---|
| **1.1** | 0 | 1 | 0 | 0 | 1 |
| **1.2** | 1 | 1 | 1 | 1 | 1 |
| **1.3** | 1 | 1 | 1 | 1 | 0 |
| **2.1** | 1 | 1 | 1 | 1 | 1 |
| **2.2** | 1 | 1 | 1 | 1 | 1 |
| **3.1** | 1 | 1 | 1 | 1 | 1 |
| **3.2** | 0 | 0 | 1 | 0 | 1 |
| **3.3** | 1 | 1 | 0 | 0 | 0 |
| **3.4** | 1 | 1 | 0 | 1 | 1 |
| **4.1.1** | 1 | 1 | 1 | 1 | 1 |
| **4.1.2** | 1 | 1 | 1 | 1 | 1 |
| **4.1.3** | na | 1 | 1 | na | 1 |
| **4.1.4** | 0 | 0 | 1 | 0 | 0 |
| **4.1.5** | 0 | 0 | 0 | 0 | 0 |
| **4.1.6** | 0 | 1 | 1 | 1 | 0 |
| **4.1.7** | 1 | 1 | 1 | 1 | 1 |
| **4.1.8** | 0 | 0 | 0 | 0 | 0 |
| **5** | 1 | 1 | 1 | 1 | 1 |
| **6.1** | 1 | 1 | 1 | 1 | 1 |
| **6.2** | 1 | 1 | 1 | 1 | 1 |
| **6.3** | 1 | 1 | 1 | 1 | 1 |
| **7** | 1 | 1 | 1 | 1 | 1 |

| | | | | | |
|---|---|---|---|---|---|
| **8.1** | na | na | na | na | 1 |
| **8.2** | na | na | na | na | 1 |
| **8.3** | na | 1 | 1 | na | na |
| **9** | 1 | 0 | 0 | 0 | 1 |
| **10.1** | 1 | 1 | 1 | 1 | 1 |
| **10.2** | na | na | na | na | na |
| **11.1** | 1 | 1 | 1 | 1 | 1 |
| **11.2** | 1 | 1 | 1 | 1 | 1 |
| **11.3** | na | na | na | na | na |
| **11.4** | 0 | 1 | 0 | 0 | 1 |
| **12.1.1** | 1 | 1 | 1 | 1 | 1 |
| **12.1.2** | 1 | 1 | 1 | 1 | 1 |
| **12.1.3** | na | 1 | 1 | na | 1 |
| **12.1.4** | 1 | 1 | 1 | 1 | 1 |
| **12.1.5** | 1 | 1 | 1 | 1 | 1 |
| **12.2** | 1 | na | na | 1 | 1 |
| **13** | 1 | 0 | 1 | 1 | 1 |
| **14.1** | 1 | 1 | 1 | 1 | 1 |
| **14.2** | 1 | 1 | 1 | 0 | 1 |
| **14.4** | 1 | na | na | 1 | 1 |
| **14.4** | 0 | 0 | 0 | 0 | 0 |
| **15** | 1 | 1 | 1 | 1 | 1 |
| **16.1** | 1 | 1 | 1 | 1 | 1 |
| **16.2** | na | na | na | na | na |

| | | | | | |
|---|---|---|---|---|---|
| **17.1** | 1 | 1 | 1 | 1 | 1 |
| **17.2** | 1 | 1 | 1 | 1 | 1 |
| **17.3** | na | na | na | na | na |
| **18** | 1 | 1 | 1 | 1 | 1 |
| **19** | na | 1 | 1 | na | 1 |
| **20.1** | 1 | 1 | 1 | 1 | 1 |
| **20.2** | 1 | 1 | 1 | 1 | 1 |
| **20.3** | 1 | 1 | 1 | 1 | 1 |
| **20.4** | 0 | 0 | 0 | 0 | 0 |
| **21** | 1 | 1 | 1 | 1 | 1 |
| **22** | 1 | 1 | 1 | 1 | 1 |
| **Total** | 80,6% | 83,7% | 81,6% | 76,6% | 84,6% |

1: yes; 0: no; na: not applicable; studies with a compliance percentage of the STROBE scale above 75% were considered to have good quality, studies with a compliance percentage between 50 and 75% were considered to have an average quality, studies with a compliance percentage below 50% were considered to have low quality