# Recolonization of Microbiomes' Influence on Autism Spectrum Disorder

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## Abstract

In recent years, there has been an increase in the number of studies conducted on the complications of gastrointestinal (GI) comorbidities associated with autism spectrum disorder (ASD) in children. Some of these GI issues include irritable bowel syndrome (IBS), diarrhea and constipation. The causes of these disorders are poorly understood. The dominant gut phyla are *Firmicutes and Bacteroidetes*, *Actinobacteria, Proteobacteria, Fusobacteria and Verrucomicrobia*. Research conducted on children with ASD compared with typical developing (TD) children showed higher ratios of *Firmicutes/Bacteroidetes* in the ASD group. *Biflobacterium, Actinobacteria* phyla, has been shown to be a good intestinal bacterium that may help with GI abnormalities. Previous studies conducted reported that children with ASD had altered gut microbiota profiles at the genus and phylum level. These abnormal alterations in the gut lead to a buildup of toxins causing intestinal permeability, leaky gut syndrome, contributing to the symptoms seen in ASD. This thesis analyzes results of potential interventions to alleviate GI and behavioral symptoms of ASD. A microbiota transfer therapy (MTT) open label clinical trial conducted an experiment with 18 children diagnosed with ASD and reported an 80% reduction in GI symptoms. A decrease in the abundance of *Eubacterium coprostanoligenes*, Firmicutes phyla, was seen after treatment. Another study, a randomized, double blinded controlled trial examined 8 children with ASD and found that an oral probiotic, *Biflobacterium Infantis,* in addition to a Bovine Colostrum Product (BCP) treatment improved behavioral and GI symptoms of ASD, although temporarily. However, the results of these experiments don't have evidence of efficacy of the treatments long term and should be examined with caution in order to develop an intervention to improve ASD symptoms.

## Overview

The human gut microbiota is defined as microorganisms, mainly bacteria and archaea, that live in the digestive tracts of vertebrates. These tiny organisms play a major role in the determination of health outcomes as well as the pathogenesis of diseases. For several years now scientists have been investigating the casual role the microbiota plays on our brain, behavior and other pathways in the human body. A mice study performed in 2004 showed mice exhibited higher levels of hormones in response to changes in stress. To reverse the response, a bacterium, *Bifidobacterium*

*infantis,* was used. This indicated the microbiota may have a role in the regulation of the central stress response system.

Autism Spectrum Disorder is a developmental disorder that interferes with the ability to interact and communicate with others. This disorder is known for causing language and communication difficulties. There is no single identified cause of autism; over years it's been found environmental in addition to genetic factors that contribute to ASD. No cure is available for autism and current treatments may be frustrating for families and the children with the disorder since they don't fully treat the symptoms of the disorder. These treatments seek to reduce the symptoms of autism allowing the child to perform daily functions with ease.

Patients with autism have a higher incidence of GI problems associated with the disorder. Parents typically alter their children's diets to adjust for their GI issue, these children are typically also picky eaters. Studies have found children with autism have gut microbiota compositions distinct from children without ASD. Mice studies show the development of autism-like behavior when normal microbiota composition is absent or altered.

Probiotics are tiny living organisms such as yeast and bacteria. The "good bacteria" these probiotics are claimed to support gut health, behavior, focus and more. Studies testing probiotics with how well it works in improving ASD and GI symptoms typically use specific bacterial genera. Improvements in GI issues like constipation, abdominal pain and diarrhea, were improved following treatment.

Elimination diets are exactly what they sound like, this method removes certain foods from your diet that your body may not be able to tolerate well. In this case it was gluten, a gluten free diet was compared to gluten diet in children with all autism. After 8 weeks on a GFD, the results

weren't significant, and no major improvement was seen between the two group's behaviors, intellectual abilities or autism symptoms.

A new experimental treatment, called microbiota fecal transfer therapy, is being tested to determine if children's ASD symptoms are alleviated over time. With this new treatment, those with ASD gut microbiota are being recolonized with microbiota from donors who are not on the spectrum. A continuous study focused on the long-term outcomes of ASD and GI symptoms following completion of the fecal transfer. Following transfer, the scientist saw an improvement in ASD like symptoms compared to baseline pretransfer, social skills also improved. The composition of the microbiota in the gut of the recipients were altered post transfer. At the two-year review experiment, the bacteria diversity was the same as post treatment.

These newer alternative methods are promising but very limited in information. The surveys and rating scales used to determine the scores of the GI and ASD symptoms were filled out by parents and not the participants directly being studied. The elimination diet may cause nutrient deficiencies in the long run. Improvements seen from the methods also may have been from outside factors, such as behavioral therapy, and not the experiment itself.

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## Introduction

Microorganisms, such as bacteria and archaea, inhabit the human gastrointestinal (GI) tract and impact health. These microorganisms are referred to as gut microbiota or microbiomes and are referred to as one of the main regulatory factors in humans (Nicholson et al., 2012). The microbiomes that populate in the human GI tract are to an extent determined by our genes, environmental conditions and diet. These microorganisms are composed of a diverse community of archaea and bacteria, differing from person to person. The first exposure occurs at birth during delivery via the birth canal and when the mother breast feeds. After birth, and throughout life, the composition of microbiota is determined by factors such as one's diet, exercise, medications and comorbidities. As humans age, other factors such as diet and environment can alternate one's microbiota composition leading to favorable health outcomes or causing harm.

In the human gut, there are anywhere from 300-500 different species of bacteria. The dominant phyla of microbiota in the gut include *Firmicutes and Bacteroidetes* (both which represent an estimated 90% of all gut microbiomes), *Actinobacteria, Proteobacteria, Fusobacteria and Verrucomicrobia* (Rinninella et al., 2019) (**Fig 1**). Higher *Bacteroidetes/Firmicutes* ratio has been shown to be linked to gut dysbiosis, a disruption to the balance of the gut microbiota leading to unhealthy outcomes, as well as liver and immune disorders (Routy et al., 2018). *Bifidobacteria*, in the *Actinobacteria* phyla, has been shown to be a good bacterium in the intestinal tract, it is associated with healthy gut health. An abundance of abnormal compositions of microbiota leads to a buildup of toxins and bad bacteria causing the intestinal wall to become more permeable. The result of this leaky gut is bacteria and toxins being able to cross into the bloodstream. The colon is occupied with obligate anaerobes such as the few listed above, thriving in environments of low

oxygen. Majority of the microbes have a mutualistic symbiotic relationship with their host, benefiting both the human and the microorganism, while a small percentage are pathogens and promote disease. In a healthy individual, both mutualistic and pathogenic microbes can coexist without generating any complications that generate diseases in humans. Once there is a disturbance to this balance-use of antibiotics for an extended period, unhealthy diet or an illness- the body becomes vulnerable to disease as the mutualistic bacteria dies off, reducing competition with the pathogenic bacteria.

Over the years strong evidence has suggested an important bidirectional connection between these microorganisms in the gut and the nervous system. The gut-brain axis is used to describe this complex relationship between the central nervous system and the microbiota. The brain has direct influence of the microbiota under the influence of signaling molecules by the neurons and immune cells. It's been established that microbiota can modulate this axis through endocrine, nervous and immune pathways (Wang et al., 2019). In this experiment, a strain of *Bifidobacterium longum (B.longum)* was used to evaluate the effect it has on social stress. Health status and brain activity was measured over the course of 4 weeks, they found *B. longum* altered the neural activity at resting state.

Autism Spectrum Disorder (ASD) is a complex neurodevelopmental condition typically marked by communication and social deficits. ASD typically develops within the first 3 years of life affecting how the child communicates, interacts socially and their daily functions. A typical characteristic of ASD is repetitive patterns of behavior and a narrow range of interest, a child may become fixated on something such as the wheels on a car truck. Symptoms vary from person to person; some may not display any of the common traits. The term "spectrum" is a wide range and

refers to a large variation and severity of skills, levels of disability and symptoms. Adults and children with ASD may be fully capable of completing daily activities on their own or may require assistance to perform basic tasks. In order to diagnose ASD, a language impairment and a thorough assessment of an intellectual disability is required. The Autism Diagnostic Interview-Revised (ADI-R) may be used in conjunction with the Autism Diagnostic Observation Schedule (ADOS) to diagnose whether a child is on the autism spectrum and to plan treatment. The ADI-R uses a trained psychiatrist or psychologist to interview the caregivers of the child asking questions pertaining to the development including any milestones, speech/language development, medical history, past and present general behavior, aggressive or destructive behaviors. The score scale is from zero to three in each diagnostic areas, with three being an abnormal result. ADOS is a standardized assessment of a child's social interaction, play, communication, their use of imagination and repetitive behaviors. The specialist interacts directly with the child and observes them in 4 x 30 minute modules (Lord et al., 2000).

A commonality seen in individuals with ASD is a wide range of comorbidities, neurological and non-neurological. In a study conducted by Ivanovic, she aimed to estimate the prevalence of psychiatric comorbidities in children with ASD. She found the prevalence of the subjects having at least one psychiatric comorbidity was about 37.8% (Ivanovic, 2021). It's been shown children with autism spectrum disorder are 7 times more likely to have gastrointestinal (GI) complications compared with peers (Isaksen et al., 2013). This includes gastroesophageal reflux, bloating, flatulence, diarrhea, abdominal problems and chronic constipation. There is a prevalence of dietary and eating problems in an early association with ASD.

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Several studies have shown that there is an alteration of gut microbiota in individuals with ASD, their bacterial diversity and population differ from people without autism. This imbalance is also linked to the occurrence of diseases other than ASD, including TII diabetes, and obesity (Bull, Plummer., 2014). There is a lower abundance of beneficial bacterium, *Bifidobacterium* and *Akkermansia,* and a higher number of *Clostridia,* a potential pathogen in patients with ASD. A study by Zhang et al examined if children with autism have an altered composition of the *Bacteroidetes/Firmicutes* ratio*,* shown to be higher in children with autism with GI disorders, and lower amounts of *Bifidobacterium* when compared to typical developing peers. They used 16s rRNA gene sequencing, a tool used to identify and classify microbes in complex mixtures such as the human gut. They used this tool to compare the composition of the collected fecal microbiota and found a higher abundance of *Bacteroidetes/Firmicutes* in the ASD group.

As of right now the treatment options for ASD are limited. There is an approved treatment for ASD, instead a combination of ways that help to minimize the behavioral and GI symptoms associated with the disorder. The best treatment options vary from person to person. Interventions and diagnosis made earlier in life lead to more positive outcomes and reduced symptoms. Nonpharmacological interventions to address the symptoms of ASD include behavioral therapy. The goal of this therapy is to reinforce positive behaviors and suppress the negative behaviors, tracking the child's progress over time. Positive behavioral support works to figure out why the child is doing a specific behavior; in doing so, the environment may be altered, new skills may be taught to help the child behave more appropriately. Discrete trial teaching is done step by step to encourage the child to use new skills by using positive feedback. Early intensive behavioral intervention focuses on an individualized approach; the therapist provides one on one behavioral

instruction tailored to the child's needs. Speech language therapy is tailored to helping children interact and communicate needs and feelings with others. This therapy helps improve verbal or spoken skills. Nonverbal communication skills are also used to teach/improve hand signals and sign language as well as using graphics to communicate. Occupational and Physical therapists can help children with personal care skills such as getting dressed, eating and grooming. Their goal is to assist the child building fine motor skills, improve strength and posture. Education and school-based therapies for children with ASD are guaranteed free under the Individuals with Disabilities Education Improvement Act, IDEA. The teaching environments are required to be designed to fit the needs and skills of the child, minimize restrictions to interactions. This method works with parents to help design the appropriate plan for the child, setting individualized goals and providing a list of the other providers working in conjunction with the child.

Microbiota transfer therapy (MTT) is the transplantation of fecal microbiota from a healthy donor into the GI tract of a patient with the purpose of treating gut dysbiosis. Over the years, MTT is currently one of the most effective gut dysbiosis interventions used in the treatment of *Clostridium difficile* infection, liver and immune disorders (Routy et al., 2018; Wortelboer et al., 2019). Several studies involving microbiota fecal transfer therapy have shown promising results in treating autism symptoms in animal models. The researchers found mice colonized with ASD microbiota had begun to show hallmark traits of autism as alternative splicing genes were displayed indicating specific bacterial profiles modulates behaviors of ASD (Sharon et al., 2019). However, the mechanism on if, and how, MTT can alleviate ASD GI and behavioral symptoms in humans is poorly understood.

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Restriction diets have unclear conclusions on if they improve behavioral and gastrointestinal symptoms. A randomized trial including only children diagnosed with ASD sought to determine if a gluten free diet influences the functioning of patients with ASD. Over the course of 8 weeks, no difference in autistic and GI symptoms were seen between the gluten free diet and gluten diet groups (Piwowarczyk et al., 2020). Many children with autism are very selective and have an already restricted diet, removing gluten may further limit what they're able to eat. Probiotics administered are effective for neurobehavioral symptoms and bowel dysfunction. Bovine colostrum product (BCP) is complex milk oligosaccharides that serves as a prebiotic in the presence of bacteria and helps Bifidobacteria, a probiotic, grow. Probiotic-BCP combination treatment that has not been an area of focus in ASD patients.

In this thesis, I will analyze the effect of recolonizing microbiota in the gut by Microbiota Transfer Therapy and probiotic-BCP has on improving behavioral indices and GI symptoms in autism.


*Figure 1.Taxonomic gut microbiota composition examples. The box outlined represents all the microbiota in the phyla Firmicutes and Bacteroidetes (Emanuele et al., 2019).*

## Current Investigations

Although there are current therapies for autism, there is no cure and the mechanism in which autism is developed is poorly understood by researchers. Prior research has shown higher incidences of gastrointestinal disorders associated with severity of autism (Vargason et al., 2019).

Two recent studies have examined the connection between gut microbiota composition and gastrointestinal disorders in Autism patients. They examine potential treatments that alter the gut diversity and have been found to alleviate and improve GI issues and symptoms typically seen in autism patients.

The first study performed by Li and colleagues examined the efficacy of fecal microbiota transplantation (FMT) on gastrointestinal issues and autism symptoms relating to behavior in patients with ASD (Li et al., 2021). Their goal was to manipulate the gut microbiota by FMT and assess the effect it had on the composition, GI and behavioral problems in children with ASD. They also sought to examine the serum levels of neurotransmitters and how they were affected by FMT. This was an open label clinical trial that used 40 children between the ages of 3-17 years diagnosed with autism by the Autism Diagnostic Interview-Revised (ADI-R) and in addition had recurrent gastrointestinal problems. In an open label, both the participants and researchers are of the drug being administered, information is not withheld. For the control, 16 additional typical developing (TD) children, same age and sex matched and with a Bristol stool score of 4 were involved. These children didn't have GI disorders and weren't on any antibiotics a month prior to the experiment. Any children with fevers, severe allergies, mental disorders, extensive problems with feeding, using probiotics or antibiotics a week before the initial screening were excluded from the experiment to eliminate flaws. The study lasted 12 weeks, at week 0, physical examination was

conducted, blood samples were collected, stool samples were examined for 16s rRNA sequencing and assessments of GI and ASD symptoms were carried out. Blood samples were examined using ELISA to quantify the serum concentrations of the neurotransmitters, GABA, DA and 5-HT. GI symptoms were assessed by parents completing the Gastrointestinal Symptoms Rating Scale (GSRS) and Bristol Stool Form Scale. The GSRS is a 7-point scale to evaluate GI symptoms, where 1 means asymptomatic, 2 means slight, 3 is mild, 4 is moderate, 5 is moderate to severe, 6 is severe, and 7 is very severe. The Bristol Stool Score is a 7-point scale used by parents to classify stool consistency. A 1 or 2 is hard consistency, 5 or 6 is soft or running consistency, and a 4 is normal. ASD symptoms were evaluated by the Childhood Autism Rating Scale (CARS), Autism Behavior Checklist (ABC) and the Social Responsiveness Scale (SRS). The first 4 weeks were the FMT treatment phase. The participants in the TD group were just monitored without any treatments or interventions for the 12 weeks. ASD symptoms, GI symptoms, blood and stool were assessed at the end of week 4. Prior to each FMT the first 4 weeks, the children were given fraction doses of GOLYTELY to clean their colon. Only one donor's stool sample was used for every participant. Screening of this individual included a full review of their medical and social history, screenings for presence of bacteria pathogens, infections, liver and renal functions as well as lipid and fasting glucose levels. After the stool was collected under anaerobic and sterile conditions, samples were combined with sterile saline and homogenized. Filtration occurred to remove large particles prior to being centrifuged and used for capsules or colonoscopy transplantation. Children with ASD had the choice of their FMT administration being oral or a colonoscopic FMT. In the oral administration group, 27 children received freeze dried capsules. The remaining 13 children who weren't capable of swallowing capsules, received the colonoscopy. Stool samples were

collected each week over the 4-week treatment phase and a LEfSe analysis was conducted. The next 8 weeks were the follow up phase. At weeks 6, 8, 10 and 12 stool samples were collected and evaluated. Blood samples and GI symptom assessments were evaluated at weeks 8 and 12. ASD symptoms assessments were administered at the end of week 12. During the observation phase, alpha and beta diversities were evaluated between the TD and ASD groups, they found at the phylum and genus levels, there was a difference in the microbiota composition between the two groups. Alpha diversity is bacterial richness within a single sample, whereas beta diversity is the identify of taxa observed among samples within a habitat. After week 12, the unweighted unifrac distance was increased to the levels pre FMT. An unifrac is used as a distance metric to compare biological communities. An unweighted unifrac measures the distance between 2 communities by calculating the fraction of the branch length that leads to descendants in either community. The researchers found that after the fourth week children had overall improvement of GI symptoms evaluated using the GSRS indicated by the average GSRS score decreasing by 35% after FMT treatment. The occurrence of type 1,2 ,6 and 7 on the Bristol Stool Form Scale was also decreased significantly by the end of treatment (**Fig 2A, 2B**). Bristol stool scores were initially seen higher in the participants that had the colonoscopy compared to the oral group. After week 4, the scores of both groups were closer to 3 and 4 indicating an improvement from both groups. ASD symptoms were evaluated using ABC, SRS and CARS and saw a significant decrease in percentages (**Fig 2C, 2D, 2F**). Once again, the children in the colonoscopy transfer subgroup had a higher score compared to their counterparts, by week 4 and the remainder of the study no significant difference was observed. Furthermore, parents of the children with ASD anxiety levels were also recorded using SAS and found a decrease in anxiety as there was an improvement in their children's GI

symptoms. As for the serum neurotransmitters level, they found an increase in the level of dopamine and a decrease in the levels of GABA and 5-HT. The biggest changes were seen at week 4 after treatment, the levels did not change further and seemed to shift back closer to their original values. A positive correlation was seen with GABA and Bristol Stool Score, while 5-HT had a negative correlation with the Bristol Stool Score indicating serum neurotransmitter alteration may have an impact on symptoms in children with ASD.

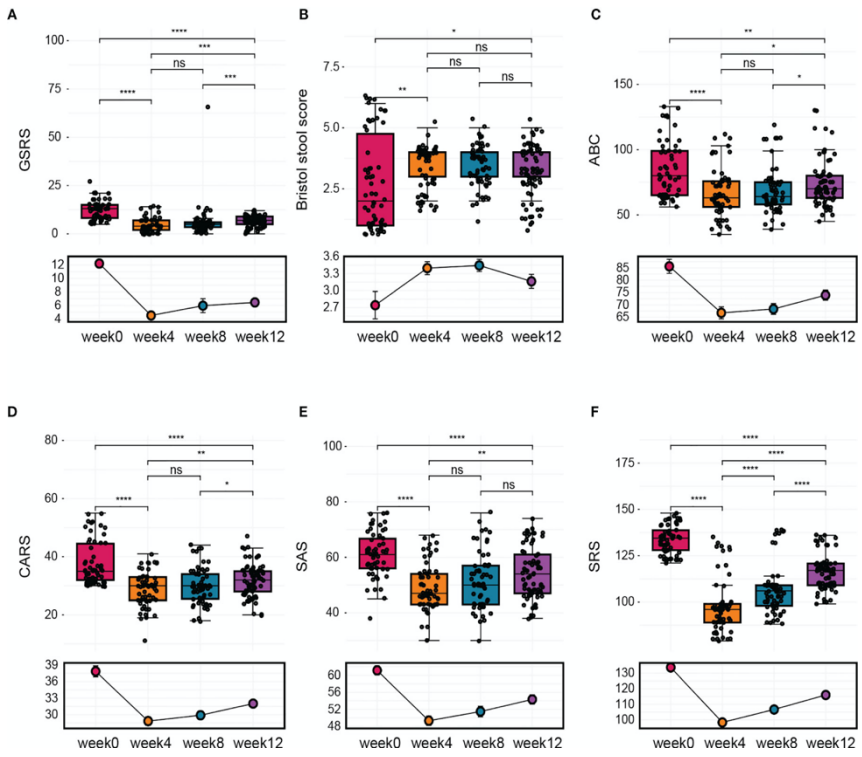


**Figure 2** At the beginning of the study a baseline score of GI symptoms and ASD symptoms were evaluated, they were reevaluated after FMT treatment at week 4 and 8 weeks after. An average of the scores were reported in the data above. **(A)** Evaluation of the changes in the Gastrointestinal Symptoms Rating Scale (GSRS) scores reported by parents prior to the experiment, after the treatment (week 4), 4 weeks and 8 weeks after the end of the treatment. Significant decrease seen at week 4, the value stays steady but slowly rising back to baseline. **(B)** Assessment of the Bristol Stool Score at baseline, after the treatment, 4 and 8 weeks after end of treatment. Significant increase seen at week 4, slow approach back to baseline at weeks 8 and 12. **(C)** Scores of Autism Behavior Checklist (ABC), evaluates the 5 common areas of problem behaviors in children with ASD. Data shown at baseline, after treatment and 4 weeks and 8 weeks post treatment. A significant decrease is seen at week 4, steady increase towards baseline seen at weeks 8 and 12. **(D)** Childhood Autism Rating Scale (CARS), 15 item scale that assesses severity of symptoms and diagnoses ASD, before treatment, right after treatment and 4 and 8 weeks after treatment ended. Significant jump after treatment at week 4, steady incline back to baseline at weeks 8 and 12. **(E)** Total Self-Rating Anxiety Scale (SAS), used by the parents to assess their anxiety, score at baseline, after treatment week 4, weeks 8 and 12. Biggest decrease seen at week 4, scores inch back towards original value. **(F)** Results of Social Responsiveness Scale (SRS) score at baseline, end of treatment, 4-week post treatment and 8 weeks post treatment follow ups. Largest drop in score at week 4, data reveres weeks 8 and 12. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001, ns=no significance.

A second systematic and controlled study by Sanctuary and colleagues was conducted with a combination of a probiotic and prebiotic supplementation to examine tolerability in children with autism and have GI disorders (Sanctuary et al., 2019). Their goal was to assess how tolerable the probiotic *Bifidobacterium infantis (B. infantis)* is in addition with bovine colostrum product (BCP) compared to BCP alone would be in evaluating GI comorbidities, microbiome and immune factors in ASD patients. This trial was randomized, double blind and controlled involving 8 subjects between the ages of 2-11 years diagnosed with ASD based on Autism Diagnostic Observation Schedule (ADOS) with a history of frequent GI symptoms that included constipation, diarrhea, abdominal pain or irritable bowel syndrome based on the Pediatric Gastrointestinal Symptoms-Rome III Version (QPGS-RIII) questionnaire diagnostic criteria (Lord et al., 2000; Walker, Caplan., 2006). Children who had other co morbid disorders, GI disease, milk allergy, compromised immunity, on antibiotics or medically prescribed diets were excluded from the study. The total length of the experiment was 12 weeks, divided into 5 weeks of probiotic-prebiotic supplements (*B. infantis* and BCP), a washout period for 2 weeks and 5 more weeks of prebiotic only supplementation (BCP). At baseline, or week 0, the CHARGE Gastrointestinal History (GIH) survey, Aberrant Behavior Checklist (ABC), Adaptive Behavior Assessment System (ABAS II), and Repetitive Behavior Scale-Revised (RBS-R) was used by parents to evaluate pre and post intervention values. Stool samples were also collected and classified based on the Bristol Stool Scale. Urine and blood samples were collected, and a medical examination was carried out by a physician. The participants served as their own control, having both treatments random in the order in which they obtained it. During the 5 weeks, the treatments were given in powder form, both

tasting the same in order to preserve the identity and avoid bias. The BCP used was Imucon, administered twice a day at 50 g. Parents were instructed to keep their children's diets' consistent and mix the powdered supplement with a cold food item as part of their diet. At the end of week 5, the questionnaires, blood, urine and stool collection were completed again. After the first 5-week arm, there was a washout period lasting 2 weeks when there were no treatments administered. The same measures that were tested at baseline were once again tested at week 7. The second 5-week arm consisted of the subjects undergoing the treatment they did not receive the first time and the same steps from the first five weeks are repeated. Microbiota communities were submitted to a genome center for examination from fecal DNA extraction provided from the stool samples. The blood samples were used to measure the changes in peripheral blood mononuclear cell cytokine expression. Researchers observed minimal side effects from both treatments. Based on the QPGS-RIII and GIH questionnaires along with the parental reporting, 87.5% of participants had improvement of some GI symptoms when on the Imucon only and 100% of participants had some improvement on the combination. However, when the parents were asked their opinion on the treatment, they saw greater GI improvements on, 75% reported on the Imucon only while 25% reported the combination treatment. There was a significant reduction of pain seen by both treatments, and reduction of diarrhea by the BCP only treatment **(Fig 3)**. The stool log saw an increase to normal consistency on the Bristol Scale after treatments. After the experiment ended, 87.5% of parents reported their children's GI symptoms began to return. A large reduction was seen in aberrant behaviors according to the ABC questionnaire, no differences in repetitive behaviors or adaptive behaviors were seen based on the RBS-R and ABAS-II questionnaires. Fecal microbiota composition was found to be the same throughout the study, a lack of changes to the

gut diversity. Four enterotypes were identified at genus level throughout the study, *Prevotella, Bifidobacteria, Bacteroides* and mixed. No treatment effect was found on the genus level. Lastly, an overall reduction of cytokines by CD4+ and CD8+ T cells were observed in intracellular expression. After combination treatment, the CD4+/IL-13+ T cells frequency was lower in stimulated cells. With BCP only treatment, CD8+/TNF-alpha T cells frequency was reduced as well.

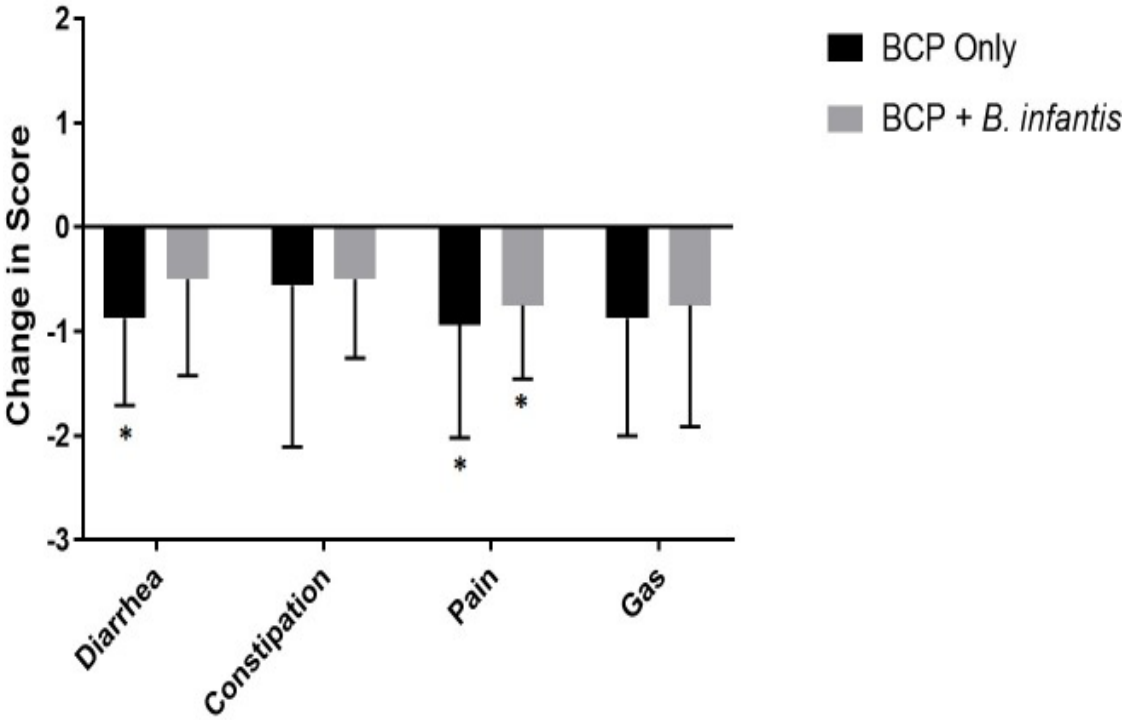


**Figure 3.** Based on the CHARGE Gastrointestinal History survey. Frequency of gastrointestinal symptoms reported by parents post experiment in both BCP only and BCP plus *B. infantis* groups. Significant reduction of pain seen by both treatments, and reduction of diarrhea by the BCP only treatment. Mean +/- SD change based on Likert scale where 0 means symptom never occurs and 4 means it always occurs. *P<0.05 denotes significant improvement, n=7 diarrhea/group, n=5 constipation/group, n=5 pain/group, n=6 gas/group.

## Discussion

Recent data presents the correlation between gut microbiota and the occurrence of autism spectrum disorder. This new evidence has shown the significance of the role the gut brain axis has on the different biological pathways in the body. In addition to the common social and communication barriers with individuals with autism, it's known that a high percentage also have gastrointestinal problems (Mulloy et al., 2009). In these trials, to better understand how recolonizing gut microbiota was able to improve behavioral and gastrointestinal (GI) incidences, Microbiota Transfer Therapy (MTT) was performed orally and by colonoscopy in the first study and a probiotic-BCP combination treatment in the latter (Li et al., 2021; Sanctuary et al., 2019).

In the study performed by Li and colleagues, they assessed the effect MTT has on intellectual disability and GI disorders of ASD children. In the follow up phase, after week 4, they observed improvements in GI symptoms such as abdominal pain, constipation, diarrhea and reflux and the GSRS scores were reduced.

Within the ASD group, there were 2 subgroups; the children that received the treatment orally and the others that had a colonoscopy. There were differences seen in the baseline between the two subgroups. The rectal group had higher Bristol stool scores, around 6 and 7 which means soft or liquid stools, indicating these children had diarrhea. They also had higher baseline ABC, CARS, and GSRS scores leading the scientists to believe those in this group had more severe GI and behavioral symptoms than the oral groups and weren't capable of swallowing capsules leading to the differences seen in the baseline. The oral subgroup had lower Bristol stool scores, around 1 and 2, indicating they had constipation. During the follow up phase of the experiment, there were no significant differences in GI or behavioral symptoms observed between the two groups

stipulating that there is equity between the two treatment routes. The Bristol stool scores of the subgroups were also in the middle around the normal range.

Past investigations have shown the connections between the microbiota composition and the effect it has on the GBA. Abnormal concentrations, or gut dysbiosis, has been seen to cause intestinal permeability causing metabolites or toxins to cross the blood-brain barrier and modulate cerebral function (Fung et al., 2017). This may contribute to the nervous system and GI complications seen in ASD (Hughes et al., 2018). In this study, during the post treatment phase, weeks 8-12, there was an increase in the dopamine (DA) serum levels and a decrease in the gamma-aminobutyric acid (GABA) and 5-HT serum levels, eluding MTT has a therapeutic effect on ASD. MTT may be able to regulate neurotransmitters that in turn modulate the CNS through the gut brain axis (GBA). GABA is the chief inhibitory neurotransmitter in humans, levels in the serum were found to be increased in ASD children compared to TD children (Marotta et al., 2020). During the experiment, they found MTT may not have affected the structure overall of the gut microbiota since the alpha diversity was not significantly changed or different from the TD group. 16S rRNA sequencing was used to identify that gut microbiota composition at the phylum and genus levels were different between the two groups, and gut dysbiosis was observed in the ASD group. This may have been due to a variation in the abundance of microorganisms in the microecology of the intestinal tract being disrupted. Since pre-existing landscape is known to impact the outcome of MTT, children in the ASD were separated into two groups, responders and non-responders, based on the amount of reduction in their GSRS score. Children that received less than 50% average reduction were classified as non-responders. Responders were found to have a lower abundance of *Eubacterium coprostanoligenes* improving their GI symptoms. *Eubacterium*

*coprostanoligenes* may be a potential regulator of the response of MTT. A positive correlation was seen in the reduction of *Eubacterium coprostanoligenes* and the reduction of GI symptoms indicating that a change in bacterial profile may lead to clinical improvements. Additionally, *Eubacterium coprostanoligenes* had a negative correlation with GABA serum levels indicating a potential influence the bacteria may have on neurotransmitters and an improvement in ASD behavioral symptoms.

Overall, the treatment was well tolerated, and effective in improving GI and behavioral symptoms in children with ASD. After treatment was over, the scores all remained consistent but slowly began to creep back to the baseline **(Fig 2A, 2B, 2C, 2D, 2E).** This may indicate that continuous treatment may need to continue for an extended period to achieve long lasting results.

In the second study conducted by Sanctuary and colleagues, a probiotic-prebiotic combination was used to analyze the improvement of ASD symptoms. The probiotic, *Bifidobacterium infantis (B. infantis),* has been known to improve gut health (O'Callaghan, Sinderen., 2016). Bovine Colostrum Product promotes growth of this probiotic by the milk oligosaccharides it contains, its abundance of immune proteins such as immunoglobulins and cytokines (Brooks et al., 2006). In this experiment, there were 8 total participants with ASD randomly assigned to the BCP, Imucon, or *B. infantis*-Imucon combination. The study had a crossover design; for the first 5 weeks, the children were given the BCP only supplement or the *B. infantis* + BCP combination treatment. The following 2 weeks was a washout period, followed by 5 weeks on the supplement they didn't receive the first time. There was high tolerability reported in each child, only mild side effects including gassiness at the beginning of treatment. Based on the results from the QPGS-RIII, GIH

questionnaires and the parent reports, there was 75% GI improvement seen with the BCP only treatment compared to 25% improvement in the combination treatment. A decrease in aberrant behaviors and diarrhea was also observed with the BCP only treatment. This result is consistent with the higher percentage of improvement seen with the GI incidences. The reason for this outcome may be that the colostrum is also modulating the growth of bacteria other than *B. infantis* in the gut. When BCP is added with the probiotic, the growth of another bacterium is not as effective. Both treatments saw a decrease in the occurrence of pain **(Fig 3)** and stool scores were around 4, the normal number, over the course of treatment.

The participants displayed high abundance of Prevotella, bifidobacterium, bacteroides or a mix, no consistency was seen in their gut microbiota. Their gut diversities were most like themselves after treatment. No significant changes to the gut microbiota were observed indicating the GI improvements weren't due to changes in the profile of the gut flora. This means the gut microbiome is affected by diet and can change within an individual. Something to consider is the children each began the treatments in different microbial states. The purpose of the washout phase was to bring the microbiota community close to their baseline; some of the subjects' gut profiles may not have returned to baseline after the washout phase. Recent randomized trial found that probiotic supplementation has shown beneficial effects in healthy individuals without changing the fecal microbiota composition (Kristensen et al., 2016). This means probiotics may not be beneficial for altering gut composition, but instead stabilizing the gut microbiota already present.

Global changes in fecal, urinary and metabolite profiles weren't observed, but a reduction in fecal ethanol and methanol levels were seen. There has been a prevalence of GI yeast infections seen in children with autism (Jyonouchi et al., 2005). The initial high fecal levels may be explained

by an overgrowth of yeast in the gut, the levels were reduced due to treatment. This would explain the improvement in GI symptoms.

There was a significant reduction of CD4+ T cells, helper T lymphocytes, after the combination treatment. The importance of this cytokine is the role it plays in allergic responses and children with ASD are known to report more food allergies alluding to the reduction of symptoms seen in the results of this experiment. Children with ASD are reported to have elevated amounts of TNF alpha express (Jyonouchi et al., 2005). After the BCP only treatment, a reduction in the CD8+ T cells were seen. CD8+ T cells are cytotoxic T cells that express TNF alpha. A reduction in these cells means the bovine colostrum was able to decrease gut pro inflammatory cytokines and improve GI function.

Treatments in the future need to consider children with ASD may be sensitive to dairy so BCP may not be appropriate treatment. There was no placebo group to compare results with, the children served as their own control to eliminate potential flaws.

In conclusion, the results were consistent with previous studies regarding the gut profile of children with ASD. The research here further supports that probiotic administered are effective for neurobehavioral symptoms and bowel dysfunction. MTT is tolerated as an effective method in the improvement of GI symptoms and ASD behaviors. Future studies may seek to obtain more data by using a wider range of patients with autism. The limited sample size and short length of both experiments supports the need for larger and longer randomized controlled trials to be conducted parallel to current research to investigate if the current findings hold up on a larger scale.

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