Universidade Estadual Paulista
"Júlio de Mesquita Filho"

Faculdade de Ciências Farmacêuticas

Efeito de Psicobióticos no Transtorno do Espectro Autista

Ana Luiza Rocha Faria Duque

Tese apresentada ao Programa de Pós-graduação em Alimentos e Nutrição para obtenção do título de Doutora em Alimentos e Nutrição.

Área de concentração: Ciências Nutricionais

Orientadora: Profa. Dra. Katia Sivieri

Araraquara
2021

Efeito de Psicobióticos no Transtorno do Espectro Autista

Ana Luiza Rocha Faria Duque

Tese apresentada ao Programa de Pós-graduação em Alimentos e Nutrição para obtenção do título de Doutora em Alimentos e Nutrição.

Área de concentração: Ciências Nutricionais

Orientadora: Profa. Dra. Katia Sivieri

Araraquara
2021

Duque, Ana Luiza Rocha Faria.
D946e Efeito de Psicobióticos no Transtorno do Espectro Autista / Ana Luiza Rocha Faria Duque. – Araraquara: [S.n.], 2021.
99 f. : il.

Tese (Doutorado) – Universidade Estadual Paulista. "Júlio de Mesquita Filho". Faculdade de Ciências Farmacêuticas. Programa de Pós Graduação em Alimentos e Nutrição. Área de Concentração em Ciências Nutricionais.

Orientadora: Katia Sivieri.

1. Autismo. 2. Probiótico. 3. Microbiota intestinal. 4. Prebiótico. 5. Eixo microbiota-intestino-cérebro. I. Sivieri, Katia, orient. II. Título.

Diretoria do Serviço Técnico de Biblioteca e Documentação - Faculdade de Ciências Farmacêuticas
UNESP - Campus de Araraquara

CAPES: 33004153070P3
Esta ficha não pode ser modificada

CERTIFICADO DE APROVAÇÃO

TÍTULO DA TESE: Efeito de Psicobióticos no Transtorno do Espectro Autista

AUTORA: ANA LUIZA ROCHA FARIA DUQUE
ORIENTADORA: KATIA SIVIERI

Aprovada como parte das exigências para obtenção do Título de Doutora em ALIMENTOS E NUTRIÇÃO, área: Ciências Nutricionais pela Comissão Examinadora:

Profa. Dra. KATIA SIVIERI (Participação Virtual)
Departamento de Ciências Biológicas / Faculdade de Ciências Farmacêuticas - UNESP- Araraquara

Profa. Dra. MARCIANE MAGNANI (Participação Virtual)
Departamento de Engenharia de Alimentos / Universidade Federal da Paraíba

Profa. Dra. TATIANA COLOMBO PIMENTEL (Participação Virtual)
Eixo de Produção Alimentícia / Instituto Federal do Paraná – Campus Paranavaí

Profa. Dra. SUSANA MARTA ISAY SAAD (Participação Virtual)
Departamento de Tecnologia Bioquímico-Farmacêutica / Faculdade de Ciências Farmacêuticas da USP

Araraquara, 27 de agosto de 2021

Agradecimentos

À Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) pelas bolsas de Doutorado (Processo nº 2017/01157-1) e de Estágio de Pesquisa no Exterior (BEPE) (Processo nº 2018/26645-1).

À Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) pelo apoio financeiro - Código de Financiamento 001.

À minha orientadora Profa. Dra. Katia Sivieri pela gentileza, paciência, disposição e apoio durante todo o projeto. Sou imensamente grata pelo aprendizado e conhecimento compartilhado durante todos esses anos.

Ao meu orientador Prof. Dr. Mauro Costa-Mattioli pela incrível oportunidade de trabalhar junto ao seu laboratório na Baylor College of Medicine. Agradeço por todo carinho e apoio durante a colaboração internacional.

A todos os membros do laboratório, em especial, à Adriana Zavarizi pela prontidão no auxílio de técnicas laboratoriais durante a execução dos experimentos.

A todos os membros do laboratório dos Estados Unidos, em especial, à Laura Ortiz, Martina Sgritta, Sean Dooling, I-Ching Wang e James Okoh pela acolhida no laboratório, oportunidade de aprender diferentes técnicas e contribuições científicas.

À Dra. Maria Angela Adorno do Departamento de Engenharia Ambiental da EESC/USP pelo auxílio nas análises de ácidos graxos de cadeia curta, à Me. Mariana Santoni do Departamento de Ciências Biológicas da FCFAr/UNESP pelo auxílio nas análises de qPCR e à Dra. Victoria Mesa pelo auxílio nas análises de bioinformática.

Aos professores e funcionários da UNESP e da Baylor College of Medicine pelos importantes ensinamentos e apoio, em especial à Claudia Molina e Marina Grasso.

Aos membros da banca examinadora pela disponibilidade e por todas as valiosas contribuições.

À FrieslandCampina Ingredients Latin America Ltda, em especial, Marcio Del Bianco, Marília Dorsa e Leonardo Appezato Filho pelo fornecimento do produto Vivinal® GOS utilizado nesse trabalho.

Agradecimentos Pessoais

Aos meus pais, Iara e Venceslau Braz, e irmãos, Tiago, Gustavo e Alex, por todo carinho, incentivo e conselhos dados ao longo da minha vida.

Ao Tiago Izeppi por todo seu amor, parceria e cumplicidade. Agradeço pela felicidade de ter alguém tão especial ao meu lado.

Aos meus queridos amigos Mateus Salgaço, Estela Mesquita, Júlia Valério, Amanda Fernandes, especialmente, Fernanda Freire, Fernanda Bianchi e Larissa Celiberto pelo incentivo e apoio durante os momentos que passamos juntos.

Aos amigos Laura, Emily, Lance, Anu e Oykü pela recepção calorosa nos Estados Unidos.

Aos meus pais, por todo amor e apoio.

Resumo

O eixo microbiota-intestino-cérebro foi recentemente reconhecido como um modulador chave de distúrbios neurológicos, incluindo o Transtorno do Espectro Autista (TEA). Evidências crescentes sugerem que a microbiota intestinal desempenha papel crítico nos sintomas gastrointestinais e déficits comportamentais em indivíduos autistas. Nesse sentido, a administração de psicobióticos (probióticos e prebióticos) tem sido investigada como uma terapia promissora no tratamento do TEA. **Objetivo:** Avaliar o efeito de psicobióticos na composição e metabolismo da microbiota intestinal de crianças com TEA usando o Simulador do Ecossistema Microbiano Humano (SEMH®), bem como na microbiota intestinal e déficits comportamentais em um modelo animal de TEA. **Métodos:** Inicialmente, foi avaliada a sobrevivência de cepas probióticas (*Limosilactobacillus* (*L*.) *reuteri* e *Bifidobacterium* (*B*.) *longum*) em cultura pura e combinadas com Vivinal® GOS sob condições gastrointestinais simuladas e, em seguida, o impacto dos probióticos (*L. reuteri* + *B. longum*), prebiótico (GOS) e simbiótico (*L. reuteri* + *B. longum* + GOS) na microbiota intestinal e no metabolismo de crianças com TEA usando o SEMH®. A análise da composição da microbiota intestinal das amostras do SEMH® foi realizada por PCR em tempo real e sequenciamento do gene 16S rRNA. O metabolismo microbiano foi determinado pela produção de amônia e ácidos graxos de cadeia curta (AGCC). Finalmente, foi investigada se a administração do GOS afetou a composição da microbiota e o comportamento em um modelo genético de autismo (*Cntnap2*$^{-/-}$). A composição da microbiota dos animais foi avaliada pelo sequenciamento do gene 16S rRNA e os comportamentos associados ao TEA foram determinados por testes de hiperatividade e de comportamentos social e repetitivo. **Resultados:** Os probióticos *L. reuteri* + *B. longum* combinados com GOS apresentaram elevada resistência gastrointestinal, sugerindo que o GOS tem um efeito protetor. De acordo com o estudo no SEMH®, o tratamento probiótico aumentou a abundância relativa de *Lactobacillus*, enquanto o tratamento prebiótico aumentou a abundância relativa de *Bifidobacterium* e diminuiu a abundância relativa de *Lachnoclostridium*. As mudanças observadas no metabolismo microbiano foram associadas ao aumento das concentrações de AGCC e níveis reduzidos de amônia, particularmente nos tratamentos prebiótico e simbiótico. No estudo em modelo animal, foi observado que o tratamento com GOS não modulou a composição da microbiota. No entanto, melhorou significativamente o comportamento social no modelo animal *Cntnap2*$^{-/-}$. **Conclusão:** Os tratamentos probiótico, prebiótico e simbiótico resultaram na modulação positiva da microbiota intestinal e atividade metabólica de crianças com TEA usando o SEMH®. Além disso, os resultados mostraram que o GOS melhorou os déficits sociais em um modelo animal de autismo.

Palavras-chave: Autismo; Probiótico; Microbiota intestinal; Prebiótico; Eixo microbiota-intestino-cérebro.

Abstract

The microbiota-gut-brain axis has been recently recognized as a key modulator of neurological disorders, including the Autism Spectrum Disorder (ASD). Increasing evidence suggests that the gut microbiota plays a crucial role in gastrointestinal symptoms and behavior deficits in autistic individuals. Thus, the administration of psychobiotics (probiotics and prebiotics) has been investigated as a promising therapy for the treatment of ASD. **Objective:** To evaluate the effect of psychobiotics on gut microbiota composition and metabolism of children with ASD using the Simulator of the Human Intestinal Microbial Ecosystem (SHIME®), as well on gut microbiota composition and behavior deficits in a mouse model for ASD. **Methods:** Initially, the survival of probiotic strains (*Limosilactobacillus* (*L.*) *reuteri* and *Bifidobacterium* (*B.*) *longum*) in pure culture and combined with Vivinal® GOS under simulated gastrointestinal conditions was evaluated, followed by the impact of probiotics (*L. reuteri* + *B. longum*), prebiotic (GOS) and synbiotic (*L. reuteri* + *B. longum* + GOS) on the gut microbiota and metabolism of children with ASD using the SHIME®. The analysis of the gut microbiota composition of samples from the SHIME® was performed using real-time PCR and 16S rRNA gene sequencing. Microbial metabolism was determined by the production of ammonium and short-chain fatty acids (SCFAs). Finally, it was investigated whether administration of GOS affected microbiota composition and behavior in a genetic model of autism (*Cntnap2*$^{-/-}$). Gut microbiota composition was evaluated using 16S rRNA gene sequencing and ASD-related behaviors were determined by hyperactivity, and social and repetitive behavior tests. **Results:** The probiotics *L. reuteri* + *B. longum* combined with GOS showed elevated gastrointestinal resistance, suggesting that GOS has a protective effect. According to the SHIME® study, probiotic treatment increased the relative abundance of *Lactobacillus*, while the prebiotic treatment increased the relative abundance of *Bifidobacterium* and decreased the relative abundance of *Lachnoclostridium*. The changes observed in microbial metabolism were associated with increased concentrations of SCFAs and reduced ammonium levels, particularly in the prebiotic and synbiotic treatments. In the animal model study, it was observed that treatment with GOS did not modulate the microbiota composition. However, GOS significantly improved social behavior in the *Cntnap2*$^{-/-}$ mice. **Conclusion:** The probiotic, prebiotic, and synbiotic treatments resulted in a positive modulation of the gut microbiota and metabolic activity of children with ASD using the SHIME®. In addition, the results showed that GOS improved social deficits in a mouse model of autism.

Keywords: Autism; Probiotic; Gut microbiota; Prebiotic; Microbiota-gut-brain axis.

Lista de Tabelas

Página

Capítulo 1.

Lista de Figuras

Página

Introdução

Capítulo 1.

Capítulo 2.

Sumário

Introdução

O trato gastrointestinal humano abriga uma comunidade dinâmica e complexa de microrganismos, conhecida como microbiota intestinal, que coevoluiu com o hospedeiro para desenvolver uma relação simbiótica (1). A colonização da microbiota ocorre nos primeiros anos de vida e gradualmente se desenvolve em um ecossistema diverso ao longo da vida adulta. O desenvolvimento e a maturação da microbiota intestinal são influenciados por diferentes fatores que variam desde o tipo de parto (cesariana versus vaginal) ao uso de antibióticos, genética do hospedeiro e dieta (2,3) (Figura 1). Estudos têm revelado que a microbiota intestinal desempenha papel chave em várias funções fisiológicas do hospedeiro, incluindo a homeostase metabólica e nutricional, a maturação e função do sistema imunológico e a manutenção da integridade da barreira intestinal (5-7). Além disso, foi demonstrado que a microbiota intestinal tem um impacto significativo extra intestinal, como na neurofisiologia e no comportamento (8,9).

Evidências crescentes sugerem uma interação entre a microbiota intestinal e o sistema nervoso central (SNC) conhecida como eixo microbiota-intestino-cérebro (10-13). A comunicação ao longo do eixo microbiota-intestino-cérebro descreve como os sinais a partir do cérebro podem influenciar atividades motoras, sensoriais e secretoras do trato gastrointestinal, e, por sua vez, como as mensagens viscerais do trato gastrointestinal podem influenciar a função cerebral (14). Esta comunicação bidirecional atua, principalmente, por meio de mecanismos endócrinos, imunes e neurais que envolvem o SNC e o sistema nervoso entérico (SNE) (12,15). No entanto, os mecanismos precisos ainda não estão completamente elucidados. As vias de comunicação entre a microbiota e o cérebro incluem a ativação do nervo vago, sistema imunológico, eixo hipotálamo-pituitária-adrenal (HPA), o metabolismo do triptofano e a

produção de neurotransmissores e metabólitos microbianos que influenciam direta e indiretamente o cérebro (Figura 2).

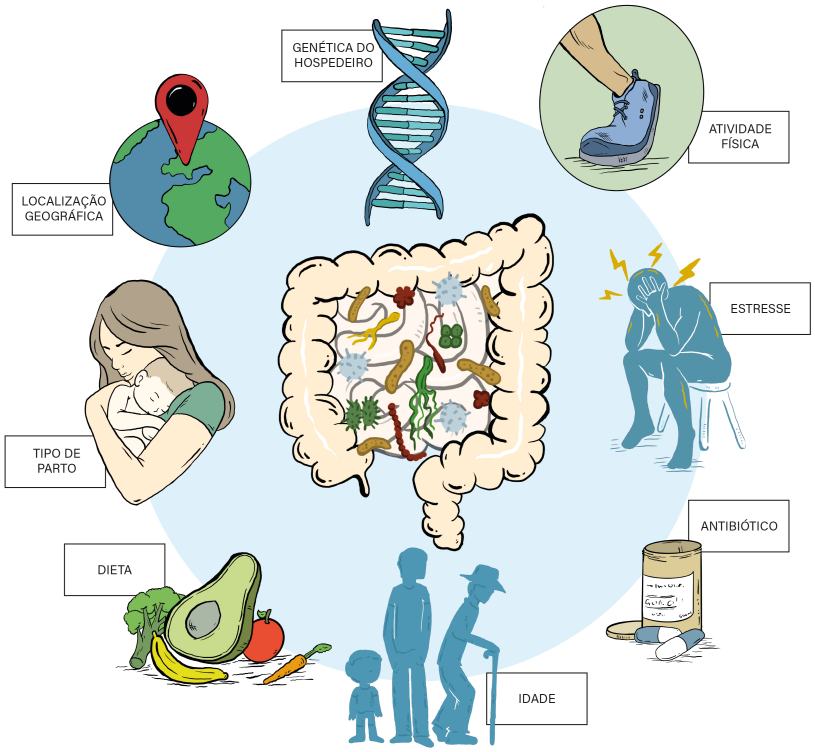

Figura 1. Fatores que influenciam a microbiota intestinal.
Fonte: Adaptado de Long-Smith et al. (4).

O nervo vago representa a principal via neural que conecta o intestino e o cérebro. As fibras do nervo vago inervam as camadas musculares e mucosas do trato gastrointestinal, detectam sinais sensoriais e, em seguida, retransmitem esses sinais para o SNC (16). A transmissão de sinais microbianos ocorre por meio da mediação de células enteroendócrinas ou pela ativação de mecanorreceptores ou quimiorreceptores no epitélio intestinal, desencadeados por estímulos químicos como hormônios, neurotransmissores e metabólitos produzidos pela microbiota intestinal (17,18).

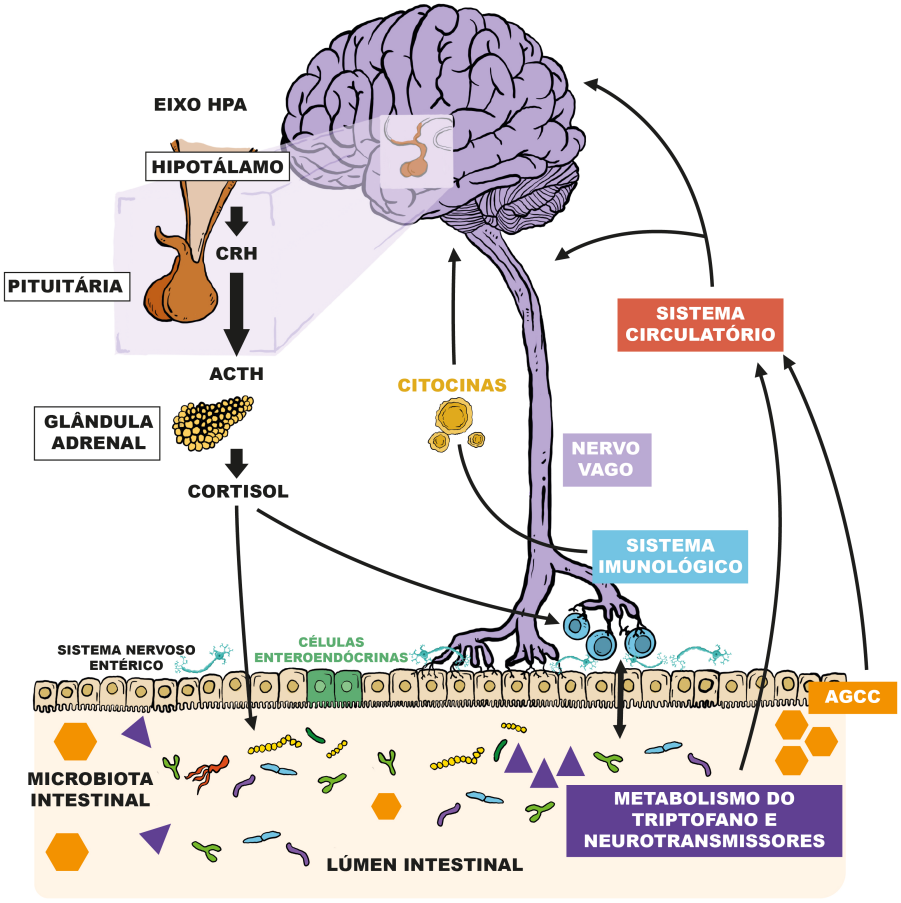

Figura 2. Mecanismos de comunicação do eixo microbiota-intestino-cérebro.

O estresse pode ativar a resposta do eixo HPA que envolve a síntese e liberação de cortisol. O cortisol regula as respostas de sinalização neuroimune que, por sua vez, afetam a integridade da barreira intestinal. Hormônios do estresse, mediadores imunológicos e neurotransmissores do SNC podem ativar células neuronais do SNE e das vias aferentes do nervo vago, e alterar a composição da microbiota intestinal (19).

A microbiota intestinal produz vários metabólitos e neurotransmissores, alguns dos quais estão envolvidos na fisiologia do cérebro e comportamento. Estudos demonstraram que certas bactérias são capazes de produzir neurotransmissores como

noradrenalina, dopamina e ácido gama-aminobutírico (GABA), o que implica os microrganismos como mediadores de moléculas de sinalização usadas pelo sistema nervoso (15,20-22). Também foi demonstrado que a microbiota intestinal influencia indiretamente a neurotransmissão serotoninérgica ao regular a disponibilidade do triptofano, o precursor da serotonina (23,24). Os ácidos graxos de cadeia curta (AGCC), principais metabólitos produzidos pela fermentação bacteriana de fibras dietéticas no cólon, podem influenciar a fisiologia do cérebro e comportamento através da ligação e ativação dos receptores de ácidos graxos livres (FFARs) que são expressos no nervo vago, bem como participar da produção de neurotransmissores no cérebro, regulando a expressão de enzimas envolvidas em sua biossíntese (25,26).

O sistema imunológico é um importante mediador da interação entre a microbiota intestinal e o cérebro por meio da produção de citocinas. As citocinas podem ser produzidas por células do sistema imunológico ou acessar o SNC por meio de transporte direto através da barreira hematoencefálica (BHE). Infecções, doenças autoimunes e lesões podem alterar a integridade da BHE, aumentando assim a acessibilidade do cérebro a produtos microbianos no sistema circulatório (27). Além disso, a ativação central de receptores de reconhecimento padrão (PRRs) por produtos bacterianos, como peptidoglicano (PGN) e lipopolissacarídeo (LPS), pode afetar diretamente o desenvolvimento do cérebro e o comportamento (28,29).

O eixo microbiota-intestino-cérebro foi reconhecido como um modulador chave de distúrbios neurológicos, incluindo o transtorno do espectro autista (TEA) (30-32). O TEA é um transtorno do neurodesenvolvimento de manifestação precoce caracterizado por comprometimentos na comunicação e nas interações sociais e pela presença de padrões restritos e repetitivos de comportamento (33). Estima-se que o TEA afeta uma em cada 54 crianças nos Estados Unidos com maior prevalência em meninos (34).

Evidências sugerem que fatores genéticos e ambientais estão associados com a etiologia do TEA. As comunidades bacterianas intestinais situam-se na interseção do hospedeiro e do meio ambiente e podem influenciar diretamente a saúde humana. A disbiose da microbiota intestinal está associada com alterações na composição e atividade metabólica das bactérias intestinais, resultando na redução de bactérias comensais e/ou no aumento da abundância de bactérias patogênicas, que podem estar associados a manifestações neurocomportamentais e sintomas gastrointestinais em indivíduos autistas (31,35-37).

Alterações na composição da microbiota intestinal têm sido relatadas em indivíduos com TEA, entretanto, é importante ressaltar que há pouco consenso sobre uma assinatura precisa da microbiota para o TEA. Tomova et al. (38) mostraram redução da razão *Bacteroidetes/Firmicutes* em crianças autistas, enquanto Zhang et al. (39) observaram resultado oposto. Gêneros como *Desulfovibrio e Clostridium*, frequentemente associados a patologias, foram encontrados em maior abundância na microbiota de crianças com TEA em comparação a crianças neurotípicas (40,41). Por outro lado, os gêneros *Bifidobacterium, Prevotella e Veillonella* foram encontrados em menor abundância na microbiota de crianças autistas (35,42-44).

Sintomas gastrointestinais como dor abdominal, diarreia, constipação e flatulência foram caracterizados como comorbidades comuns em indivíduos com TEA (45,46). Curiosamente, foi relatado que os sintomas gastrointestinais estão fortemente associados à gravidade do TEA (47,48). De fato, crianças autistas que apresentam problemas gastrointestinais muitas vezes exibem comportamentos significativamente mais elevados de irritabilidade, ansiedade e isolamento social (49).

Nesse sentido, a modulação da microbiota intestinal é uma estratégia potencialmente interessante que pode alterar comportamentos influenciados pelo SNC

(9,50). Assim, a administração de psicobióticos vem sendo apontada como uma promissora abordagem terapêutica na redução de sintomas gastrointestinais e comportamentais associados ao TEA (51-53). Os psicobióticos foram inicialmente definidos como probióticos que, quando ingeridos em quantidades adequadas, produzem efeitos psiquiátricos positivos na psicopatologia (54). Sarkar et al. (55) propuseram a inclusão de prebióticos na definição de psicobióticos, uma vez que os prebióticos produzem alterações específicas na composição e atividade microbiana do cólon, como o crescimento de bactérias comensais. Os psicobióticos podem atuar na regulação dos neurotransmissores e proteínas, incluindo GABA, serotonina e glutamato que desempenham papéis importantes no controle do comportamento, funções cognitivas, aprendizagem e processos de memória (56).

Especificamente, os probióticos são microrganismos vivos que, quando administrados em quantidades adequadas, conferem benefícios à saúde do hospedeiro (57). As bactérias probióticas mais frequentemente estudadas são *Bifidobacterium* spp. e *Lactobacillus* spp.. Os efeitos benéficos atribuídos aos probióticos incluem a modulação da composição da microbiota intestinal, inibição de potenciais patógenos às células epiteliais do cólon, estimulação do sistema imunológico e manutenção da função da barreira epitelial intestinal (57-59). Os probióticos também atuam na regulação do eixo microbiota-intestino-cérebro. A administração de *Lactobacillus rhamnosus* JB-1 mostrou redução de comportamentos semelhantes à ansiedade e depressão em camundongos. Além disso, esse microrganismo alterou a expressão de receptores GABA no cérebro (60). Bercik et al. (61) mostraram que o *Bifidobacterium longum* NCC3001 melhorou o comportamento semelhante à ansiedade em camundongos com colite infecciosa.

A administração de probióticos também tem sido investigada como estratégia

para melhorar os sintomas relacionados ao TEA. Hsiao et al. (62) demonstraram que a administração de *Bacteroides fragilis* em modelo animal de ativação imune materna, caracterizado por exibir comportamentos semelhantes ao autismo, melhorou a integridade da barreira intestinal e reduziu significativamente déficits comportamentais como ansiedade e comportamento repetitivo. Além disso, foi demonstrado que a administração do probiótico *Lactobacillus reuteri* reverteu déficits sociais associados ao TEA e restaurou níveis de ocitocina - hormônio que influencia o comportamento social - em modelo animal (63). Em um estudo clínico, a suplementação com probióticos por 4 meses, compreendendo cepas de *Lactobacillus*, *Bifidobacterium* e *Streptococcus*, normalizou a razão *Bacteroidetes/Firmicutes* e as contagens de *Desulfovibrio* spp. e *Bifidobacterium* spp. na microbiota fecal de crianças autistas (38). Shaaban et al. (51) mostraram que crianças com TEA tratadas por 3 meses com um coquetel probiótico (*Bifidobacterium longum*, *Lactobacillus acidophilus* e *Lactobacillus rhamnosus*) apresentaram melhora nos sintomas comportamentais e gastrointestinais associados ao TEA. Embora essas observações clínicas sejam promissoras, devem ser interpretadas com cautela, uma vez que estudos clínicos são atualmente limitados.

Os prebióticos, por sua vez, são considerados substratos utilizados seletivamente por microrganismos hospedeiros que conferem benefícios à saúde (64). Inulina, fruto-oligossacarídeos (FOS) e galacto-oligossacarídeos (GOS) são os prebióticos mais documentados na saúde humana (65). Os principais efeitos dos prebióticos estão relacionados com o impacto na composição e atividade da microbiota intestinal, estimulando o crescimento de bactérias comensais como *Bifidobacterium* e *Lactobacillus*, bem como a produção de AGCC (66).

Estudos também mostram que os prebióticos podem apresentar efeitos neuroendócrinos. Os prebióticos FOS e GOS mostraram efeito ansiolítico e

antidepressivo em camundongos com estresse crônico que foi associado a alterações na composição da microbiota intestinal e produção de metabólitos (67). Schmidt et al. (68) demonstraram que a intervenção prebiótica com Bimuno® (B-GOS®) por três semanas diminuiu os níveis de cortisol salivar em indivíduos saudáveis, sugerindo que a administração de prebióticos pode modular o eixo HPA. O efeito da intervenção prebiótica também foi investigada no contexto do TEA. O prebiótico B-GOS® teve um impacto positivo na composição e no perfil metabólico da microbiota intestinal usando um modelo de fermentação *in vitro* inoculado com amostras fecais de crianças autistas (69). Posteriormente, esses pesquisadores mostraram que a combinação de uma dieta livre de glúten e caseína com o B-GOS® resultou na melhora do comportamento social em crianças com TEA (53).

Já os simbióticos são definidos como uma "mistura compreendendo microrganismos vivos e substrato(s) utilizado(s) seletivamente por microrganismos hospedeiros que confere benefício à saúde do hospedeiro" (70). Em um estudo duplo-cego, controlado por placebo, Wang et al. (71) mostraram que a intervenção com probióticos (*Bifidobacterium infantis* Bi-26, *Lactobacillus rhamnosus* HN001, *Bifidobacterium lactis* BL-04 e *Lactobacillus paracasei* LPC-37) + FOS melhorou os sintomas do TEA em crianças autistas e foi eficaz na modulação da composição da microbiota intestinal e na produção de AGCC. Apesar dos resultados desse estudo serem promissores, o efeito da administração de simbióticos nas disfunções gastrointestinais e comportamentais do TEA tem sido pouco explorado.

Recentemente, o transplante da microbiota fecal (TMF) também vem sendo investigado como uma terapia potencial para indivíduos com TEA. Em um estudo clínico, 18 crianças com TEA tratadas com TMF durante 8 semanas apresentaram melhora nos sintomas gastrointestinais e comportamentais associados ao autismo (72). Além disso,

mudanças importantes na microbiota intestinal dos mesmos participantes, incluindo aumentos significativos na diversidade bacteriana e abundância relativa de *Bifidobacterium* e *Prevotella*, foram mantidas no estudo de acompanhamento de 2 anos após a interrupção do tratamento com TMF (73). No entanto, é importante reconhecer que os estudos apresentaram limitações quanto ao tamanho da amostra e desenho experimental. Embora a replicação e validação desses achados em uma população mais ampla e heterogênea sejam necessárias, esses estudos forneceram evidências preliminares promissoras para demonstrar que a microbiota pode de fato ser uma abordagem viável como estratégia de tratamento para o TEA.

Os estudos da microbiota intestinal podem ser realizados utilizando modelos *in vitro* ou *in vivo*. Os modelos de fermentação *in vitro* permitem estudar de forma representativa a microbiota intestinal humana sob condições ambientais controladas. Além disso, podem monitorar a composição e atividade microbiana, fornecendo informações sobre as etapas do processo de fermentação nas diferentes regiões consecutivas do cólon (74,75). Essa abordagem permite explorar a influência de uma ampla variedade de fatores sobre a microbiota intestinal, como componentes dietéticos (probióticos e prebióticos), patógenos microbianos, produtos farmacêuticos e substâncias tóxicas (76). Nos últimos anos, nosso grupo vem desenvolvendo várias pesquisas utilizando o Simulador do Ecossistema Microbiano Humano (SEMH®) como ferramenta de avaliação da microbiota intestinal, incluindo o impacto do *Enterococcus faecium* CRL 183 (77) e da pectina cítrica combinada com *Bifidobacterium longum* BB-46 (78) na microbiota intestinal, bem como o efeito prebiótico do FOS (79), bebida “multifuncional” de leite cabra fermentado (80) e sorvete probiótico (81) na microbiota intestinal.

Estudos *in vivo*, em animais ou humanos, constituem uma abordagem

representativa para investigar as interações entre a microbiota e o hospedeiro. Os modelos animais são importantes ferramentas em pesquisas relacionadas ao TEA, uma vez que podem exibir endofenótipos neuropatológicos e comportamentais semelhantes ao autismo (82). Vários modelos animais têm sido utilizados para explorar as múltiplas vias de comunicação entre a microbiota intestinal e o cérebro, e mostrar como a sinalização química, neuronal e imune podem influenciar fenótipos semelhantes ao TEA (62,63,83,84). Sgritta et al. (85), por exemplo, mostraram que o *Lactobacillus reuteri* reverteu déficits sociais em vários modelos animais de TEA, incluindo modelos genéticos, ambientais e idiopáticos. Esses achados apoiam a ideia de que terapias microbianas podem melhorar endofenótipos específicos associados ao TEA.

Em conjunto, essas evidências destacam que estratégias baseadas na microbiota estão emergindo como promissoras abordagens terapêuticas na redução de sintomas associados ao TEA. Dessa maneira, o objetivo do presente trabalho foi avaliar o efeito de psicobióticos na composição e metabolismo da microbiota intestinal de crianças com TEA usando o SEMH®, bem como na microbiota intestinal e déficits comportamentais em um modelo animal de TEA.

Capítulo 1.

Effect of probiotic, prebiotic, and synbiotic on the gut microbiota of autistic children using an *in vitro* gut microbiome model

Artigo publicado na Revista Food Research International

Effect of probiotic, prebiotic, and synbiotic on the gut microbiota of autistic children using an *in vitro* gut microbiome model

Ana Luiza Rocha Faria Duque[a], Fernanda Manaia Demarqui[b], Mariana Marchi Santoni[b], Cleslei Fernando Zanelli[b], Maria Angela Tallarico Adorno[c], Dragan Milenkovic[d,e], Victoria Mesa[f,g], Katia Sivieri[a*]

[a] Department of Food and Nutrition, School of Pharmaceutical Sciences, São Paulo State University (UNESP), Araraquara, Brazil.

[b] Department of Biological Sciences, School of Pharmaceutical Sciences, São Paulo State University (UNESP), Araraquara, Brazil.

[c] Department of Hydraulics and Sanitation, School of Engineering of São Carlos, University of São Paulo (USP), São Carlos, Brazil.

[d] Department of Internal Medicine, UC Davis School of Medicine, University of California, Davis, United States.

[e] INRAE, UNH, Université Clermont Auvergne, St Genes Champanelle, France.

[f] Faculty of Pharmacy, Paris University, Paris, France.

[g] Food and Human Nutrition Research Group, University of Antioquia, Medellín, Colombia.

[*] Corresponding author:

Katia Sivieri, Department of Food and Nutrition, School of Pharmaceutical Sciences, São Paulo State University (UNESP), Rodovia Araraquara-Jaú Km 1, 14800-903, Araraquara, São Paulo, Brazil.

Phone: +55 16 3301 6931. E-mail: katia.sivieri@unesp.br

Abstract

Imbalances in gut microbiota composition occur in individuals with autism spectrum disorder (ASD). The administration of probiotics, prebiotics, and synbiotics is emerging as a potential and promising strategy for regulating the gut microbiota and improving ASD-related symptoms. We first investigated the survival of the probiotics *Limosilactobacillus* (*L*.) *reuteri* and *Bifidobacterium* (*B*.) *longum* alone, mixed and combined with a galacto-oligosaccharide (GOS) under simulated gastrointestinal conditions. Next, we evaluated the impact of probiotics (*L. reuteri* + *B. longum*), prebiotic (GOS), and synbiotic (*L. reuteri* + *B. longum* + GOS) on gut microbiota composition and metabolism of children with ASD using an *in vitro* fermentation model (SHIME®). The combination of *L. reuteri*, *B. longum*, and GOS showed elevated gastrointestinal resistance. The probiotic, prebiotic, and synbiotic treatments resulted in a positive modulation of the gut microbiota and metabolic activity of children with ASD. More specifically, the probiotic treatment increased the relative abundance of *Lactobacillus*, while the prebiotic treatment increased the relative abundance of *Bifidobacterium* and decreased the relative abundance of *Lachnoclostridium*. Changes in microbial metabolism were associated with increased short-chain fatty acid concentrations and reduced ammonium levels, particularly in the prebiotic and synbiotic treatments.

Keywords: *Limosilactobacillus reuteri*; Galacto-oligosaccharide; *Bifidobacterium longum*; SHIME®; Microbiota modulation; Autism spectrum disorder.

1. Introduction

Autism Spectrum Disorder (ASD) is a neurodevelopmental disorder characterized by impairments in social interaction and communication, as well as restricted and repetitive behaviors (American Psychiatric Association, 2013). The prevalence of ASD has increased in recent years, representing a serious public health problem (Lyall et al., 2017; Xu et al., 2019). The World Health Organization (WHO) (2021) estimates that one in 160 children worldwide has ASD. The etiology of ASD is believed to involve a combination of genetic and environmental risk factors (Fattorusso et al., 2019; Tordjman et al., 2014). Several findings support the role of gut microbiota as an interface between environmental and genetic risk factors associated with ASD (Vuong & Hsiao, 2017). Gastrointestinal (GI) symptoms are common comorbidities in individuals with ASD and include constipation, diarrhea, and abdominal pain (Chaidez et al., 2014; Holingue et al., 2018). Additionally, increased susceptibility to intestinal inflammation (McElhanon et al., 2014) and altered gut permeability (de Magistris et al., 2010) have been documented in individuals with ASD.

A growing body of evidence shows that changes in the gut microbiota can impact immune, metabolic, and nervous system development and function via the microbiota-gut-brain axis, supporting the idea of a bidirectional communication system linking gut and brain (Carabotti et al., 2015; Cryan et al., 2019; Martin et al., 2018; Morais et al., 2021). The gut microbiota is known to play an important role in maintaining normal GI tract functions and immune homeostasis. The occurrence of GI symptoms in autistic individuals suggests gut microbiota involvement in the GI pathophysiology of ASD (Strati et al., 2017; Vuong & Hsiao, 2017).

Individuals with ASD harbor an altered gut microbiota relative to neurotypical individuals. The bacterial genus *Bifidobacterium* has been found in lower abundance in

the gut microbiota of autistic children compared to neurotypical controls (Adams et al., 2011; De Angelis et al., 2013; Wang et al., 2011). More specifically, Wang et al. (2020) reported a significantly lower relative abundance of *B. longum* in children with autism. Fecal microbiome profiling has shown a higher abundance of *Clostridium* and *Desulfovibrio* species in subjects with ASD (Finegold et al., 2010; Parracho et al., 2005; Song et al., 2004). Imbalances in the gut microbiota of autistic individuals may also contribute to an altered production of metabolites such as short-chain fatty acids (SCFAs). Increased levels of propionic acid in the stools of children with ASD have also been reported (Wang et al., 2012). The intraventricular administration of propionic acid to animals has been shown to result in some ASD-like behaviors (MacFabe et al., 2011; Thomas et al., 2012). Propionic acid is a major SCFA produced by ASD-associated GI bacteria such as *Clostridia*, *Bacteroides*, and *Desulfovibrio* (MacFabe, 2015). Although altered gut microbiota is often seen in autistic individuals, a consensus among studies has not yet been established.

Gut microbiota modulation is a potential strategy either to reduce the abundance of harmful microorganisms and related metabolites that may negatively alter central nervous system (CNS)-driven behaviors or to increase the abundance of microorganisms and metabolites that may support normal CNS-driven behavior (Sharon et al., 2016; Sherwin et al., 2019). Thus, the administration of pro- and prebiotics is emerging as a non-invasive therapeutic alternative for treating neurological disorders, including ASD (Abdellatif et al., 2020; Grimaldi et al., 2018; Hsiao et al., 2013; Santocchi et al., 2020). The *L. reuteri* ATCC 23272 has improved despair and anxiety-like behaviors in chronically stressed mice (Marin et al., 2017). Studies have found that treatment with the probiotic species *L. reuteri* has reversed social deficits in several mouse models of autism (Buffington et al., 2016; Sgritta et al., 2019). Moreover, the bacteria *B. longum*, a well-

documented probiotic, has been shown to protect against autoimmune diseases such as inflammatory bowel disease and to normalize anxiety-like behavior induced by chronic gut inflammation in mice (Bercik et al., 2011; Zhang et al., 2017). The oral administration of *B. longum* to rats under "stressed" conditions revealed that the treatment influenced the functioning of the hypothalamic-pituitary-adrenal axis and reduced the anxiolytic behavior (Haas et al., 2020).

Prebiotics, specifically galacto-oligosaccharides (GOSs), have also shown potential to manage ASD symptoms and comorbidities. Specifically, Grimaldi et al. (2017) found significant changes in gut microbiota composition and metabolism after treatment with the prebiotic B-GOS® using an *in vitro* gut model system inoculated with fecal samples from autistic children. The authors subsequently reported that a combination of a gluten- and casein-free diet with the prebiotic B-GOS® resulted in an improvement in social behavior symptoms of children with ASD (Grimaldi et al., 2018).

In vitro gut fermentation models are excellent tools for assessing the microbiota and GI environment under controlled conditions (Van de Wiele et al., 2015). Research using GI simulators is an essential complement to *in vivo* studies and provides insights into different steps of the fermentation process by allowing a dynamic sampling over time in different consecutive regions of the colon (Venema & Van den Abbeele, 2013). The Simulator of the Human Intestinal Microbial Ecosystem (SHIME®) is a dynamic *in vitro* system that simulates the different parts of the GI tract and thus enables the study of gut microbiota composition and metabolites production, as well as the potential effects of pro- and prebiotics on the gut microbiota (Bianchi et al., 2018; Molly et al., 1994; Pham & Mohajeri, 2018; Rodrigues et al., 2020).

Taken together, these findings highlight that administration of pro- and prebiotics may be a promising approach for modulating the gut microbiota and improving ASD-

related symptoms. Thus, the current study sought to evaluate the survival of the probiotic strains of *L. reuteri* and *B. longum* alone, mixed and combined with GOS in simulated GI conditions and to then evaluate the impact of select treatment on the gut microbiota composition and metabolism of children with ASD using an *in vitro* fermentation model (SHIME®).

2. Material and methods

2.1. Cultures and substrate

The prebiotic Vivinal® GOS was supplied by FrieslandCampina Ingredients (Vinhedo, Brazil). *Limosilactobacillus reuteri* ATCC 23272 (André Tosello Foundation, Campinas, Brazil) and *Bifidobacterium longum* BB-46 (Christian Hansen, Valinhos, Brazil) were purchased as fresh cultures and stored at −80 °C in De Man, Rogosa and Sharpe (MRS) broth (Merck, Germany) and glycerol.

These probiotics were activated in MRS broth at 37 °C for 16 h. The cultures were centrifuged, washed, and resuspended in sterile saline solution (NaCl 0.85%). Populations of *L. reuteri* were determined by plating on MRS agar after 48 h of anaerobic incubation at 37 °C. Populations of *B. longum* were determined by plating on MRS agar with the addition of sodium propionate (3 g/L) (Sigma-Aldrich, USA) and lithium chloride (2 g/L) (Merck, Germany), followed by anaerobic incubation at 37 °C for 72 h.

2.2. Survival of L. reuteri and B. longum in simulated GI conditions

The survival of probiotic strains was assessed as previously described by Buriti, Castro, and Saad (2010), with minor modifications (Table 1). Briefly, each probiotic strain (alone, mixed and combined with GOS) in NaCl solution (0.85% w/v) was transferred to sterilized flasks. Pepsin from porcine gastric mucosa (3 g/L) (Sigma-Aldrich, USA) and

amano lipase G from *Penicillium camemberti* (0.9 mg/L) (Sigma-Aldrich, USA) were added to the samples, and the pH was adjusted to 2.0–2.5 using a 1 N HCl solution (Merck, Germany). The flasks were incubated at 37 °C and shaken at 150 rpm in a metabolic water bath (Dubnoff MA-095, Marconi, Brazil) for 2 h to simulate the gastric phase. Next, bovine bile (10 g/L) (Sigma-Aldrich, USA) and porcine pancreatin (1 g/L) (Sigma-Aldrich, USA) were added to the samples, and the pH was adjusted to 4.5–5.5 using an alkaline solution (150 mL of 1 N NaOH, 11.16 g of $PO_4H_2Na \cdot 2H_2O$, and distilled water up to 1 L). The flasks were incubated at 37 °C and shaken at 150 rpm for 2 h to simulate enteric phase I. Finally, bile and pancreatin were adjusted to maintain the concentrations of 10 g/L and 1 g/L, respectively, and the pH was increased to 6.5–7.5 using the same alkaline solution. The flasks were incubated again at 37 °C and shaken at 150 rpm for 2 h to simulate enteric phase II, thus completing the 6 h of assay. Samples were collected before the assay (0 h) and after each phase of the assay (2 h, 4 h, and 6 h) in triplicate to enumeration of colony-forming unites (CFU) of *L. reuteri* and *B. longum*.

Additionally, the survival rate proposed by Guo et al. (2009) was calculated using the following equation:

Survival rate (%) = (log CFU N_1 / log CFU N_0) x 100

where N_1 = the total viable count of probiotic strains after exposure to *in vitro* simulated GI conditions (6 h) and N_0 = the total viable count of probiotic strains before exposure to *in vitro* simulated GI conditions (0 h).

2.3. SHIME® model

The SHIME® is an *in vitro* GI system consisting of five double-jacketed vessels set up in sequence to simulate the stomach, small intestine, ascending colon (AC), transverse colon (TC), and descending colon (DC) (Molly et al., 1994). The system was

maintained at 37 °C under anaerobic conditions sustained by sealing the vessels and applying nitrogen flow. The pH and retention time of each vessel was controlled to match the physiological conditions of the colon regions as previously described by Possemiers et al. (2004).

2.3.1. Short-term SHIME® run

The most promising treatment selected from the simulated GI conditions assay (Section 2.2) was evaluated on gut microbiota composition and metabolism of a child with ASD using the SHIME® as an *in vitro* batch incubation model. In this experiment, the fecal sample was obtained from a 4-year-old autistic child who had not used antibiotics, probiotics, or prebiotics in the 6 months prior to the study. The autistic child was classified with mild autism according to the Childhood Autism Rating Scale (CARS). The fecal inoculum was prepared using an aliquot of 20 g of the fecal sample diluted in 200 mL of phosphate buffer (0.05 mol/L of monosodium phosphate, 0.05 mol/L of disodium phosphate, and 0.1% sodium thioglycolate - pH 6.5) and homogenized in a stomacher (model 130, Nova Ética, Brazil) for 10 min. Next, the diluted fecal sample was centrifuged for 15 min at 3000 rpm, and 40 mL of supernatant was added to the vessel representing the AC (Possemiers et al., 2010). The fecal inoculum was stabilized over a 2-week period in a carbohydrate-based medium to allow the microbiota to adapt to the colon conditions (control period). After stabilization, the SHIME® run included a treatment period. In the treatment period, 10^8 CFU/mL of *L. reuteri* and *B. longum*, as well as 5 g of GOS were added to the system and incubated for 72 h (Figure 1). Colon samples were taken during batch incubation (0, 24, 48, and 72 h) to analyze metabolic activity and microbiota composition.

2.3.2. Long-term SHIME® run

The fecal inoculum was prepared as previously described by Possemiers et al. (2010). The AC, TC, and DC of the SHIME® were inoculated with fecal samples from three children with ASD between 4 and 5 years of age who had not used antibiotics, probiotics, or prebiotics in the 6 months prior to the study. The autistic children were classified with mild-severe autism according to the CARS. The experimental protocol was performed in the SHIME® for 7 weeks. After fecal inoculation, a 2-week stabilization period was performed to allow the microbial community to adapt to each colon condition (Control 1). After stabilization, 1-week treatments were consecutively administered to the system with a pause of 1 week between each treatment to stabilize the microbiota (Control 2 and Control 3). Based on the results of the short-term SHIME® run, a probiotic treatment (*L. reuteri* + *B. longum*), a prebiotic treatment (GOS), and a synbiotic treatment (*L. reuteri* + *B. longum* + GOS) were administered to the system. The probiotic cultures (10^8 CFU/mL of each strain), the prebiotic GOS (2.5 g), as well as the synbiotic (10^8 CFU/mL of each strain + 2.5 g of GOS) were administered with the carbohydrate-based medium twice a day. Samples from the AC, TC, and DC were collected at the end of each experimental period to analyze metabolic activity and microbiota composition. The SHIME® experiment was performed in duplicate (Figure 2).

2.4. Quantitative real-time PCR (qPCR)

The bacterial composition in the *in vitro* colonic model was assessed using quantitative real-time PCR (qPCR). The DNA was extracted using a QIAamp Fast DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and then quantified using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA). Next, qPCR was performed using Power SYBR Green Master Mix

(Applied Biosystems, USA), with analysis in a 7500 Real-Time PCR System (Applied Biosystems, USA). Specific primers were selected to detect bacteria of interest that might be involved in ASD pathogenesis and are shown in Table 2. The qPCR reaction had a final volume of 20 µL, which consisted of 1 µL of DNA template, 10 µL of SYBR Green, and primers at a final concentration of 0.5 µM. Each sample was analyzed in duplicate. Amplification conditions were 50 °C for 2 min, 95 °C for 10 m, followed by 40 cycles at 95 °C for 15 s and 60 °C for 60 s. The relative abundance of each bacterial group was computed according to the comparative C_T method proposed by Schmittgen and Livak (2008), where ΔCt = (C_T bacterial group – C_T universal bacteria), using universal bacteria as a normalizer.

2.5. 16S rRNA gene sequencing

Samples were collected from the AC, TC, and DC and were then lyophilized and stored at −80°C until prepared for sequencing. Bacterial genomic DNA was extracted using the DNeasy PowerLyzer PowerSoil kit (Qiagen, Valencia, USA) according to the manufacturer's instructions. The 16S rRNA V3-V4 regions were amplified by two-step PCR and sequenced on the MiSeq platform (Illumina) using the 300 bp paired-end protocol. The primers used to amplify the V3-V4 regions of the 16S rRNA gene were 319 F − 806 R. The 16S rRNA gene read pairs were demultiplexed in FASTQ files and trimmed. The bioinformatic analysis of the sequences was performed in the Quantitative Insights Into Microbial Ecology (QIIME2) program, version 2019.7. The DADA2 method was implemented to detect and correct sequencing noises, remove chimeric sequences, and group the sequences into operational taxonomic units (OTUs). To classify the sequences according to their taxonomic information, the q2-feature-classifier plugin was

used based on the VSEARCH alignment method (Rognes et al., 2016) with the SILVA v132 database (Quast et al., 2013) and with a sequence identity percentage of 97%.

2.6. Ammonium and SCFAs

Ammonium levels were determined using a specific ion meter (model HI 4101, Hanna Instruments, USA) combined with an ammonia ion selective electrode (Orion 95-12 model, Thermo Fisher Scientific, USA). An aliquot of 0.2 mL of an ammonia ionic strength adjustor solution (Orion) was added to each 10 mL of the sample, and the ammonium level was measured in triplicate (Bianchi et al., 2014).

To analyze the SCFAs, 2 mL samples were centrifuged at 14000 rpm for 5 min, and 300 µL of the supernatant was diluted in 1700 µL of ultrapure water. Next, 1 g of NaCl was added, as well as 100 µL of crotonic acid (700 mg/L), 70 µL of isobutanol (1 g/L), and 200 µL of 2 M H_2SO_4. The SCFAs were analyzed using a GC-2010 gas chromatograph (Shimadzu, Kyoto, Japan) equipped with a split/splitless injector, a flame ionization detector, and a CombiPAL autosampler for headspace analysis. The SCFAs were separated using a HP-INNOWax column (30 m × 0.25 mm × 0.25 µm) (Agilent Technologies, USA). Hydrogen was used as the carrier gas at a flow rate of 1.45 mL/min. The temperature of both the injector and detector was 240 °C (Adorno et al., 2014). The analysis was performed in triplicate.

2.7. Statistical analysis

Data were presented as mean ± standard deviation (SD). The paired t-test, one-way analysis of variance (ANOVA), and Tukey's test were used to analyze the results, with $p < 0.05$ considered statistically significant. Statistical analysis was performed using GraphPad Prism software, version 8.0 (La Jolla, USA). The 16S rRNA gene sequencing

analyses were conducted in RStudio, version 3.2.4 (R Core Team, 2018) utilizing the phyloseq package (McMurdie, Holmes, & Watson, 2013) to import sample data and calculate alpha and beta diversity metrics. The significance of categorical variables was determined using the non-parametric Wilcoxon test for two category comparisons or the Kruskal-Wallis test when comparing three or more categories. Principal coordinate plots relied on the PERMANOVA test to estimate p values (Fierer et al., 2010). The p values were adjusted for multiple comparisons using the FDR algorithm (Benjamini et al., 2001). Correlation analysis between microbial groups and the metabolism products was performed using Spearman correlation test using the cor.mtest function from the corrplot stats package (Wei & Simko, 2021).

3. Results

3.1. Survival of L. reuteri and B. longum under simulated GI conditions

We first verified the survival of *L. reuteri* and *B. longum* alone, mixed and combined with GOS in *in vitro* adverse GI conditions, which were specifically established as low pH, the presence of pepsin and pancreatin, and tolerance to bile salts (Table 3). Survival of all probiotic strains decreased significantly over the course of the gastric and enteric I and II phases. We also observed pronounced reductions in the *L. reuteri* and *B. longum* survival during enteric phases I and II, which was likely due to the presence of bile salts and pancreatin affecting cell membranes and impacting the viability of these microorganisms. Moreover, survival of most of the probiotic strains combined with GOS was higher than in the pure cultures. This finding suggests that GOS has a protective effect. The highest survival rates were exhibited by *L. reuteri* when combined with GOS (74%), by *L. reuteri* when combined with *B. longum* and GOS (71%), and by *B. longum* when combined with *L. reuteri* and GOS (68%) (Table 3). The effects of probiotics are

known to be strain-specific. Thus, we hypothesized that the use of different probiotic strains in combination could be more effective than a single strain. In this sense, a combination of two probiotic strains with a prebiotic could lead to a broader range of bacterial stimulation in the colon. Based on the viability results, the combination of *L. reuteri* + *B. longum* + GOS was selected for further analysis using the SHIME® in a short-term run.

3.2. Microbiota composition and metabolic activity in short-term SHIME® run

Changes to microbiota composition over the incubation periods with the synbiotic treatment were analyzed using qPCR that targeted specific bacterial groups (Figure 3). We observed that the relative abundance of *Bifidobacterium* spp. and of the *Lactobacillus* group increased significantly while the *Clostridium difficile* and the *Clostridium perfringen*s group decreased slightly but not significantly over the course of the incubation period. The *Desulfovibrio desulfuricans* group significantly decreased for 48 h of incubation and was then found to be higher at 72 h, with no significant difference relative to 0 h.

The effect of the synbiotic treatment on the saccharolytic and proteolytic fermentation of the gut microbiota was also evaluated based on the production of SCFAs and ammonium, respectively (Figure 4). A significant increase in acetic and propionic acid production was observed over the course of the incubation periods (Figure 4A). Butyric acid was also analyzed (data not shown), but the results were below the detection limit. Ammonium concentrations significantly decreased over the course of the incubation periods (Figure 4B). The promising results from this step of the study provided a good starting point for further investigations into the effect of synbiotic on the gut microbiota of ASD children in a long-term SHIME® run.

3.3. Microbiota composition in long-term SHIME® run

The microbiota composition and microbial community structure were analyzed using 16S rRNA gene sequencing. The microbiota was mainly represented by the phyla *Firmicutes*, *Actinobacteria*, *Bacteroidetes*, and *Proteobacteria* in the three colon regions during the control periods (Figure 5). Changes were seen in the relative abundance of the main phyla following the treatment administration. The probiotic treatment increased the relative abundance of *Firmicutes* and decreased *Actinobacteria* and *Bacteroidetes* abundance in the three colon regions. Also, an increase in *Verrucomicrobia* phylum abundance was observed in the TC and the DC (Figure 5). The increase in *Firmicutes* phylum abundance observed was largely the result of the increase in *Lactobacillaceae* family abundance, as well as of the decrease in *Bifidobacteriaceae* and *Bacteroidaceae* family abundance in the three colon regions (Figure 6).

The prebiotic and synbiotic treatments increased *Actinobacteria* abundance and decreased *Bacteroidetes* and *Verrucomicrobia* abundance in the three colon regions. In addition, the prebiotic treatment decreased *Firmicutes* abundance in the TC and the DC, while the symbiotic treatment increased *Firmicutes* abundance in the same colons (Figure 5). The prebiotic and synbiotic treatments also increased *Bifidobacteriaceae* family abundance and decreased *Bacteroidaceae* abundance in the three colon regions. Additionally, *Lactobacillaceae* abundance decreased in the prebiotic treatment and increased in the synbiotic treatment (Figure 6).

More specifically, we evaluated whether the treatments changed the main genera found in the microbiota of children with ASD (Figure 7). The probiotic treatment significantly increased *Lactobacillus* abundance. *Akkermansia*, *Blautia*, and *Desulfovibrio* abundance increased, while *Bifidobacterium*, *Bacteroides*, *Lachnoclostridium*,

Veillonella, and *Prevotella* abundance decreased with no significant difference. The prebiotic treatment significantly increased *Bifidobacterium* abundance, as well as *Blautia* and *Veillonella* abundance, though not significantly. In contrast, *Lachnoclostridium* abundance significantly decreased while the *Lactobacillus*, *Akkermansia*, *Desulfovibrio*, *Bacteroides*, and *Prevotella* abundance decreased with no significant difference. Finally, the synbiotic treatment increased *Bifidobacterium*, *Lactobacillus*, *Akkermansia*, and *Blautia* abundance while *Bacteroides*, *Lachnoclostridium*, *Desulfovibrio*, *Prevotella*, and *Veillonella* abundance decreased with no significant difference (Figure 7). These results reinforce the idea that the different treatments evaluated can modulate the microbiota of children with ASD in different ways.

The alpha diversity analysis showed that all of the treatments (probiotic, prebiotic, and synbiotic) reduced richness and diversity, as evidenced by the Chao1 and Shannon indices relative to the controls (Figures 8A and 8B). In addition, richness and diversity indices decreased significantly in the AC relative to the TC and DC (Figures 8C and 8D). The beta diversity analysis based on the Bray-Curtis dissimilarity revealed significant differences in the microbial community between the treatments ($p = 0.013$), as well as between the colon regions ($p = 0.003$) (Figure 9A). Moreover, weighted UniFrac distances revealed significant differences between the treatments ($p = 0.013$) (Figure 9B), while un-weighted UniFrac distances exhibited no differences ($p = 0.537$) (Figure 9C). Taken together, the beta diversity analyses revealed differing microbial profiles between the treatments, indicating changes in the microbial community structure caused by treatments in all three colon regions.

3.4. Microbial metabolism in long-term SHIME® run

Metabolic activity involving SCFAs and ammonium production is shown in Figure 10. The probiotic treatment decreased acetic acid concentrations and increased butyric acid concentrations in all regions of the colon. Specifically, acetic acid decreased significantly in the AC while butyric acid significantly increased in the TC. In addition, propionic acid concentrations decreased in the AC and increased slightly in the TC and the DC with no significant difference. The prebiotic and synbiotic treatments increased all SCFA concentrations in the three regions of the colon. More specifically, the prebiotic and synbiotic treatments significantly increased butyric acid concentrations in the AC and the DC. Acetic acid concentrations also increased significantly in the TC and the DC in the prebiotic treatment and in all three regions in the synbiotic treatment (Figures 10A-10C). In the probiotic treatment, ammonium concentrations increased in the AC with no significant difference and decreased significantly in the TC and the DC. On the other hand, both prebiotic and synbiotic treatments significantly decreased ammonium concentrations in all three regions of the colon (Figure 10D).

Correlation analysis was performed to identify associations between the main bacterial genera in the microbiota of children with ASD and the production of ammonium and SCFAs (Figure 11). Ammonium production negatively correlated with *Bifidobacterium*, *Veillonella*, and *Agathobacter* ($p < 0.05$) and positively correlated with *Akkermansia*, *Bacteroides*, *Desulfovibrio*, *Parasutterella*, and *Subdoligranulum* ($p < 0.05$). Butyric acid positively correlated with *Bifidobacterium*, *Blautia*, *Prevotella*, *Agathobacter*, and *Subdoligranulum* ($p < 0.05$), while acetic acid positively correlated with *Bifidobacterium* ($p < 0.05$). Propionic acid negatively correlated with *Enterobacter* and positively correlated with *Subdoligranulum* ($p < 0.05$). Interestingly, the *Lachnoclostridium* genus was negatively correlated with all SCFA production ($p < 0.05$) (Figure 11).

4. Discussion

The ability to survive adverse conditions in the GI tract is a key requirement for probiotics to exert their beneficial effects on the colon (Food and Agriculture Organization of the United Nations [FAO] & WHO, 2002). Survival of most of the probiotic strains combined with GOS was found to be higher than in the pure cultures, suggesting that GOS has a protective effect. According to Hernandez-Hernandez et al. (2012), the use of prebiotic ingredients may be a good alternative to support the growth of probiotic strains and improve their survival through the GI tract. Krasaekoopt and Watcharapoka (2014) reported that the presence of GOS in the microencapsulation of *Lactobacillus acidophilus* (LA 5) and *Lactobacillus casei* (LC 01) provided protection and enhanced the viability of these probiotics in a simulated digestive system. Our results indicate that GOS combined with *L. reuteri* + *B. longum* increased the probiotics' ability to resist the simulated GI conditions.

The gut microbiota has been suggested to play a potential role in ASD. Individuals with ASD have been found to have differences in gut microbiota composition relative to neurotypical individuals (De Angelis et al., 2013; Finegold et al., 2010; Kang et al., 2019; Liu et al., 2019; Strati et al., 2017; Zhang et al., 2018). The frequent occurrence of GI symptoms and evidence of immune imbalances in these individuals also suggest the involvement of the microbiota in ASD (Onore et al., 2012; Vuong & Hsiao, 2017). In addition, cellular components produced by gut bacteria, such as peptidoglycan, lipopolysaccharide, and flagellin, are recognized by pattern-recognition receptors (PRRs) on epithelial and immune cells. They produce cytokines, hormones, and other molecular signals, which serve as neurotransmitters within the CNS (Cerdó et al., 2017). Microbiota-based strategies have demonstrated the potential to regulate CNS-driven behaviors

(Sherwin et al., 2019; Vuong et al., 2017). Preclinical and clinical studies have shown that the administration of pro- and prebiotics can be a potential therapeutic strategy for treating neurological disorders such as ASD (Buffington et al., 2016; Grimaldi et al., 2018; Hsiao et al., 2013; Sgritta et al., 2019; Tomova et al., 2015). More specifically, Grimaldi et al. (2017) investigated the influence of the prebiotic B-GOS® on an *in vitro* gut model system inoculated with feces from autistic children. B-GOS® administration positively modulated the bacterial populations and metabolic profile, which showed an increase in the abundance of *Bifidobacterium*, as well as higher acetate and butyrate levels. Similar results were also observed in our study, in which the abundance of *Bifidobacterium* increased following prebiotic and synbiotic treatments.

Lower *Bifidobacterium* and *Blautia* levels have been reported in individuals with ASD (Inoue et al., 2016; Wang et al., 2011). We observed that *Lactobacillus* abundance increased following probiotic treatment, and *Bifidobacterium* and *Blautia* abundance increased after the prebiotic and synbiotic treatments. The increase in these genera can be considered positive, since lower levels of beneficial bacteria seen in ASD individuals may be involved in the pathogenesis of the disease (Liu et al., 2019). It is known that some species of *Bifidobacterium* produce gamma-aminobutyric acid (GABA) (Srikantha & Mohajeri, 2019), which is involved in the metabolism of glutamate, an excitatory neurotransmitter in the brain (Shen, 2013). Consequently, glutamate concentrations negatively correlate with the severity of the anxiety, social, and behavioral disorders typical of ASD (Horder et al., 2018; Iglesias-Vázquez et al., 2020; Wierońska et al., 2011). *Blautia* plays an important role in preventing inflammation by upregulating intestinal regulatory T cells and producing SCFAs (Liu et al., 2021). In addition, *Lactobacillus* can contribute to the regulation of intestinal epithelial functions by maintaining the epithelial barrier and enhancing protective immune responses (Yan & Polk, 2020). Bacteria such

as *Desulfovibrio* and *Clostridium* are often present in higher numbers in autistic subjects than in controls and are related to the severity of ASD symptoms (Finegold et al., 2017; Luna et al., 2017; Tomova et al., 2015). *Desulfovibrio* is known to produce lipopolysaccharide (LPS) and hydrogen sulfide, which could be toxic to intestinal cells under certain circumstances (Finegold et al., 2010). The role of Clostridia in ASD has been also explored. Kandeel et al. (2020) emphasized the potential correlation between gut colonization by Clostridia and the probability of developing or exacerbating ASD in children, demonstrating that *Clostridium* may play a potential role in the etiology of ASD. Some *Clostridium* species have also been correlated with constipation in ASD and could produce pro-inflammatory exotoxins (Strati et al., 2017). Our results showed a decrease in *Clostridium perfringens* and *Desulfovibrio desulfuricans* group over the course of the synbiotic treatment in the short-term SHIME® run, a decrease in the genus *Desulfovibrio* in the prebiotic and synbiotic treatments, and a decrease in *Lachnoclostridium* in all treatments administered in the long-term SHIME® run. According to Yutin and Galperin (2013), the *Lachnoclostridium* genus is a new proposed classification for several *Clostridium* species. We also observed a decrease in the *Bacteroides* genus in all treatments. *Bacteroides* has been reported to be abundant in ASD subjects and is related to the production of propionic acid, which, at elevated levels, can induce an inflammatory response seen in ASD cases (Abdelli et al., 2019; De Angelis et al., 2013; Macfabe, 2015). Taken together, our results suggest that the treatments evaluated in this study may reduce populations of these ASD-associated bacteria.

Changes in the gut microbiota composition can result in altered metabolic profiles, impacting the nutrients and microbial metabolites bioavailability (Sharon et al., 2014, 2019). Lower concentrations of SCFAs have been found in the gut microbiota of autistic children; these levels may be partly related to lower saccharolytic fermentation by

beneficial bacteria, to prolonged transit time due to constipation or common GI disorders in ASD, and/or to increased gut permeability (Adams et al., 2011). Williams et al. (2011) also reported impaired carbohydrate digestion and transport as well as mucosal dysbiosis in the gut of autistic children. Our study showed more evident results in the production of SCFAs following the administration of prebiotic and synbiotic treatments. Vivinal GOS® is a prebiotic of the galacto-oligosaccharide type, considered a substrate that is used selectively by host microorganisms, generally associated with the stimulation of beneficial bacteria. This substrate is fermented by intestinal bacteria resulting in the production of metabolites such as SCFAs (Gibson et al., 2017). The increase in SCFA production by prebiotic and synbiotic treatments applied herein can be considered positive, since some studies have shown that children with ASD exhibit lower SCFA concentrations relative to neurotypical children (Adams et al., 2011; De Angelis et al., 2013; Grimaldi et al., 2017). This is likely due not only to a compromised microbiota characterized by changes in composition (such as reduced levels of *Bifidobacterium*) (Adams et al., 2011), but also to reduced metabolic activity of intestinal bacteria. In line with our findings, Grimaldi et al. (2017) showed that administering B-GOS® in an *in vitro* model simulating the gut of autistic children increased acetate and butyrate concentrations. More recently, Wang et al. (2020) observed a significant increase in SCFAs in children with ASD after an intervention combining probiotics and fructo-oligosaccharide.

SCFAs are the main metabolites produced in the colon by bacterial fermentation of dietary carbohydrates and play a crucial role in intestinal health, serving as a source of energy for the colon's epithelial cells (Koh et al., 2016). Additionally, SCFAs are microbial metabolites that can cross the blood-brain barrier and affect CNS functions, brain development, and behavior (Dalile et al., 2019; Macfabe, 2015; Silva et al., 2020). Interestingly, we observed that increases in *Bifidobacterium* and *Blautia* abundance were

positively correlated with acetic and butyric acid production, while a decrease in *Lachnoclostridium* abundance was negatively correlated with SCFA production, results which are considered positive. *Bifidobacterium* and *Blautia* play an important role in SCFA production (Liu et al., 2021; Rivière et al., 2016), while the genus *Lachnoclostridium* has been associated with ASD, specifically in subjects with GI symptoms (Luna et al., 2017).

Proteolytic activity in the gut can produce potentially toxic metabolites such as ammonia. Wang et al. (2012) observed higher fecal ammonia concentrations in children with ASD, while other studies have reported elevated plasma ammonia levels in children with ASD (Clark-Taylor & Clark-Taylor, 2004; Filipek et al., 2004). Since most ammonia enters the blood from the GI tract (Felipo & Butterworth, 2002), higher fecal ammonia concentrations in ASD may translate into higher plasma levels (Wang et al., 2012). When present in high concentrations, ammonia can negatively impact the morphology of epithelial cells in the colon and increase gut permeability (Davila et al., 2013; Hughes et al., 2008). Our results showed significant decreases in ammonium concentrations following the treatments administered in both the short- and long-term SHIME® runs. Decreases in ammonium may be largely attributed to the reduced *Bacteroides* and *Desulfovibrio* abundance and increased *Bifidobacterium* abundance observed after the administration of the probiotic, prebiotic, and synbiotic treatments. Though several studies have investigated the relationship between microbial metabolites and ASD, the mechanisms involved in this association have not been fully elucidated.

5. Conclusions

The growing prevalence of ASD has encouraged researchers to develop microbiota-based strategies to prevent and treat this disorder. Our results showed that

the combination of *L. reuteri* + *B. longum* + GOS exhibited elevated GI resistance. According to the SHIME® results, the probiotic, prebiotic, and synbiotic treatments positively modulated the gut microbiota and metabolic activity of ASD children. More specifically, probiotic treatment increased the relative abundance of *Lactobacillus*, while the prebiotic treatment increased the relative abundance of *Bifidobacterium* and decreased the relative abundance of *Lachnoclostridium*. The changes seen in microbial metabolism were associated with increased concentrations of SCFAs and reduced ammonium levels, particularly in the prebiotic and synbiotic treatments.

Declaration of Competing Interest

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

Acknowledgments

The authors wish to thank Marcio Del Bianco, Marília Nardy Dorsa, and Leonardo Appezato Filho for providing the Vivinal® GOS product (FrieslandCampina Ingredients Latin America Ltda., Brazil) used in this study.

Funding

This work was supported by the São Paulo Research Foundation (FAPESP) [2017/01157-1 and 2018/26645-1] and the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) [Finance code 001].

References

Abdellatif, B., McVeigh, C., Bendriss, G., & Chaari, A. (2020). The promising role of probiotics in managing the altered gut in autism spectrum disorders. *International Journal of Molecular Sciences, 21*(11), 4159. https://doi.org/10.3390/ijms21114159.

Abdelli, L. S., Samsam, A., & Naser, S. A. (2019). Propionic acid induces gliosis and neuro-inflammation through modulation of PTEN/AKT pathway in autism spectrum disorder. *Scientific Reports, 9*, 8824. https://doi.org/10.1038/s41598-019-45348-z.

Adams, J. B., Johansen, L. J., Powell, L. D., Quig, D., & Rubin, R. A. (2011). Gastrointestinal flora and gastrointestinal status in children with autism–comparisons to typical children and correlation with autism severity. *BMC Gastroenterology, 11*, 22. https://doi.org/10.1186/1471-230X-11-22.

Adorno, M., Hirasawa, J., & Varesche, M. (2014). Development and validation of two methods to quantify volatile acids (C2–C6) by GC/FID: Headspace (automatic and manual) and liquid-liquid extraction (LLE). *American Journal of Analytical Chemistry, 5*, 406–414. https://doi.org/10.4236/ajac.2014.57049.

American Psychiatric Association. (2013). Diagnostic and statistical manual of mental disorders (DSM-5) (5th ed.). https://doi.org/10.1176/appi.books.9780890425596.

Benjamini, Y., Drai, D., Elmer, G., Kafkafi, N., & Golani, I. (2001). Controlling the false discovery rate in behavior genetics research. *Behavioural Brain Research, 125*(1–2), 279–284. https://doi.org/10.1016/s0166-4328(01)00297-2.

Bercik, P., Park, A. J., Sinclair, D., Khoshdel, A., Lu, J., Huang, X., Deng, Y., Blennerhassett, P. A., Fahnestock, M., Moine, D., Berger, B., Huizinga, J. D., Kunze, W., McLean, P. G., Bergonzelli, G. E., Collins, S. M., & Verdu, E. F. (2011). The anxiolytic effect of Bifidobacterium longum NCC3001 involves vagal pathways for gut-brain communication. *Neurogastroenterology and Motility: the official journal of the European Gastrointestinal Motility Society, 23*(12), 1132–1139. https://doi.org/10.1111/j.1365-2982.2011.01796.x.

Bianchi, F., Larsen, N., de Mello Tieghi, T., Adorno, M., Kot, W., Saad, S., Jespersen, L., & Sivieri, K. (2018). Modulation of gut microbiota from obese individuals by in vitro fermentation of citrus pectin in combination with Bifidobacterium longum BB-46. *Applied Microbiology and Biotechnology, 102*(20), 8827–8840. https://doi.org/10.1007/s00253-018-9234-8.

Bianchi, F., Rossi, E. A., Sakamoto, I. K., Adorno, M. A. T., Van de Wiele, T., & Sivieri, K. (2014). Beneficial effects of fermented vegetal beverages on human gastrointestinal microbial ecosystem in a simulator. *Food Research International, 64*, 43–52. https://doi.org/10.1016/j.foodres.2014.05.072.

Boon, N., Top, E. M., Verstraete, W., & Siciliano, S. D. (2003). Bioaugmentation as a tool to protect the structure and function of an activated-sludge microbial community against a 3-Chloroaniline shock load. *Applied and Environmental Microbiology, 69*(3), 1511–1520. https://doi.org/10.1128/AEM.69.3.1511-1520.2003.

Buffington, S. A., Di Prisco, G. V., Auchtung, T. A., Ajami, N. J., Petrosino, J. F., & Costa- Mattioli, M. (2016). Microbial reconstitution reverses maternal diet-induced social and synaptic deficits in offspring. *Cell, 165*(7), 1762–1775. https://doi.org/10.1016/j.cell.2016.06.001.

Buriti, F. C., Castro, I. A., & Saad, S. M. (2010). Viability of Lactobacillus acidophilus in synbiotic guava mousses and its survival under in vitro simulated gastrointestinal conditions. *International Journal of Food Microbiology, 137*(2–3), 121–129. https://doi.org/10.1016/j.ijfoodmicro.2009.11.030.

Carabotti, M., Scirocco, A., Maselli, M. A., & Severi, C. (2015). The gut-brain axis: Interactions between enteric microbiota, central and enteric nervous systems. *Annals of Gastroenterology, 28*(2), 203–209.

Cerdó, T., Ruíz, A., Suárez, A., & Campoy, C. (2017). Probiotic, prebiotic, and brain development. *Nutrients, 9*(11), 1247. https://doi.org/10.3390/nu9111247.

Chaidez, V., Hansen, R. L., & Hertz-Picciotto, I. (2014). Gastrointestinal problems in children with autism, developmental delays or typical development. *Journal of Autism and Developmental Disorders, 44*(5), 1117–1127. https://doi.org/10.1007/s10803-013-1973-x.

Clark-Taylor, T., & Clark-Taylor, B. E. (2004). Is autism a disorder of fatty acid metabolism? Possible dysfunction of mitochondrial beta-oxidation by long chain acyl-CoA dehydrogenase. *Medical Hypotheses, 62*(6), 970–975. https://doi.org/10.1016/j.mehy.2004.01.011.

Cryan, J. F., O'Riordan, K. J., Cowan, C. S. M., Sandhu, K. V., Bastiaanssen, T. F. S., Boehme, M., ... Dinan, T. G. (2019). The microbiota-gut-brain axis. *Physiological Reviews, 99*(4), 1877–2013. https://doi.org/10.1152/physrev.00018.2018.

Dalile, B., Van Oudenhove, L., Vervliet, B., & Verbeke, K. (2019). The role of short-chain fatty acids in microbiota-gut-brain communication. *Nature Reviews Gastroenterology & Hepatology, 16*(8), 461–478. https://doi.org/10.1038/s41575-019-0157-3.

Davila, A. M., Blachier, F., Gotteland, M., Andriamihaja, M., Benetti, P. H., Sanz, Y., & Tomé, D. (2013). Intestinal luminal nitrogen metabolism: Role of the gut microbiota and consequences for the host. *Pharmacological research, 68*(1), 95–107. https://doi.org/10.1016/j.phrs.2012.11.005.

De Angelis, M., Piccolo, M., Vannini, L., Siragusa, S., De Giacomo, A., Serrazzanetti, D. I., ... Heimesaat, M. (2013). Fecal microbiota and metabolome of children with autism and pervasive developmental disorder not otherwise specified. *PloS ONE, 8*(10), e76993. https://doi.org/10.1371/journal.pone.0076993.

de Magistris, L., Familiari, V., Pascotto, A., Sapone, A., Frolli, A., Iardino, P., Carteni, M., De Rosa, M., Francavilla, R., Riegler, G., Militerni, R., & Bravaccio, C. (2010). Alterations of the intestinal barrier in patients with autism spectrum disorders and in their first-degree relatives. *Journal of Pediatric Gastroenterology and Nutrition, 51*(4), 418–424. https://doi.org/10.1097/MPG.0b013e3181dcc4a5.

Fattorusso, A., Di Genova, L., Dell'Isola, G. B., Mencaroni, E., & Esposito, S. (2019). Autism spectrum disorders and the gut microbiota. *Nutrients, 11*(3), 521. https://doi.org/10.3390/nu11030521.

Felipo, V., & Butterworth, R. F. (2002). Neurobiology of ammonia. *Progress in Neurobiology, 67*(4), 259–279. https://doi.org/10.1016/s0301-0082(02)00019-9.

Fierer, N., Lauber, C. L., Zhou, N., McDonald, D., Costello, E. K., & Knight, R. (2010). Forensic identification using skin bacterial communities. *Proceedings of the National Academy of Sciences of the United States of America, 107*(14), 6477–6481. https://doi.org/10.1073/pnas.1000162107.

Filipek, P. A., Juranek, J., Nguyen, M. T., Cummings, C., & Gargus, J. J. (2004). Relative carnitine deficiency in autism. *Journal of Autism and Developmental Disorders, 34*(6), 615–623. https://doi.org/10.1007/s10803-004-5283-1.

Finegold, S. M., Dowd, S. E., Gontcharova, V., Liu, C., Henley, K. E., Wolcott, R. D., Youn, E., Summanen, P. H., Granpeesheh, D., Dixon, D., Liu, M., Molitoris, D. R., & Green, J. A. (2010). Pyrosequencing study of fecal microflora of autistic and control children. *Anaerobe, 16*(4), 444–453. https://doi.org/10.1016/j.anaerobe.2010.06.008.

Finegold, S. M., Summanen, P. H., Downes, J., Corbett, K., & Komoriya, T. (2017). Detection of Clostridium perfringens toxin genes in the gut microbiota of autistic children. *Anaerobe, 45*, 133–137. https://doi.org/10.1016/j.anaerobe.2017.02.008.

Food and Agriculture Organization of the United Nations & World Health Organization. (2002). Joint FAO/WHO working group on drafting guidelines for the evaluation of probiotics in food. London, ON, Canada: Food and Agriculture Organization of the United Nations and World Health Organization.

Gibson, G. R., Hutkins, R., Sanders, M. E., Prescott, S. L., Reimer, R. A., Salminen, S. J., Scott, K., Stanton, C., Swanson, K. S., Cani, P. D., Verbeke, K., & Reid, G. (2017). Expert consensus document: The International Scientific Association for Probiotics and Prebiotics (ISAPP) consensus statement on the definition and scope of prebiotics. *Nature Reviews Gastroenterology & Hepatology, 14*(8), 491–502. https://doi.org/10.1038/nrgastro.2017.75.

Grimaldi, R., Cela, D., Swann, J. R., Vulevic, J., Gibson, G. R., Tzortzis, G., Costabile, A., & Marchesi, J. (2017). In vitro fermentation of B-GOS: Impact on faecal bacterial populations and metabolic activity in autistic and non-autistic children. *FEMS Microbiology Ecology, 93*(2), fiw233. https://doi.org/10.1093/femsec/fiw233.

Grimaldi, R., Gibson, G. R., Vulevic, J., Giallourou, N., Castro-Mejia, J. L., Hansen, L. H., Leigh Gibson, E., Nielsen, D. S., & Costabile, A. (2018). A prebiotic intervention study in children with autism spectrum disorders (ASDs). *Microbiome, 6*(1). https://doi.org/10.1186/s40168-018-0523-3.

Guo, Z., Wang, J., Yan, L., Chen, W., Liu, X.-M., & Zhang, H.-P. (2009). In vitro comparison of probiotic properties of Lactobacillus casei Zhang, a potentialnew probiotic, with selected probiotic strains. *LWT - Food Science and Technology, 42*(10), 1640–1646. https://doi.org/10.1016/j.lwt.2009.05.025.

Haas, G. S., Wang, W., Saffar, M., Mooney-Leber, S. D., & Brummelte, S. (2020). Probiotic treatment (Bifidobacterium longum subsp. longum 35624TM) affects stress responsivity in male rats after chronic corticosterone exposure. *Behavioural Brain Research, 393*. https://doi.org/10.1016/j.bbr.2020.112718.

Hernandez-Hernandez, O., Muthaiyan, A., Moreno, F. J., Montilla, A., Sanz, M. L., & Ricke, S. C. (2012). Effect of prebiotic carbohydrates on the growth and tolerance of Lactobacillus. *Food Microbiology, 30*(2), 355–361. https://doi.org/10.1016/j.fm.2011.12.022.

Holingue, C., Newill, C., Lee, L.-C., Pasricha, P. J., & Daniele Fallin, M. (2018). Gastrointestinal symptoms in autism spectrum disorder: A review of the literature on ascertainment and prevalence. *Autism Research: Official journal of the International Society for Autism Research, 11*(1), 24–36. https://doi.org/10.1002/aur.v11.110.1002/aur.1854.

Horder, J., Petrinovic, M. M., Mendez, M. A., Bruns, A., Takumi, T., Spooren, W., Barker, G. J., Künnecke, B., & Murphy, D. G. (2018). Glutamate and GABA in autism spectrum disorder-a translational magnetic resonance spectroscopy study in man and rodent models. *Translational Psychiatry, 8*(1), 106. https://doi.org/10.1038/s41398-018-0155-1.

Hsiao, E. Y., McBride, S. W., Hsien, S., Sharon, G., Hyde, E. R., McCue, T., Codelli, J. A., Chow, J., Reisman, S. E., Petrosino, J. F., Patterson, P. H., & Mazmanian, S. K. (2013). Microbiota modulate behavioral and physiological abnormalities associated with neurodevelopmental disorders. *Cell, 155*(7), 1451–1463. https://doi.org/10.1016/j.cell.2013.11.024.

Hughes, R., Kurth, M. J., McGilligan, V., McGlynn, H., & Rowland, I. (2008). Effect of colonic bacterial metabolites on Caco-2 cell paracellular permeability in vitro. *Nutrition and cancer, 60*(2), 259–266. https://doi.org/10.1080/01635580701649644.

Iglesias-Vázquez, L., Van Ginkel Riba, G., Arija, V., & Canals, J. (2020). Composition of gut microbiota in children with autism spectrum disorder: A systematic review and meta-analysis. *Nutrients, 12*(3), 792. https://doi.org/10.3390/nu12030792.

Inoue, R., Sakaue, Y., Sawai, C., Sawai, T., Ozeki, M., Romero-Pérez, G. A., & Tsukahara, T. (2016). A preliminary investigation on the relationship between gut microbiota and gene expressions in peripheral mononuclear cells of infants with autism spectrum disorders. *Bioscience, Biotechnology, and Biochemistry, 80*(12), 2450–2458. https://doi.org/10.1080/09168451.2016.1222267.

Kandeel, W. A., Meguid, N. A., Bjørklund, G., Eid, E. M., Farid, M., Mohamed, S. K., Wakeel, K. E., Chirumbolo, S., Elsaeid, A., & Hammad, D. Y. (2020). Impact of Clostridium bacteria in children with autism spectrum disorder and their anthropometric measurements. *Journal of molecular neuroscience, 70*(6), 897–907. https://doi.org/10.1007/s12031-020-01482-2.

Kang, D. W., Adams, J. B., Coleman, D. M., Pollard, E. L., Maldonado, J., McDonough-Means, S., Caporaso, J. G., & Krajmalnik-Brown, R. (2019). Long-term benefit of Microbiota Transfer Therapy on autism symptoms and gut microbiota. *Scientific Reports, 9*(1), 5821. https://doi.org/10.1038/s41598-019-42183-0.

Koh, A., De Vadder, F., Kovatcheva-Datchary, P., & Bäckhed, F. (2016). From dietary fiber to host physiology: Short-chain fatty acids as key bacterial metabolites. *Cell, 165*(6), 1332–1345. https://doi.org/10.1016/j.cell.2016.05.041.

Krasaekoopt, W., & Watcharapoka, S. (2014). Effect of addition of inulin and galactooligosaccharide on the survival of microencapsulated probiotics in alginate beads coated with chitosan in simulated digestive system, yogurt and fruit juice. *LWT - Food Science and Technology, 57*(2), 761–766. https://doi.org/10.1016/j.lwt.2014.01.037.

Liu, F., Li, J., Wu, F., Zheng, H., Peng, Q., & Zhou, H. (2019). Altered composition and function of intestinal microbiota in autism spectrum disorders: A systematic review. *Translational*. *Psychiatry, 9*(43). https://doi.org/10.1038/s41398-019-0389-6.

Liu, X., Mao, B., Gu, J., Wu, J., Cui, S., Wang, G., Zhao, J., Zhang, H., & Chen, W. (2021). *Blautia*-a new functional genus with potential probiotic properties? *Gut Microbes, 13* (1), 1–21. https://doi.org/10.1080/19490976.2021.1875796.

Luna, R. A., Oezguen, N., Balderas, M., Venkatachalam, A., Runge, J. K., Versalovic, J., Veenstra-VanderWeele, J., Anderson, G. M., Savidge, T., & Williams, K. C. (2017). Distinct

microbiome-neuroimmune signatures correlate with functional abdominal pain in children with autism spectrum disorder. *Cellular and Molecular Gastroenterology and Hepatology, 3*(2), 218–230. https://doi.org/10.1016/j.jcmgh.2016.11.008.

Lyall, K., Croen, L., Daniels, J., Fallin, M. D., Ladd-Acosta, C., Lee, B. K., Park, B. Y., Snyder, N. W., Schendel, D., Volk, H., Windham, G. C., & Newschaffer, C. (2017). The changing epidemiology of autism spectrum disorders. *Annual Review of Public Health, 38*(1), 81–102. https://doi.org/10.1146/annurev-publhealth-031816-044318.

MacFabe, D. F. (2015). Enteric short-chain fatty acids: Microbial messengers of metabolism, mitochondria, and mind: Implications in autism spectrum disorders. *Microbial Ecology in Health and Disease, 26*, 28177. https://doi.org/10.3402/mehd.v26.28177.

MacFabe, D. F., Cain, N. E., Boon, F., Ossenkopp, K. P., & Cain, D. P. (2011). Effects of the enteric bacterial metabolic product propionic acid on object-directed behavior, social behavior, cognition, and neuroinflammation in adolescent rats: Relevance to autism spectrum disorder. *Behavioural Brain Research, 217*(1), 47–54. https://doi.org/10.1016/j.bbr.2010.10.005.

Marin, I. A., Goertz, J. E., Ren, T., Rich, S. S., Onengut-Gumuscu, S., Farber, E., Wu, M., Overall, C. C., Kipnis, J., & Gaultier, A. (2017). Microbiota alteration is associated with the development of stress-induced despair behavior. *Scientific Reports, 7*, 43859. https://doi.org/10.1038/srep43859.

Martin, C. R., Osadchiy, V., Kalani, A., & Mayer, E. A. (2018). The brain-gut-microbiome axis. *Cellular and Molecular Gastroenterology and Hepatology, 6*(2), 133–148. https://doi.org/10.1016/j.jcmgh.2018.04.003.

McElhanon, B. O., McCracken, C., Karpen, S., & Sharp, W. G. (2014). Gastrointestinal symptoms in autism spectrum disorder: A meta-analysis. *Pediatrics, 133*(5), 872–883. https://doi.org/10.1542/peds.2013-3995.

McMurdie, P. J., Holmes, S., & Watson, M. (2013). phyloseq: An R package for reproducible interactive analysis and graphics of microbiome census data. *PloS ONE, 8*(4), e61217. https://doi.org/10.1371/journal.pone.0061217.

Molly, K., Woestyne, M. V., Smet, I. D., & Verstraete, W. (1994). Validation of the simulator of the human intestinal microbial ecosystem (SHIME) reactor using microorganism-associated activities. *Microbial Ecology in Health and Disease, 7*(4), 191–200. https://doi.org/10.3109/08910609409141354.

Morais, L. H., Schreiber, H. L., & Mazmanian, S. K. (2021). The gut microbiota-brain axis in behaviour and brain disorders. *Nature Reviews Microbiology, 19*(4), 241–255. https://doi.org/10.1038/s41579-020-00460-0.

Onore, C., Careaga, M., & Ashwood, P. (2012). The role of immune dysfunction in the pathophysiology of autism. *Brain, Behavior, and Immunity, 26*(3), 383–392. https://doi.org/10.1016/j.bbi.2011.08.007.

Parracho, H. M., Bingham, M. O., Gibson, G. R., & McCartney, A. L. (2005). Differences between the gut microflora of children with autistic spectrum disorders and that of healthy children. *Journal of Medical Microbiology, 54*(Pt 10), 987–991. https://doi.org/10.1099/jmm.0.46101-0.

Pham, V. T., & Mohajeri, M. H. (2018). The application of in vitro human intestinal models on the screening and development of pre- and probiotics. *Beneficial Microbes, 9*(5), 725–742. https://doi.org/10.3920/BM2017.0164.

Possemiers, S., Marzorati, M., Verstraete, W., & Van de Wiele, T. (2010). Bacteria and chocolate: A successful combination for probiotic delivery. *International Journal of Food Microbiology, 141*(1–2), 97–103. https://doi.org/10.1016/j.ijfoodmicro.2010.03.008.

Possemiers, S., Verthé, K., Uyttendaele, S., & Verstraete, W. (2004). PCR-DGGE-based quantification of stability of the microbial community in a simulator of the human intestinal microbial ecosystem. *FEMS Microbiology Ecology, 49*(3), 495–507. https://doi.org/10.1016/j.femsec.2004.05.002.

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., & Glöckner, F. O. (2013). The SILVA ribosomal RNA gene database project: Improved data processing and web-based tools. *Nucleic Acids Research, 41*(Database issue), D590–D596. https://doi.org/10.1093/nar/gks1219.

R Core Team. (2018). *R: A language and environment for statistical computing*. Vienna, Austria: R Foundation for Statistical Computing. Retrieved from https://www.R-project.org.

Rinttilä, T., Kassinen, A., Malinen, E., Krogius, L., & Palva, A. (2004). Development of an extensive set of 16S rDNA-targeted primers for quantification of pathogenic and indigenous bacteria in faecal samples by real-time PCR. *Journal of Applied Microbiology, 97*(6), 1166–1177. https://doi.org/10.1111/jam.2004.97.issue-610.1111/j.1365-2672.2004.02409.x.

Rivière, A., Selak, M., Lantin, D., Leroy, F., & De Vuyst, L. (2016). Bifidobacteria and butyrate-producing colon bacteria: Importance and strategies for their stimulation in the human gut. *Frontiers in Microbiology, 7*, 979. https://doi.org/10.3389/fmicb.2016.00979.

Rodrigues, V. C.d. C., Duque, A. L. R. F., Fino, L.d. C., Simabuco, F. M., Sartoratto, A., Cabral, L., Noronha, M. F., Sivieri, K., & Antunes, A. E. C. (2020). Modulation of the intestinal microbiota and the metabolites produced by the administration of ice cream and a dietary supplement containing the same probiotics. *The British Journal of Nutrition, 124*(1), 57–68. https://doi.org/10.1017/S0007114520000896.

Rognes, T., Flouri, T., Nichols, B., Quince, C., & Mahé, F. (2016). VSEARCH: A versatile open source tool for metagenomics. *PeerJ, 4*. https://doi.org/10.7717/peerj.2584.

Santocchi, E., Guiducci, L., Prosperi, M., Calderoni, S., Gaggini, M., Apicella, F., Tancredi, R., Billeci, L., Mastromarino, P., Grossi, E., Gastaldelli, A., Morales, M. A., & Muratori, F. (2020). Effects of probiotic supplementation on gastrointestinal, sensory and core symptoms in autism spectrum disorders: A randomized controlled trial. *Frontiers in Psychiatry, 11*, Article 550593. https://doi.org/10.3389/fpsyt.2020.550593.

Schmittgen, T. D., & Livak, K. J. (2008). Analyzing real-time PCR data by the comparative C(T) method. *Nature Protocols, 3*(6), 1101–1108. https://doi.org/10.1038/nprot.2008.73.

Sgritta, M., Dooling, S. W., Buffington, S. A., Momin, E. N., Francis, M. B., Britton, R. A., & Costa-Mattioli, M. (2019). Mechanisms underlying microbial-mediated changes in social behavior in mouse models of autism spectrum disorder. *Neuron, 101*(2), 246–259.e6. https://doi.org/10.1016/j.neuron.2018.11.018.

Sharon, G., Cruz, N. J., Kang, D. W., Gandal, M. J., Wang, B., Kim, Y. M., ... Mazmanian, S. K. (2019). Human gut microbiota from autism spectrum disorder promote behavioral symptoms in mice. *Cell, 177*(6), 1600–1618.e17. https://doi.org/10.1016/j.cell.2019.05.004.

Sharon, G., Garg, N., Debelius, J., Knight, R., Dorrestein, P. C., & Mazmanian, S. K. (2014). Specialized metabolites from the microbiome in health and disease. *Cell Metabolism, 20*(5), 719–730. https://doi.org/10.1016/j.cmet.2014.10.016.

Sharon, G., Sampson, T. R., Geschwind, D. H., & Mazmanian, S. K. (2016). The central nervous system and the gut microbiome. *Cell, 167*(4), 915–932. https://doi.org/10.1016/j.cell.2016.10.027.

Shen, J. (2013). Modeling the glutamate-glutamine neurotransmitter cycle. *Frontiers in Neuroenergetics, 5*(1). https://doi.org/10.3389/fnene.2013.00001.

Sherwin, E., Bordenstein, S. R., Quinn, J. L., Dinan, T. G., & Cryan, J. F. (2019). Microbiota and the social brain. *Science, 366*(6465), eaar2016. https://doi.org/10.1126/science.aar2016.

Silva, Y. P., Bernardi, A., & Frozza, R. L. (2020). The role of short-chain fatty acids from gut microbiota in gut-brain communication. *Frontiers in Endocrinology, 11*, 25. https://doi.org/10.3389/fendo.2020.00025.

Song, Y., Liu, C., & Finegold, S. M. (2004). Real-time PCR quantitation of clostridia in feces of autistic children. *Applied and Environmental Microbiology, 70*(11), 6459–6465. https://doi.org/10.1128/AEM.70.11.6459-6465.2004.

Srikantha, P., & Mohajeri, M. H. (2019). The possible role of the microbiota-gut-brain-axis in autism spectrum disorder. *International Journal of Molecular Sciences, 20*(9), 2115. https://doi.org/10.3390/ijms20092115.

Strati, F., Cavalieri, D., Albanese, D., De Felice, C., Donati, C., Hayek, J., Jousson, O., Leoncini, S., Renzi, D., Calabro`, A., & De Filippo, C. (2017). New evidences on the altered gut microbiota in autism spectrum disorders. *Microbiome, 5*(1), 24. https://doi.org/10.1186/s40168-017-0242-1.

Thomas, R. H., Meeking, M. M., Mepham, J. R., Tichenoff, L., Possmayer, F., Liu, S., & MacFabe, D. F. (2012). The enteric bacterial metabolite propionic acid alters brain and plasma phospholipid molecular species: Further development of a rodent model of autism spectrum disorders. *Journal of Neuroinflammation, 9*(153). https://doi.org/10.1186/1742-2094-9-153

Tomova, A., Husarova, V., Lakatosova, S., Bakos, J., Vlkova, B., Babinska, K., & Ostatnikova, D. (2015). Gastrointestinal microbiota in children with autism in Slovakia. *Physiology & Behavior, 138*, 179–187. https://doi.org/10.1016/j. physbeh.2014.10.033.

Tordjman, S., Somogyi, E., Coulon, N., Kermarrec, S., Cohen, D., Bronsard, G., Bonnot, O., Weismann-Arcache, C., Botbol, M., Lauth, B., Ginchat, V., Roubertoux, P., Barburoth, M., Kovess, V., Geoffray, M. M., & Xavier, J. (2014). Gene × Environment interactions in autism spectrum disorders: Role of epigenetic mechanisms. *Frontiers in Psychiatry, 5*, 53. https://doi.org/10.3389/fpsyt.2014.00053.

Van de Wiele, T., Van den Abbeele, P., Ossieur, W., Possemiers, S., & Marzorati, M. (2015). The Simulator of the Human Intestinal Microbial Ecosystem (SHIME®). In K. Verhoeckx, P. Cotter, I. López-Expósito, C. Kleiveland, T. Lea, A. Mackie, T. Requena, D. Swiatecka, & H. Wichers (Eds.), *The impact of food bioactives on health: In vitro and ex vivo models* (pp. 305–317). Springer.

Venema, K., & Van den Abbeele, P. (2013). Experimental models of the gut microbiome. *Best Practice & Research Clinical Gastroenterology, 27*(1), 115–126. https://doi.org/10.1016/j.bpg.2013.03.002.

Vuong, H. E., & Hsiao, E. Y. (2017). Emerging roles for the gut microbiome in autism spectrum disorder. *Biological Psychiatry, 81*(5), 411–423. https://doi.org/10.1016/j.biopsych.2016.08.024.

Vuong, H. E., Yano, J. M., Fung, T. C., & Hsiao, E. Y. (2017). The microbiome and host behavior. *Annual Review of Neuroscience, 40*(1), 21–49. https://doi.org/10.1146/annurev-neuro-072116-031347.

Wang, L., Christophersen, C. T., Sorich, M. J., Gerber, J. P., Angley, M. T., & Conlon, M. A. (2011). Low relative abundances of the mucolytic bacterium Akkermansia muciniphila and Bifidobacterium spp. in feces of children with autism. *Applied and Environmental Microbiology, 77*(18), 6718–6721. https://doi.org/10.1128/AEM.05212-11.

Wang, L., Christophersen, C. T., Sorich, M. J., Gerber, J. P., Angley, M. T., & Conlon, M. A. (2012). Elevated fecal short chain fatty acid and ammonia concentrations in children with autism spectrum disorder. *Digestive Diseases and Sciences, 57*(8), 2096–2102. https://doi.org/10.1007/s10620-012-2167-7.

Wang, Y., Li, N., Yang, J.-J., Zhao, D.-M., Chen, B., Zhang, G.-Q., Chen, S., Cao, R.-F., Yu, H., Zhao, C.-Y., Zhao, L.u., Ge, Y.-S., Liu, Y.i., Zhang, L.-H., Hu, W., Zhang, L., & Gai, Z.-T. (2020). Probiotics and fructo-oligosaccharide intervention modulate the microbiota-gut brain axis to improve autism spectrum reducing also the hyper-serotonergic state and the dopamine metabolism disorder. *Pharmacological Research, 157*, 104784. https://doi.org/10.1016/j.phrs.2020.104784.

Wei, T., & Simko, V. (2021). R package "corrplot": Visualization of a correlation matrix (Version 0.88). https://github.com/taiyun/corrplot.

Wierońska, J., Stachowicz, K., Nowak, G., & Pilc, A. (2011). The loss of glutamate-GABA harmony in anxiety disorders. https://doi.org/10.5772/19919.

Williams, B. L., Hornig, M., Buie, T., Bauman, M. L., Cho Paik, M., Wick, I., ... Jacobson, S. (2011). Impaired carbohydrate digestion and transport and mucosal dysbiosis in the intestines of children with autism and gastrointestinal disturbances. *PloS ONE, 6*(9), e24585. https://doi.org/10.1371/journal.pone.0024585.

World Health Organization. (2021). Autism spectrum disorders. Retrieved from https://www.who.int/news-room/fact-sheets/detail/autism-spectrum-disorders. Accessed June 20, 2021.

Xu, G., Strathearn, L., Liu, B., O'Brien, M., Kopelman, T. G., Zhu, J., Snetselaar, L. G., & Bao, W. (2019). Prevalence and treatment patterns of autism spectrum disorder in the United States, 2016. *JAMA pediatrics, 173*(2), 153–159. https://doi.org/10.1001/jamapediatrics.2018.4208.

Yan, F., & Polk, D. B. (2020). Probiotics and probiotic-derived functional factors-mechanistic insights into applications for intestinal homeostasis. *Frontiers in Immunology, 11*, 1428. https://doi.org/10.3389/fimmu.2020.01428.

Yutin, N., & Galperin, M. Y. (2013). A genomic update on clostridial phylogeny: Gram-negative spore formers and other misplaced clostridia. *Environmental Microbiology, 15*(10), 2631–2641. https://doi.org/10.1111/1462-2920.12173.

Zhang, M., Ma, W., Zhang, J., He, Y., & Wang, J. (2018). Analysis of gut microbiota profiles and microbe-disease associations in children with autism spectrum disorders in China. *Scientific Reports, 8*(13981). https://doi.org/10.1038/s41598-018-32219-2.

Zhang, M., Zhou, L., Zhang, S., Yang, Y., Xu, L., Hua, Z., & Zou, X. (2017). Bifidobacterium longum affects the methylation level of forkhead box P3 promoter in 2, 4, 6-trinitrobenzenesulphonic acid induced colitis in rats. *Microbial Pathogenesis, 110*, 426–430. https://doi.org/10.1016/j.micpath.2017.07.029.

Table 1. Treatments exposed to simulated gastrointestinal conditions

Probiotics alone, mixed and combined with GOS
Limosilactobacillus reuteri
Bifidobacterium longum
Limosilactobacillus reuteri + *Bifidobacterium longum*
Limosilactobacillus reuteri + GOS
Bifidobacterium longum + GOS
Limosilactobacillus reuteri + *Bifidobacterium longum* + GOS

Table 2. Primer sequences used in quantitative real-time PCR

Bacterial group	Oligonucleotide sequence	Reference
Bifidobacterium spp.	F: 5'-TCGCGTC(C/T)GGTGTGAAAG-3' R: 5'-CCACATCCAGC(A/G)TCCAC-3'	Rinttilä, Kassinen, Malinen, Krogius, and Palva (2004)
Lactobacillus group	F: 5'-AGCAGTAGGGAATCTTCCA-3' R: 5'-CACCGCTACACATGGAG-3'	Rinttilä et al. (2004)
Clostridium perfringens group	F: 5'-ATGCAAGTCGAGCGA(G/T)G-3' R: 5'-TATGCGGTATTAATCT(C/T)CCTTT-3'	Rinttilä et al. (2004)
Clostridium difficile	F: 5'-TTGAGCGATTTACTTCGGTAAAGA-3' R: 5'-CCATCCTGTACTGGCTCACCT-3'	Rinttilä et al. (2004)
Desulfovibrio desulfuricans group	F: 5'-GGTACCTTCAAAGGAAGCAC-3' R: 5'-GGGATTTCACCCCTGACTTA-3'	Rinttilä et al. (2004)
Universal bacteria	F: 5'-ACTCCTACGGGAGGCAGCAG-3' R: 5'-ATTACCGCGGCTGCTGG-3'	Boon et al. (2003)

F: forward; R: reverse.

Table 3. Population (log CFU/mL) and survival rate (%) of *B. longum* and *L. reuteri* alone, mixed and combined with GOS under simulated gastrointestinal conditions

Treatments	Initial time	Gastric phase	Enteric phase I	Enteric phase II	Survival rate (%)
B. longum	$9.08^{Aa} \pm 0.04$	$5.34^{Be} \pm 0.08$	$4.72^{Dd} \pm 0.12$	$5.08^{Cd} \pm 0.04$	56%
B. longum + GOS	$9.14^{Aa} \pm 0.09$	$6.17^{Bd} \pm 0.12$	$3.98^{Cf} \pm 0.03$	$4.09^{Cf} \pm 0.09$	45%
L. reuteri	$9.22^{Aa} \pm 0.05$	$7.31^{Bc} \pm 0.03$	$5.24^{Cc} \pm 0.04$	$5.31^{Cc} \pm 0.08$	58%
L. reuteri + GOS	$8.46^{Ab} \pm 0.08$	$7.21^{Bc} \pm 0.04$	$5.72^{Db} \pm 0.12$	$6.27^{Cb} \pm 0.08$	74%
L. reuteri (combined with *B. longum*)	$9.08^{Aa} \pm 0.06$	$6.32^{Bd} \pm 0.07$	$4.32^{Ce} \pm 0.07$	$4.33^{Ce} \pm 0.08$	48%
B. longum (combined with *L. reuteri*)	$9.19^{Aa} \pm 0.04$	$6.27^{Bd} \pm 0.05$	$5.28^{Cc} \pm 0.06$	$5.35^{Cc} \pm 0.08$	58%
L. reuteri (combined with *B. longum* + GOS)	$9.22^{Aa} \pm 0.02$	$8.59^{Ba} \pm 0.07$	$6.43^{Ca} \pm 0.04$	$6.54^{Ca} \pm 0.09$	71%
B. longum (combined with *L. reuteri* + GOS)	$9.21^{Aa} \pm 0.04$	$8.26^{Bb} \pm 0.16$	$6.31^{Ca} \pm 0.05$	$6.27^{Cb} \pm 0.05$	68%

B. longum: *Bifidobacterium longum*; *L. reuteri*: *Limosilactobacillus reuteri*; GOS: galacto-oligosaccharide. Initial time = 0 h; Gastric phase = 2 h; Enteric phase I = 4 h; Enteric phase II = 6 h.
*Different upper-case letters on the same line indicate statistical difference according to Tukey's post-test ($p < 0.05$). Different lower-case letters on the same column indicate statistical difference according to Tukey's post-test ($p < 0.05$).
†Data are presented as mean ± SD (n = 3).

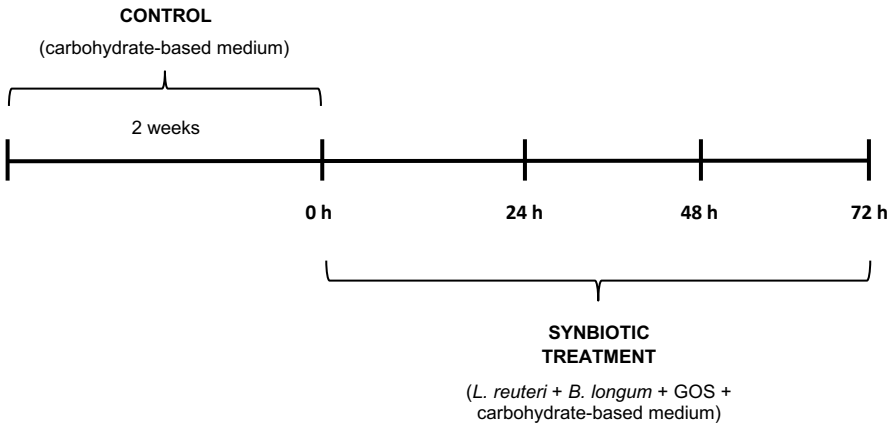

Figure 1. Schematic representation of the experimental design used in the short-term SHIME® run.

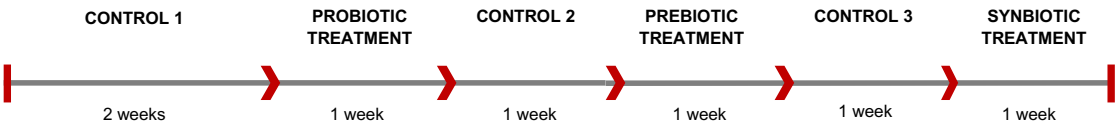

Figure 2. Schematic representation of the experimental design used in the long-term SHIME® run. Control 1 (carbohydrate-based medium); Probiotic treatment (*L. reuteri* + *B. longum* + carbohydrate-based medium); Control 2 (carbohydrate-based medium); Prebiotic treatment (GOS + carbohydrate-based medium); Control 3 (carbohydrate-based medium); and Synbiotic treatment (*L. reuteri* + *B. longum* + GOS + carbohydrate-based medium).

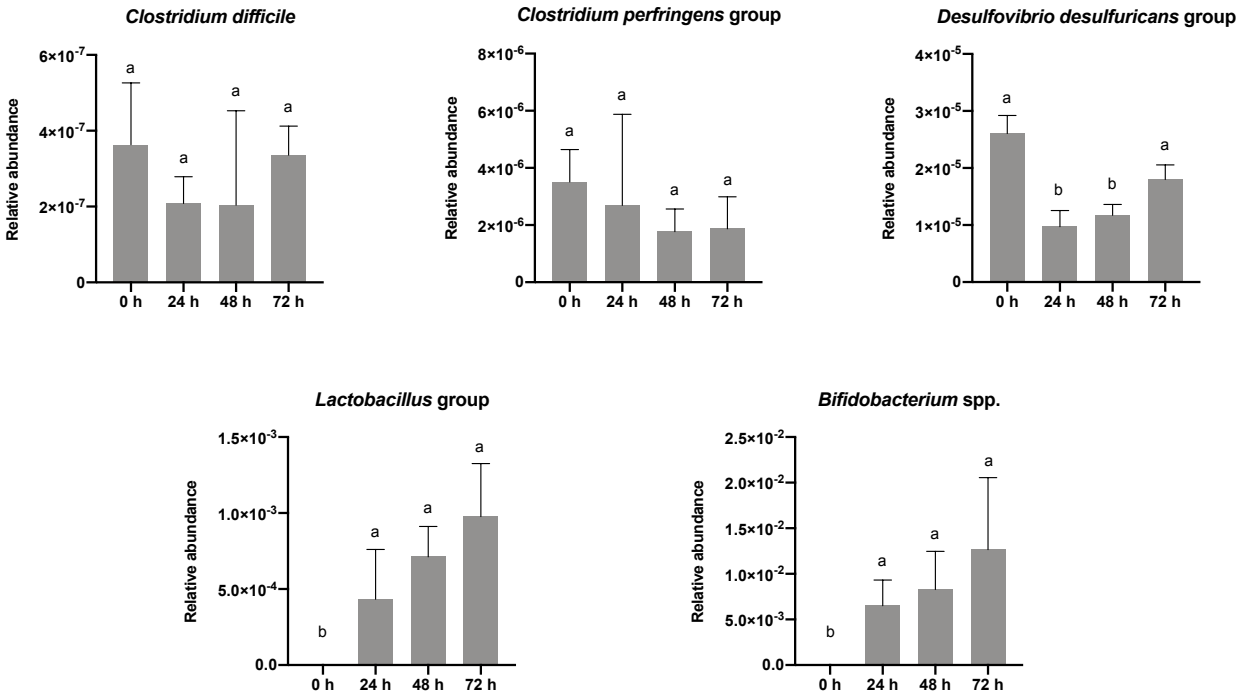

Figure 3. Relative abundance (ΔCt) of bacterial groups in the microbiota of child with autism spectrum disorder (ASD) using the SHIME® model over the course of synbiotic incubation periods (n = 4). Data are presented as mean ± SD. Different letters indicate statistical difference between incubation periods according to ANOVA and Tukey's post-test ($p < 0.05$).

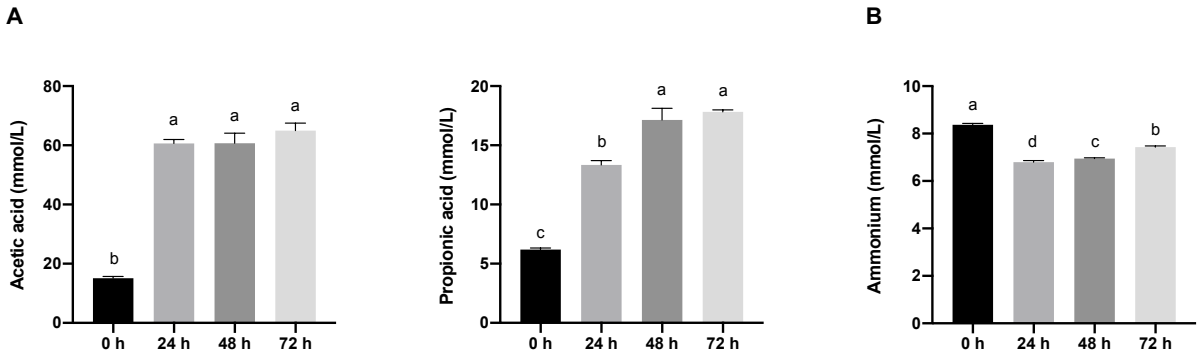

Figure 4. (**A**) Short-chain fatty acid and (**B**) ammonium concentrations in the microbiota of child with autism spectrum disorder (ASD) using the SHIME® model over the course of synbiotic incubation periods (n = 3). Data are presented as mean ± SD. Different letters indicate statistical difference between incubation periods according to ANOVA and Tukey's post-test ($p < 0.05$).

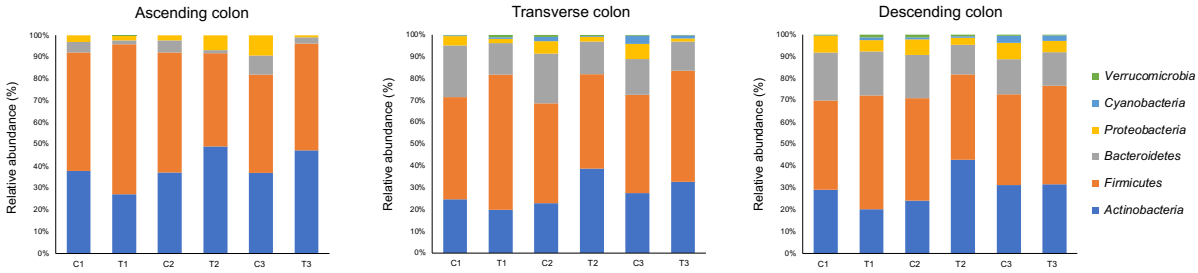

Figure 5. Relative abundance of different phyla in the microbiota of children with autism spectrum disorder (ASD) using the SHIME® model over the course of the probiotic, prebiotic, and synbiotic treatments. C1: Control 1 (control related to treatment 1); T1: Probiotic treatment (*L. reuteri* + *B. longum*); C2: Control 2 (control related to treatment 2); T2: Prebiotic treatment (GOS); C3: Control 3 (control related to treatment 3); T3: Synbiotic treatment (*L. reuteri* + *B. longum* + GOS).

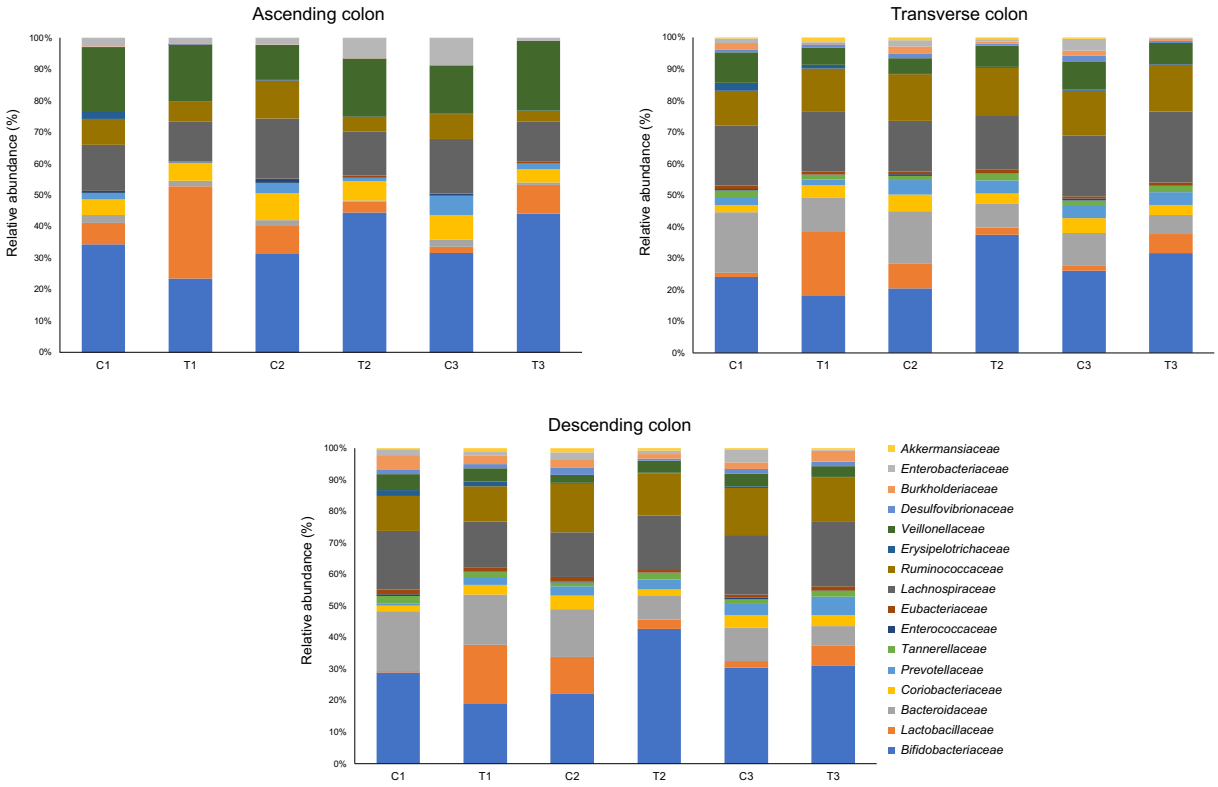

Figure 6. Relative abundance of different families in the microbiota of children with autism spectrum disorder (ASD) using the SHIME® model over the course of the probiotic, prebiotic, and synbiotic treatments. C1: Control 1 (control related to treatment 1); T1: Probiotic treatment (*L. reuteri* + *B. longum*); C2: Control 2 (control related to treatment 2); T2: Prebiotic treatment (GOS); C3: Control 3 (control related to treatment 3); T3: Synbiotic treatment (*L. reuteri* + *B. longum* + GOS).

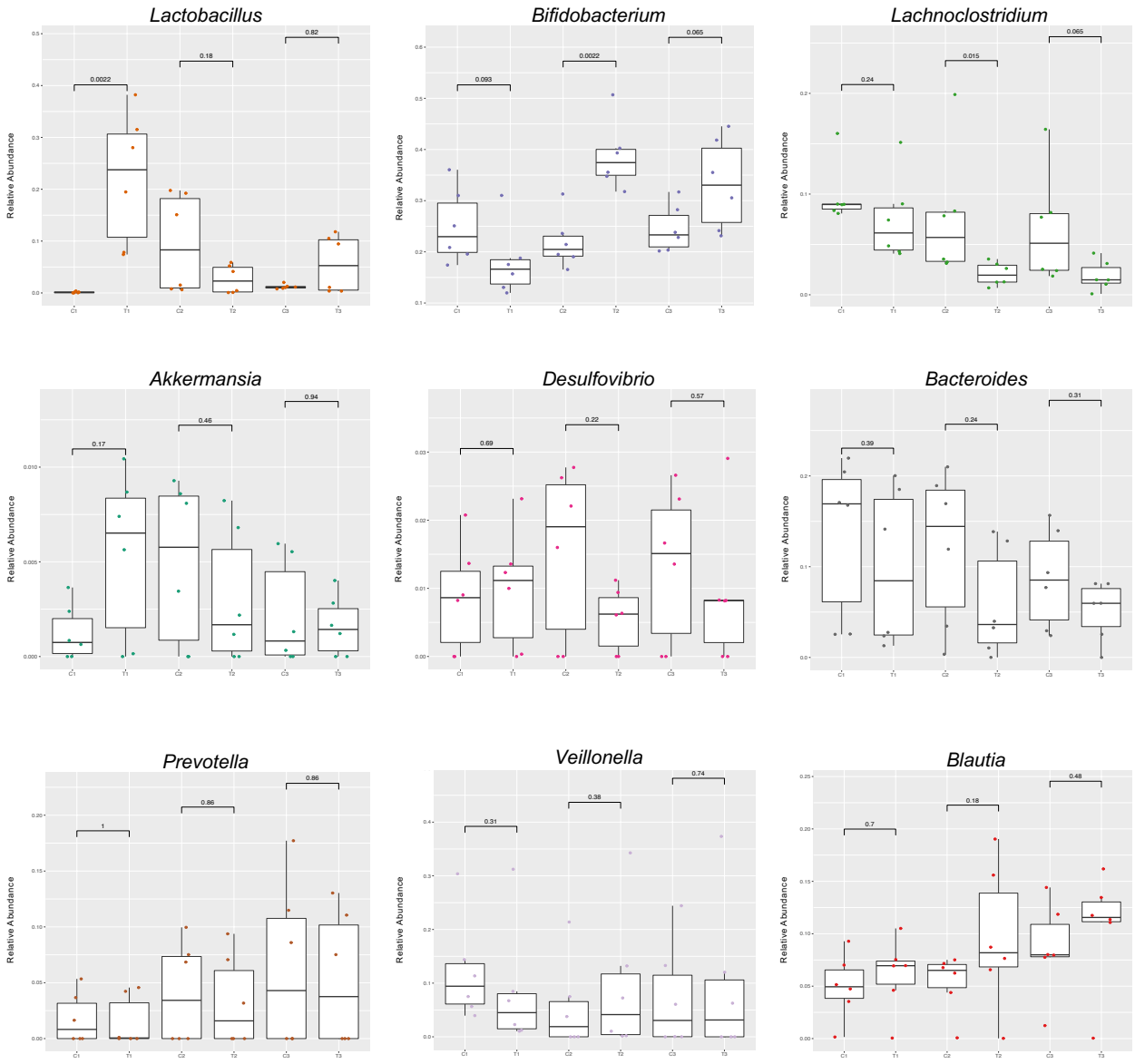

Figure 7. Relative abundance of bacterial genera in the microbiota of children with autism spectrum disorder (ASD) using the SHIME® model over the course of the probiotic, prebiotic, and synbiotic treatments. Differences in means were analyzed using the Wilcoxon test. C1: Control 1 (control related to treatment 1); T1: Probiotic treatment (*L. reuteri* + *B. longum*); C2: Control 2 (control related to treatment 2); T2: Prebiotic treatment (GOS); C3: Control 3 (control related to treatment 3); T3: Synbiotic treatment (*L. reuteri* + *B. longum* + GOS).

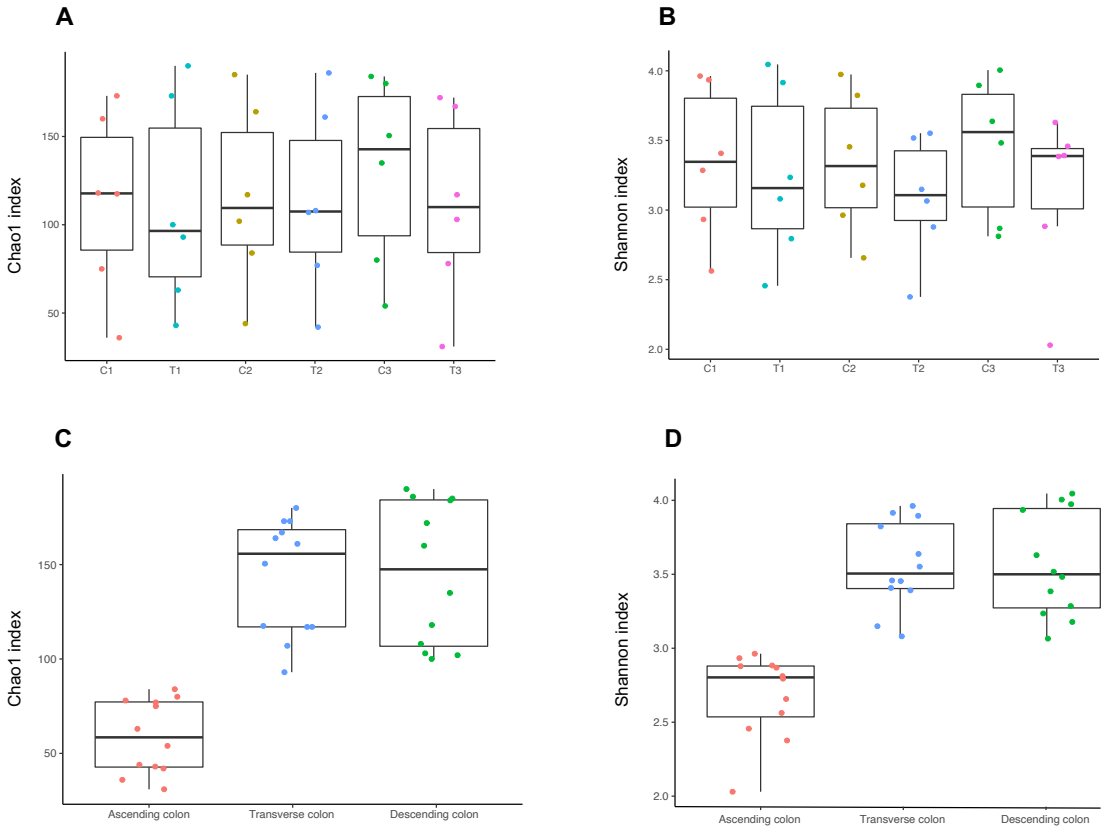

Figure 8. Alpha diversity metrics of the microbiota of children with autism spectrum disorder (ASD) using the SHIME® model. (**A**) Chao1 index ($p = 0.985$) and (**B**) Shannon diversity index ($p = 0.889$) over the course of the probiotic, prebiotic, and synbiotic treatments. (**C**) Chao1 index ($p < 0.001$) and (**D**) Shannon diversity index ($p < 0.001$) in the ascending, transverse, and descending colons. C1: Control 1 (control related to treatment 1); T1: Probiotic treatment (*L. reuteri* + *B. longum*); C2: Control 2 (control related to treatment 2); T2: Prebiotic treatment (GOS); C3: Control 3 (control related to treatment 3); T3: Synbiotic treatment (*L. reuteri* + *B. longum* + GOS).

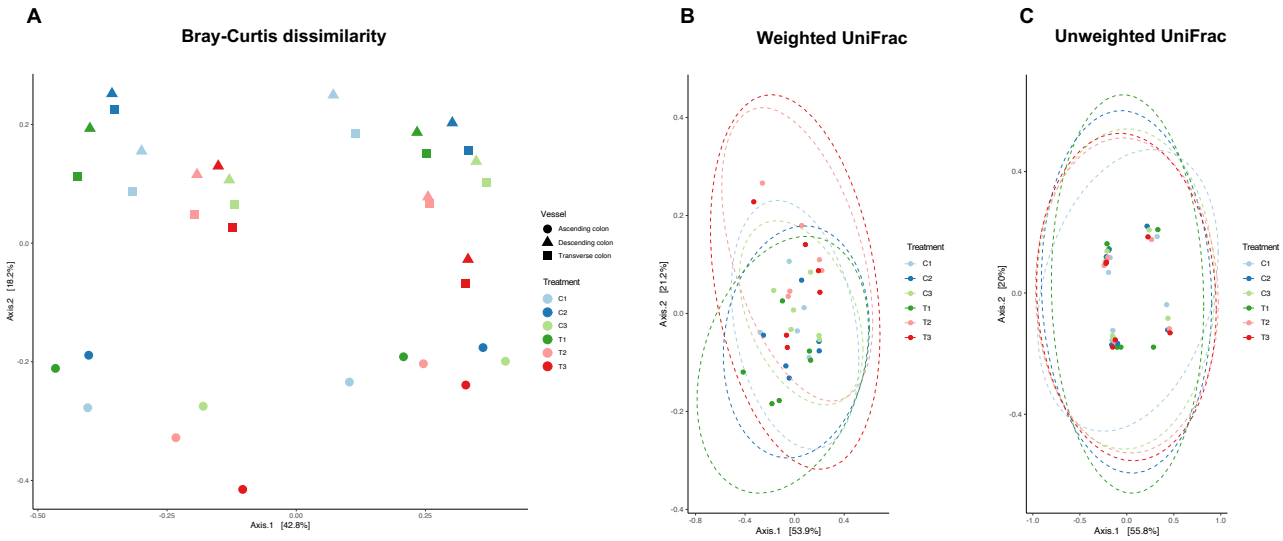

Figure 9. Principal coordinates analysis (PCoA) of bacterial beta diversity based on (**A**) Bray-Curtis dissimilarity, and (**B**) weighted and (**C**) unweighted UniFrac distances of the microbiota of children with autism spectrum disorder (ASD). Control 1 (control related to treatment 1); T1: Probiotic treatment (*L. reuteri* + *B. longum*); C2: Control 2 (control related to treatment 2); T2: Prebiotic treatment (GOS); C3: Control 3 (control related to treatment 3); T3: Synbiotic treatment (*L. reuteri* + *B. longum* + GOS).

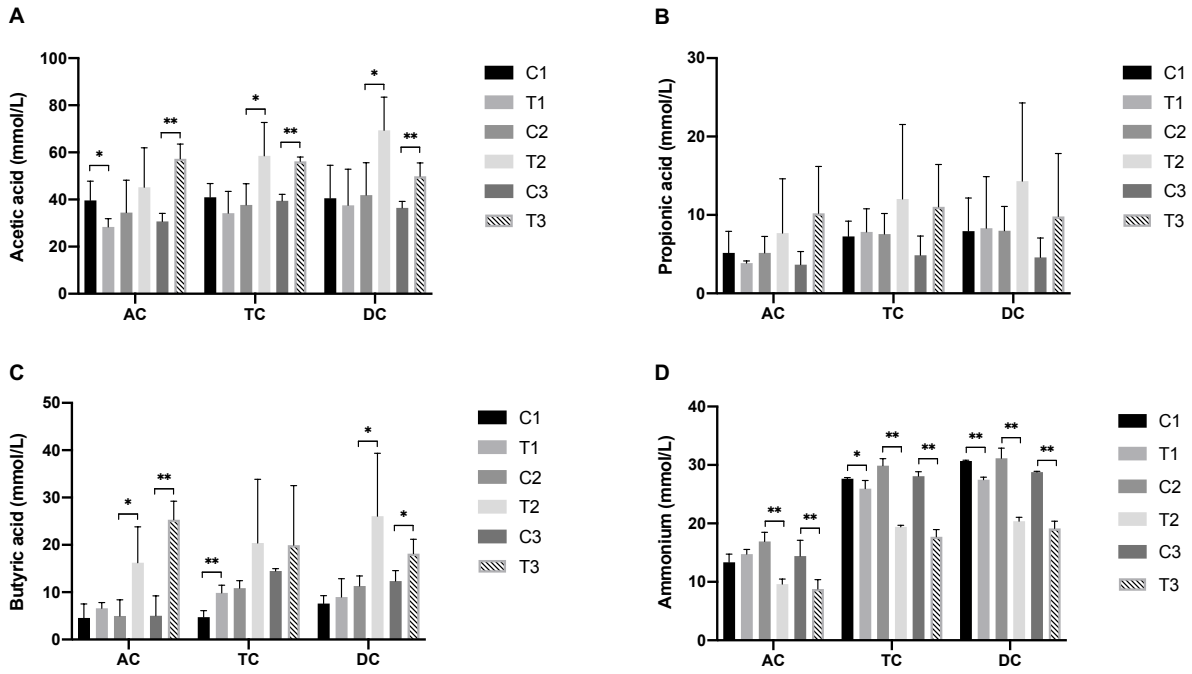

Figure 10. Short-chain fatty acid and ammonium concentrations in the ascending (AC), transverse (TC), and descending (DC) colons inoculated with fecal microbiota from children with autism spectrum disorder (ASD) over the course of the probiotic, prebiotic, and synbiotic treatments. (**A-C**) short-chain fatty acid concentration (n = 4). (**D**) ammonium concentration (n = 6). Data are presented as mean ± SD. Asterisks (*) represent significant difference relative to controls according to the paired t-test (*$p < 0.05$; **$p < 0.01$). C1: Control 1 (control related to treatment 1); T1: Probiotic treatment (*L. reuteri* + *B. longum*); C2: Control 2 (control related to treatment 2); T2: Prebiotic treatment (GOS); C3: Control 3 (control related to treatment 3); T3: Synbiotic treatment (*L. reuteri* + *B. longum* + GOS).

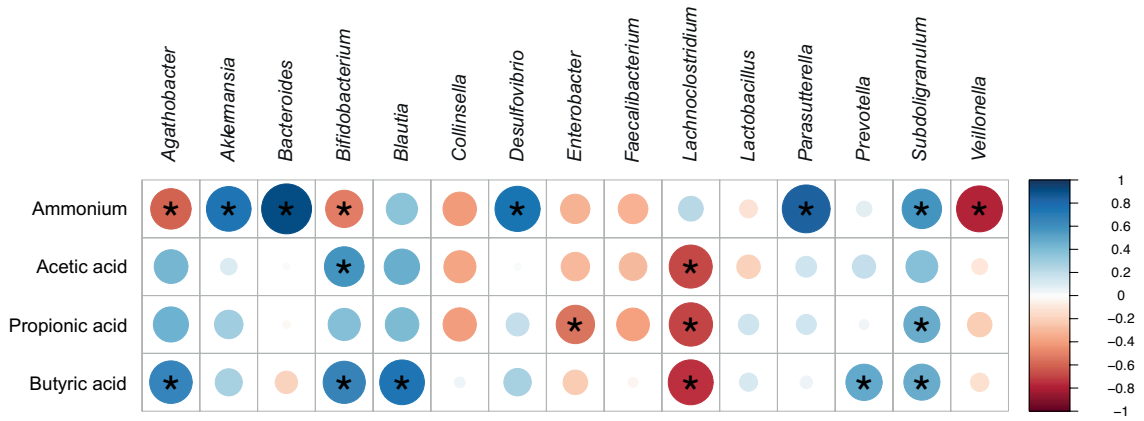

Figure 11. Correlation analysis between different bacterial genera and metabolite production in the samples obtained from the SHIME® model. The color indicates the magnitude of correlation. Asterisks (*) indicate significant correlations according to Spearman's correlation ($p < 0.05$).

Capítulo 2.

Impact of prebiotic on gut microbiota and social behaviors in a mouse model of autism spectrum disorder

Impact of prebiotic on gut microbiota and social behaviors in a mouse model of autism spectrum disorder

Ana Luiza Rocha Faria Duque [1]

Sean Willian Dooling [2,3,4]

Martina Sgritta [2,3]

I-Ching Wang [2,3]

Katia Sivieri [1]

Mauro Costa-Mattioli [2,3,4]

[1] Department of Food and Nutrition, School of Pharmaceutical Sciences, São Paulo State University (UNESP), Araraquara, Brazil.

[2] Department of Neuroscience, Baylor College of Medicine, Houston, Texas, USA.

[3] Memory and Brain Research Center, Baylor College of Medicine, Houston, Texas, USA.

[4] Department of Molecular & Human Genetics, Baylor College of Medicine, Houston, Texas, USA.

Abstract

A large body of preclinical literature supports the notion of a bidirectional communication system linking the gut and the brain, known as the microbiota-gut-brain axis. Recent evidence indicates that gut microbes can modulate central nervous system (CNS)-driven behaviors in a very powerful way. Thus, the administration of prebiotics, which support gut microbes, has been investigated as novel non-invasive therapeutic alternative to treat neurological disorders. The aim of this study was to evaluate the impact of the prebiotic galacto-oligosaccharide (GOS) on the gut microbiota composition and behavioral deficits in a genetic mouse model for autism spectrum disorder (*Cntnap2*$^{-/-}$ mice). Briefly, mice were divided into 3 treatment groups: 1: Wild-type (WT) control mice; 2: *Cntnap2*$^{-/-}$ mice treated with vehicle; 3: *Cntnap2*$^{-/-}$ mice treated with GOS. We measured whether the different treatments: a) altered the gut microbiota composition by using 16S rRNA gene sequence analysis, and b) reversed the autism symptoms (hyperactivity, social and repetitive behaviors). Briefly, we found that the bacterial composition and phylogenetic profile of the microbial communities differed significantly between WT and *Cntnap2*$^{-/-}$ groups; however, GOS had no effect in modulating gut microbiota composition. Additionally, we observed that *Cntnap2*$^{-/-}$ + vehicle mice showed hyperactivity symptoms and impaired social behavior compared to WT mice. The GOS treatment significantly improved social behavior but did not affect locomotor activity or repetitive behavior in a genetic mouse model for autism.

Keywords: Autism; Microbiota-gut-brain axis; Galacto-oligosaccharide; Autism-like behavior; Social deficits.

1. Introduction

Autism spectrum disorder (ASD) is a neurodevelopmental disorder characterized by deficits in communication and social interactions and the presence of stereotyped behaviors (1). The etiology of ASD remains unclear, but the gut microbiota has been suggested to play a role in ASD (2). Individuals with ASD often present gastrointestinal dysfunction including increased intestinal permeability (3,4). Intriguingly, there is a positive correlation between the severity of behavioural and gastrointestinal symptoms (5,6), which suggests a communication between the gut and the brain (7).

Several studies report that the composition of the gut microbiota differs between individuals with ASD and neurotypical individuals (8-12). Recent evidence indicates that gut microbes have a very powerful influence on central nervous system (CNS)-driven behaviours (13,14). More specifically, experiments in mouse models have shown that gut microbes can modulate endophenotypes traditionally associated with brain-centered disorders, including ASD (15-17). The *Cntnap2*$^{-/-}$ mice are considered a genetic mouse model for ASD (18,19). Consistent with some of the behavioral abnormalities observed in individuals with mutations in CNTNAP2, loss of Cntnap2 (contactin-associated protein-like 2, which encodes the protein Caspr2) (20,21), in mice leads to social deficits and hyperactivity (18,19,22-24).

The administration of pro- and prebiotics as a strategy to modulate the microbiota-gut-brain axis has been suggested as a potential therapeutic approach to ameliorate both gastrointestinal and behavioural abnormalities associated with ASD (25-29). However, more research is needed to determine the efficacy of these treatment strategies, especially regarding prebiotics.

Prebiotics are defined as a substrate that is selectively utilized by host microorganisms, usually associated with the stimulation of beneficial bacteria such as

Bifidobacterium and/or *Lactobacillus* spp., conferring a health benefit. The prebiotics most extensively documented to have health benefits in humans are the non-digestible oligosaccharides fructans (fructo-oligosaccharides (FOS) and inulin) and galactans (galacto-oligosaccharides (GOS)) (30). Many bacteria ferment these prebiotics to produce short-chain fatty acids (SCFA), which can play a key role in neuro-immunoendocrine regulation (31,32). Interestingly, mice fed with a combination of GOS and FOS showed reduced stress-induced corticosterone release and depression-like and anxiety-like behavior, suggesting that these changes could be mediated partially by SCFAs (33). More importantly, Grimaldi et al. (26) reported a significant improvement in anti-social behavior of children with ASD after a 6-week intervention of B-GOS® combined with an exclusion diet.

Considering the current interest in the role of the gut microbiota in behavioral neuroscience, investigations of how the prebiotics affect the microbiota-gut-brain axis can help decipher how alterations in the microbiota can positively impact behavioral disorders related to autism. Thus, in this study we investigated whether administration of GOS affects behavior and microbiota composition in a genetic mouse model for ASD.

2. Material and methods

2.1. Animals

WT C57BL/6J and *Cntnap2*$^{-/-}$ (*Cntnap2*^{tm1Pele}) mice were obtained from Jackson Laboratories (Bar Harbor, ME) and colonies of these mice were maintained at Baylor College of Medicine. Subject mice were separated by sex at weaning and housed at 2-4 mice per cage. All mice were kept on a 12 hr light/dark cycle and had access to food and water *ad libitum*. Animal care and experimental procedures were approved by Baylor

College of Medicine's Institutional Animal Care and Use Committee in accordance with all guidelines set forth by the U.S. National Institutes of Health.

2.2. Prebiotic treatment

The prebiotic galacto-oligosaccharide Vivinal® GOS was supplied by FrieslandCampina Ingredients (Vinhedo, Brazil). GOS or phosphate buffered saline (PBS) (vehicle) were added to the drinking water daily to minimize dosage variability. The experimental group received prebiotic (0.3-0.4 g of GOS/mouse/day), while the control group received equal volume of PBS. Mice consumed the treated water *ad libitum* during the treatment period. Fecal sample collection and behavioral assays were initiated 4 weeks after the beginning of the treatment period (Figure 1). Treatment duration and dosage were based on previous studies that showed behavioral and neurochemical effects in rodents after treatments with prebiotics (33-35).

2.3. 16S rRNA gene sequence

Fresh fecal samples were collected from mice, snap frozen and stored at -80°C until prepared for sequencing. 16S rRNA gene sequencing was performed by the Center for Metagenomic & Microbiome Research at Baylor College of Medicine as previously described (15,27). Methods were adapted from protocols developed for the NIH-Human Microbiome Project (36,37). Bacterial genomic DNA was extracted using MO BIO PowerSoil DNA Isolation Kit (MO BIO Laboratories). The 16S rDNA V4 region was amplified by PCR and sequenced in the MiSeq platform (Illumina) using the 2x250 bp paired-end protocol yielding pair-end reads that overlap almost completely. The primers used for amplification contain adapters for MiSeq sequencing and single-end barcodes allowing pooling and direct sequencing of PCR products. The 16S rRNA gene read pairs

were demultiplexed based on the unique molecular barcodes, and reads were merged using USEARCH v7.0.1090 (38), allowing zero mismatches and a minimum overlap of 50 bases. Merged reads were trimmed at first base with Q5. A quality filter was applied to the resulting merged reads. Reads containing above 0.05 expected errors were discarded. 16S rRNA gene sequences were clustered into Operational Taxonomic Units (OTUs) at a similarity cutoff value of 97% using the UPARSE algorithm (39). OTUs were mapped using an optimized version of the SILVA Database (40) containing only the 16S v4 region to determine taxonomies. Abundances were recovered by mapping the demultiplexed reads to the UPARSE OTUs. A custom script constructed a rarefied OTU table from the output files generated in the previous two steps for downstream analyses of alpha-diversity, beta-diversity, and phylogenetic trends.

2.4. Behavioral tests

All behavior tests were conducted on 7-11 week old mice. Mice were habituated to the experimenter for 3 days prior to the start of the behavioral test. All tests were conducted during the light cycle.

2.4.1. Open field

Mice were placed in an open arena (40 x 40 x 20 cm) and allowed to explore freely for 10 minutes. Their position was continually monitored using the tracking software AnyMaze. Distance traveled, speed, and time spent in the center of the arena were recorded and automatically measured throughout the task. The center of the arena was defined as the interior 20 x 20 cm area (27).

2.4.2. Three-chamber social test

The three-chamber test was performed as previously described (15,27,41). Mice were first habituated for a 10-minutes period in an empty 60 x 40 x 23 cm Plexiglas arena divided into three interconnected chambers of equal size (left, center, right). Sociability was evaluated during a second 10-minutes period in which the subject could interact either with an empty wire cup (Empty) or a wire cup containing a stranger mouse (Mouse 1). The interaction time was determined by measuring the time the subject mouse spent sniffing or climbing upon either the empty cup or the cup containing the stranger mouse. Empty cup/stranger mouse placement in the left or right chamber during the sociability test was alternated between trials in order to avoid bias. Preference for social novelty was assayed in a third 10-minutes period, by introducing a second stranger mouse (Mouse 2) into the previously empty wire cup. The time spent interacting with either Mouse 1 or Mouse 2 was measured as mentioned above. Interaction time was recorded using the automated AnyMaze software by trained, independent observers who were blind to the treatment group.

2.4.3. Reciprocal social interaction

Mice were placed in a 25 x 25 x 25 cm Plexiglas arena with an unfamiliar conspecific matched according to genotype, age, sex, and treatment. Trained, independent observers recorded the time a pair of mice socially interacted, using the AnyMaze software. Interactions included close following, touching, nose-to-nose sniffing, nose-to-anus sniffing, and/or crawling over/under each other. The human observers were blind to the treatment group (15,27).

2.4.4. Self-grooming

The self-grooming behavior test was performed as previously described with modifications (42). Mice were placed individually in a standard-sized cage and habituated for a 5-minutes period. Following this habituation period, cumulative time spent grooming all body regions was evaluated for a 10-minutes period. The self-grooming behavior was recorded using the AnyMaze software by trained, independent observers who were blind to the treatment group.

2.4.5. T-maze spontaneous alternation task

The spontaneous alternation task was performed as previously described (43,44). Mice were placed in a black Plexiglas T-maze apparatus with walls 25 cm high and arms 30 cm long and 9 cm wide. A removable central partition was used during the sample phase but not in the test phase of each trial. Guillotine doors were positioned at the entrance to each goal arm. Both doors were raised at the beginning of the sample phase and the mouse was placed at the end of the start arm facing away from the goal arms. Each mouse was allowed to make a free choice between the two goal arms; after its tail had cleared the door of the chosen arm, the door was closed, and the mouse was allowed to explore the arm for 30 s. The mouse was then returned to the end of the start arm, with the central partition removed and both guillotine doors raised, signaling the beginning of the test phase. Again, the mouse was allowed to make a free choice between the two goal arms. This sequence (trial) was repeated 10 times per day for two days with each mouse. The percentage of alternation was averaged over the two days. Trials that were not completed within 90 s were terminated and disregarded during analysis.

2.4.6. Nest-building test

Mice were housed individually with food and water in home cages approximately 1 h before the dark phase. Nesting material (Nestlets), 5-cm pressed cotton square, (Ancare, Bellmore, NY, USA) was placed in each cage. The weight and nest quality were measured on the following morning. The nest quality was evaluated using 5-point nest-rating scale: (1) Nestlet not noticeably touched; (2) Nestlet partially torn; (3) Nestlet mostly shredded but often no identifiable nest site; (4) An identifiable but flat nest; (5) A (near) perfect nest. Any unshredded material left (untorn Nestlet pieces) after a bout of nesting also was weighed, providing a semi-independent objective assay of nesting ability. The examiner was blind to the treatment group (45).

2.5. Statistical analysis

Data were presented as mean ± SEM. Statistical analyses performed include one- or two-way ANOVA with Bonferroni post hoc analysis to correct for multiple comparisons, unless otherwise indicated. p values were presented in the figure legends. $p < 0.05$ was considered statistically significant. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. GraphPad's Prism 8 (La Jolla, CA) software was used to perform statistical analyses and generate graphical data representations. The analyses for 16S rRNA gene sequencing were conducted in RStudio 0.99.292, utilizing the phyloseq package (46) to import sample data and calculate alpha and beta-diversity metrics. 16S rRNA gene sequencing data was analyzed using Silva 115 and 123 (40). Analyses were performed on datasets that were rarefied 1,000x, then averaged and rounded. Significance of categorical variables were determined using the non-parametric Kruskal-Wallis test for three or more categories comparisons. Principal coordinate plots employed the Monte Carlo

permutation test to estimate p values. p values were adjusted for multiple comparisons with the FDR algorithm (47).

3. Results

3.1. Microbiota composition

Individuals with ASD often present an altered gut microbiota composition (17,48). To examine whether *Cntnap2*$^{-/-}$ mice display alterations on gut microbial composition and if GOS treatment induces alterations on gut microbiota, we analyzed the bacterial composition and community structure in the feces of *Cntnap2*$^{-/-}$ mice and wild-type (WT) littermates by 16S rRNA gene sequencing. The bacterial composition as assessed by weighted and unweighted UniFrac distances revealed that WT mice harbor an intestinal microbial composition distinct from *Cntnap2*$^{-/-}$ mice (Figures 2A and 2B). By contrast, there were no differences in alpha diversity, or within group variation, such as the number of OTUs, Shannon Diversity, and Chao1 richness (Figure 3).

Analysis of differentially abundant taxa at the phylum level analysis showed some differences between the groups (Figure 4). *Firmicutes*, *Actinobacteria* and *Bacteroidetes* represented the most abundant phyla whereas *Verrucomicrobia* and *Proteobacteria* were the least abundant in both WT and *Cntnap2*$^{-/-}$ mice. We observed a higher relative abundance of *Firmicutes* in WT compared to *Cntnap2*$^{-/-}$ + vehicle mice while *Bacteroidetes* was more abundant in *Cntnap2*$^{-/-}$ + vehicle mice. In addition, WT mice showed elevated *Verrucomicrobia* relative abundance compared to *Cntnap2*$^{-/-}$ mice ($p = 0.002$). We also found that *Cntnap2*$^{-/-}$ + GOS mice showed higher levels of *Proteobacteria* compared to *Cntnap2*$^{-/-}$ + vehicle mice ($p = 0.046$), whereas no significant difference in WT mice was observed (Figure 4). These results suggest that the increase of *Proteobacteria* levels is not related to the GOS treatment.

3.2. Open field test

Attention-deficit hyperactivity disorder (ADHD) and anxiety are common comorbidities of ASD (49,50). Open field test involves mapping an animal's movement in an open arena to measure locomotion and anxiety-related behavior (51). When we assessed the locomotor activity in WT and *Cntnap2*$^{-/-}$ mice, we observed that *Cntnap2*$^{-/-}$ + vehicle mice were hyperactive compared to WT mice ($p<0.05$), as determined by both parameters higher distance traveled and speed. Consistent with our results, Peñagarikano et al. (19) reported a high rate of hyperactivity symptoms in *Cntnap2*$^{-/-}$ mice compared to WT. However, there was no significant effect of GOS administration on locomotor activity levels in *Cntnap2*$^{-/-}$ mice. No significant changes in anxiety-related behavior, as measured by the time spent in the center of the arena, were observed comparing WT and *Cntnap2*$^{-/-}$ mice (Figure 5). Despite the administration of either FOS + GOS (33) and Sialyllactose (34) have been shown to reduce anxiety-like behaviors in stress-induced mice, GOS did not significantly affect anxiety-like behaviors in the *Cntnap2*$^{-/-}$ mice in our experiments.

3.3. Social behavior

Social deficits are one of the most salient features of individuals with ASD (1) and *Cntnap2*$^{-/-}$ mice show impaired social behavior as previously reported (18). We first assessed social behavior in WT and *Cntnap2*$^{-/-}$ mice using the three-chamber test for sociability and social novelty. To test the sociability, we compared the time that the experimental mouse spent interacting with either a stranger mouse (Mouse 1) or an empty wired cup (Empty). We observed that WT mice displayed normal sociability as reflected by their preferential interaction with the stranger mouse. *Cntnap2*$^{-/-}$ + vehicle

mice showed no preference for the stranger mouse over the empty cup, indicating impaired sociability. In contrast, *Cntnap2*$^{-/-}$ + vehicle mice displayed normal preference for social novelty as they spent more time interacting with the novel mouse (Mouse 2) than with the familiar one (Mouse 1). Interestingly, the treatment with GOS significantly improved sociability in *Cntnap2*$^{-/-}$ mice (Figure 6A).

We also assessed reciprocal social interactions by recording the amount of time a pair of mice, unfamiliar with each other, spent interacting in a neutral arena. We observed that *Cntnap2*$^{-/-}$ + vehicle had fewer reciprocal social interactions when compared to WT mice ($p<0.05$), indicating an impaired social behavior. Moreover, the treatment with GOS reversed the social deficits in *Cntnap2*$^{-/-}$ mice (Figure 6B). Thus, these results indicate that treatment with GOS reverses the social deficits in *Cntnap2*$^{-/-}$ mice. Burokas et al. (33) showed that prebiotic administration had no effect for sociability and preference for social novelty in WT mice but did rescue social deficits in a chronic social defeat paradigm. In line with our results, previous studies have shown that probiotics treatments, specifically treatment with the commensal bacterial species *L. reuteri* could reverse social deficits in genetic, environmental, and idiopathic models of ASD (15,27,28).

3.4. Self-grooming behavior, T-maze spontaneous alternation and nest-building

Along with deficits in social communication and interaction, repetitive behavior comprises the defining core features of ASD (52). Repetitive behaviors are often seen in some mouse models of ASDs (42,53). We assessed the repetitive behavior in mice using two tests: spontaneous self-grooming and T-maze spontaneous alternation. First, we measured the time that mice spent engaged in self-grooming behaviors. Our results

showed that *Cntnap2*$^{-/-}$ + vehicle mice did not exhibit repetitive behaviors compared to WT mice and GOS treatment did not show effect in *Cntnap2*$^{-/-}$ mice (Figure 7A).

Next, to test behavioral inflexibility in mice, we measured the willingness of mice to explore a new environment in a T-maze as previously described by Johnson et al. (44). While exploring the maze, mice normally tend to alternate between the two arms. However, if the mice tend to repeatedly explore the same arm of the maze, it is an indication of behavioral inflexibility. Our results showed that *Cntnap2*$^{-/-}$ mice did not exhibit deficits in spontaneous alternation ($p>0.05$) and GOS treatment did not influence the inflexibility behavior ($p>0.05$) (Figure 7B).

Finally, we assessed mice for their ability to build nests. Nest building has been reported as disrupted in mice with hyperactivity (54,55). However, we observed that *Cntnap2*$^{-/-}$ + vehicle mice did not show significantly differences in nesting behavior compared to WT mice and GOS treatment did not show effect in *Cntnap2*$^{-/-}$ mice (Figure 7C).

4. Discussion

Both genetic and environmental factors play a crucial role in the etiology of neurodevelopmental disorders including ASD (56). Individuals with ASD often present an altered microbiota composition (12,57,58). Indeed, while some subjects with ASD present an increased *Firmicutes/Bacteroidetes* ratio (12,29), others show opposite results (57,59). Differences on the profile of the microbial communities using several ASD mouse model were also reported (15,16,27). Sgritta et al. (27) have not found significant difference in abundance of *Firmicutes/Bacteroidetes* ratio in *Shank3B*$^{-/-}$ mice, but the BTBR and VPA mice models showed an increased *Bacteroidetes/Firmicutes* ratio compared to control mice. In our study, no major differences were observed at the phylum

level for WT and *Cntnap2*$^{-/-}$ mice. Thus, further analyses are needed to elucidate the role of the gut microbiota on the *Cntnap2*$^{-/-}$ model. Although many studies have explored the composition of ASD-related microbiota, the results are still controversial. Possibly due to variations in sampling strategies and methodologies employed or the broad phenotypical variability of ASD in both children and animal models (12).

In addition to changes in gut microbiota composition, gastrointestinal issues may contribute to ASD behavioral symptoms (60,17). In this sense, a growing body of evidence supports the notion of a bidirectional communication system linking the gut and the brain, known as the microbiota-gut-brain axis (17,61,62). More specifically, experiments in mouse models have shown that gut microbes can modulate endophenotypes traditionally associated with brain-centered disorders, such as anxiety-like behavior and social deficits commonly reported in ASD individuals (15-17,27). Here, we used the *Cntnap2*$^{-/-}$ genetic mouse model for ASD because it exhibits behavioral abnormalities including social deficits and hyperactivity (18,19). We also found that *Cntnap2*$^{-/-}$ mice displayed hyperactivity and social deficits when compared to WT mice in addition to some changes in gut microbiota composition.

There is growing interest in profiling the gut bacteria to potentially treat autistic symptomatology and comorbidities by modulating the gut microbiota by administering pro- and prebiotics (48). Prebiotics are often used as modulators of the gut microbiota and immune system (63). However, few studies have been focused on the effects of prebiotics on the CNS (35,64) and behavior (34). Here, we showed that GOS treatment significantly improved social deficits in *Cntnap2*$^{-/-}$ mice but did not modulate the gut microbiota composition. In contrast with our results, several studies in humans and animal models have reported that treatments with prebiotics could improve not only the composition of gut microbiota but also complex behaviours (26,33,65). Although the

precise mechanism by which prebiotics promote these effects has not been widely explored, it is accepted that gut bacteria initiate signals that are transmitted from the gut to the CNS mediated via neural, immune and metabolic routes (7,14).

Prebiotics can modulate the gut microbiota, serving as a substrate that is selectively utilized by host microorganisms which in turn ferment them into SCFAs (30). SCFAs are microbial metabolites that have shown neuroactive properties. These metabolites can cross the blood-brain barrier and modulate CNS functions, brain development and behavior (32,66). The SCFAs may be indirectly able of influencing brain physiology and behavior through binding to and activating free fatty acid receptors (FFARs) expressed on the vagus nerve (14). Burokas et al. (33) showed that the prebiotics GOS and FOS were able to modulate stress-related behaviors in mice, which was associated with alterations in gut microbiota composition and metabolite production. These behavioral changes may have been partially mediated by SCFAs. Previous studies have investigated SCFAs and their relation to ASD (8,57,65). Although the association between these bacterial metabolites and ASD remains unclear, its role in ASD must be considered for future research in humans and animal models of ASD.

5. Conclusion

Taken together, our results showed that GOS had no effect in modulating gut microbiota but improved social deficits in a mouse model for ASD. This study suggests a beneficial role of GOS for some behavioral symptoms associated with ASD. However, additional preclinical and clinical studies will be necessary to evaluate the potential effects of prebiotics in ASD and may help elucidate the mechanisms involved in the communication of the microbiota-gut-brain axis.

References

1. American Psychiatric Association - APA Diagnostic and statistical manual of mental disorders (DSM-5). 5th ed. Washington DC: Am Psyc Assoc. 2013.

2. Li Q, Zhou J-M. The microbiota-gut-brain axis and its potential therapeutic role in autism spectrum disorder. Neuroscience. 2016;324:131-139.

3. Holingue C, Newill C, Lee LC, Pasricha PJ, Daniele FM. Gastrointestinal symptoms in autism spectrum disorder: A review of the literature on ascertainment and prevalence. Autism Res. 2018;11(1):24-36.

4. Hsiao EY. Gastrointestinal issues in autism spectrum disorder. Harv Rev Psychiatry. 2014;22:104-111.

5. Adams JB, Johansen LJ, Powell LD, Quig D, Rubin RA. Gastrointestinal flora and gastrointestinal status in children with autism - comparisons to typical children and correlation with autism severity. BMC Gastroenterol. 2011;11:22.

6. McElhanon BO, McCracken C, Karpen S, Sharp WG. Gastrointestinal symptoms in autism spectrum disorder: A meta-analysis. Pediatrics. 2014;133:872-883.

7. Morais LH, Schreiber HL, Mazmanian SK. The gut microbiota–brain axis in behaviour and brain disorders. Nat Rev Microbiol. 2021;19:241-255.

8. Kang DW, Ilhan ZE, Isern NG, Hoyt DW, Howsmon DP, Shaffer M, et al. Differences in fecal microbial metabolites and microbiota of children with autism spectrum disorders. Anaerobe. 2018;49:121-131.

9. Liu F, Li J, Wu F, Zheng H, Peng Q, Zhou H. Altered composition and function of intestinal microbiota in autism spectrum disorders: a systematic review. Transl Psychiatry. 2019;9:43.

10. Luna RA, Oezguen N, Balderas M, Venkatachalam A, Runge JK, Versalovic J, et al. Distinct microbiome-neuroimmune signatures correlate with functional abdominal pain in children with autism spectrum disorder. Cell Mol Gastroenterol Hepatol. 2016;11(2):218-230.

11. Son JS, Zheng LJ, Rowehl LM, Tian X, Zhang Y, Zhu W, et al. Comparison of fecal microbiota in children with autism spectrum disorders and neurotypical siblings in the simons simplex collection. PLoS One. 2015;10:e0137725.

12. Strati F, Cavalieri D, Albanese D, De Felice C, Donati C, Hayek J, et al. New evidences on the altered gut microbiota in autism spectrum disorders. Microbiome. 2017;5(1):24.

13. Sharon G, Cruz NJ, Kang DW, Gandal MJ, Wang B, Kim YM, et al. Human gut microbiota from autism spectrum disorder promote behavioral symptoms in mice. Cell. 2019;30(6):1600-1618.

14. Sherwin E, Bordenstein SR, Quinn JL, Dinan TG, Cryan JF. Microbiota and the social brain. Science. 2019;366(6465):eaar2016.

15. Buffington SA, Di Prisco GV, Auchtung TA, Ajami NJ, Petrosino JF, Costa-Mattioli M. Microbial reconstitution reverses maternal diet-induced social and synaptic deficits in offspring. Cell. 2016;165(7):1762-1775.

16. Hsiao EY, McBride SW, Hsien S, Sharon G, Hyde ER, McCue T, et al. Microbiota modulate behavioral and physiological abnormalities associated with neurodevelopmental disorders. Cell. 2013;155(7):1451-63.

17. Vuong HE, Hsiao EY. Emerging roles for the gut microbiome in autism spectrum disorder. Biol Psychiatry. 2017;81:411-423.

18. Peñagarikano O, Abrahams BS, Herman EI, Winden KD, Gdalyahu A, Dong H, et al. Absence of CNTNAP2 leads to epilepsy, neuronal migration abnormalities, and core autism-related deficits. Cell. 2011;147(1):235-46.

19. Peñagarikano O, Lázaro MT, Lu XH, Gordon A, Dong H, Lam HA, et al. Exogenous and evoked oxytocin restores social behavior in the Cntnap2 mouse model of autism. Sci Transl Med. 2015;7(271):271ra8.

20. Poliak S, Gollan L, Martinez R, Custer A, Einheber S, Salzer JL, et al. Caspr2, a new member of the neurexin superfamily, is localized at the juxtaparanodes of myelinated axons and associates with K+ channels. Neuron. 1999;24(4):1037-1047.

21. Poliak S, Gollan L, Salomon D, Berglund EO, Ohara R, Ranscht B, et al. Localization of Caspr2 in myelinated nerves depends on axon-glia interactions and the generation of barriers along the axon. J Neurosci. 2001;21(19):7568-7575.

22. Bakkaloglu B, O'Roak BJ, Louvi A, Gupta AR, Abelson JF, Morgan TM, et al. Molecular cytogenetic analysis and resequencing of contactin associated protein-like 2 in autism spectrum disorders. Am J Hum Genet. 2008;82(1):165-173.

23. O'Roak BJ, Deriziotis P, Lee C, Vives L, Schwartz JJ, Girirajan S, et al. Exome sequencing in sporadic autism spectrum disorders identifies severe de novo mutations. Nat Genet. 2011;43(6):585-589.

24. Buffington SA, Dooling SW, Sgritta M, Noecker C, Murillo OD, Felice DF, et al. Dissecting the contribution of host genetics and the microbiome in complex behaviors. Cell. 2021;184(7):1740-1756.e16.

25. Doenyas C. Gut microbiota, inflammation, and probiotics on neural development in autism spectrum disorder. Neuroscience. 2018;374:271-286.

26. Grimaldi R, Gibson GR, Vulevic, J, Giallourou N, Castro-Mejía JL, Hansen LH, et al. A prebiotic intervention study in children with autism spectrum disorders (ASDs). Microbiome. 2018;6:133.

27. Sgritta M, Dooling SW, Buffington SA, Momin EN, Francis MB, Britton RA, et al. Mechanisms underlying microbial-mediated changes in social behavior in mouse models of autism spectrum disorder. Neuron. 2019;101(2):246-259.

28. Tabouy L, Getselter D, Ziv O, Karpuj M, Tabouy T, Lukic I, et al. Dysbiosis of microbiome and probiotic treatment in a genetic model of autism spectrum disorders. Brain Behav Immun. 2018;73:310-319.

29. Tomova A, Husarova V, Lakatosova S, Bakos J, Vlkova B, Babinska K, Ostatnikova D. Gastrointestinal microbiota in children with autism in Slovakia. Physiol Behav. 2015;138:179-87.

30. Gibson G, Hutkins R, Sanders ME, Prescott SL, Reimer RA, Salmien SJ, et al. Expert consensus document: The International Scientific Association for Probiotics and Prebiotics (ISAPP) consensus statement on the definition and scope of prebiotics. Nat Rev Gastroenterol Hepatol. 2017;14:491-502.

31. Dalile B, Van Oudenhove L, Vervliet B, Verbeke K. The role of short-chain fatty acids in microbiota–gut–brain communication. Nat Rev Gastroenterol Hepatol. 2019;16:461-478.

32. Silva YP, Bernardi A, Frozza RL. The role of short-chain fatty acids from gut microbiota in gut-brain communication. Front Endocrinol (Lausanne). 2020;11:25.

33. Burokas A, Arboleya S, Moloney RD, Peterson VL, Murphy K, Clarke G. Targeting the microbiota-gut-brain axis: Prebiotics have anxiolytic and antidepressant-like effects and reverse the impact of chronic stress in mice. Biol Psychiatry. 2017;82(7):472-487.

34. Tarr AJ, Galley JD, Fisher SE, Chichlowski M, Berg BM, Bailey MT. The prebiotics 3'sialyllactose and 6'sialyllactose diminish stressor-induced anxiety-like behavior and colonic microbiota alterations: Evidence for effects on the gut-brain axis. Brain Behav Immun. 2015;50:166-177.

35. Savignac HM, Couch Y, Stratford M, Bannerman DM, Tzortzis G, Anthony DC, et al. Prebiotic administration normalizes lipopolysaccharide (LPS)-induced anxiety and cortical 5-HT2A receptor and IL-1β levels in male mice. Brain Behav Immun. 2016;52:120-131.

36. Human Microbiome Project Consortium. Structure, function and diversity of the healthy human microbiome. Nature. 2012;486:207-214.

37. Methé BA, Nelson KE, Pop M, Creasy HH, Giglio MG, Huttenhower C, et al. A framework for human microbiome research. Nature. 2012;486(7402):215-21.

38. Edgar RC. Search and clustering orders of magnitude faster than BLAST. Bioinformatics. 2010;26:2460-2461.

39. Edgar R. UPARSE: Highly accurate OTU sequences from microbial amplicon reads. Nat Methods. 2013;10:996-998.

40. Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, Peplies J, Glöckner FO. The SILVA ribosomal RNA gene database project: Improved data processing and web-based tools. Nucleic Acids Res. 2013;41:D590-6.

41. Silverman JL, Yang M, Lord C, Crawley JN. Behavioural phenotyping assays for mouse models of autism. Nat Rev Neurosci. 2010;11:490-502.

42. McFarlane HG, Kusek GK, Yang M, Phoenix JL, Bolivar VJ, Crawley JN. Autism-like behavioral phenotypes in BTBR T+tf/J mice. Genes Brain Behav. 2008;7:152-163.

43. Deacon RM, Rawlins JN. T-maze alternation in the rodent. Nat Protoc. 2006;1:7-12.

44. Johnson JL, Stoica L, Liu Y, Zhu PJ, Bhattacharya A, Buffington SA, et al. Inhibition of Upf2-dependent nonsense-mediated decay leads to behavioral and neurophysiological abnormalities by activating the immune response. Neuron. 2019;104(4):665-679.

45. Deacon RM. Assessing nest building in mice. Nat Protoc. 2006;1:1117-1119.

46. McMurdie PJ, Holmes S. phyloseq: An R package for reproducible interactive analysis and graphics of microbiome census data. PLoS ONE. 2013;8(4):e61217.

47. Benjamini Y, Drai D, Elmer G, Kafkafi N, Golani I. Controlling the false discovery rate in behavior genetics research. Behav Brain Res. 2001;125:279-284.

48. Iglesias-Vázquez L, Van Ginkel Riba G, Arija V, Canals J. Composition of gut microbiota in children with autism spectrum disorder: A systematic review and meta-analysis. Nutrients. 2020;12(3):792.

49. Kirsch AC, Huebner ARS, Mehta SQ, Howie FR, Weaver AL, Myers SM, et al. Association of comorbid mood and anxiety disorders with autism spectrum disorder. JAMA Pediatrics. 2020;174(1):63-70.

50. Lee DO, Ousley OY. Attention-deficit hyperactivity disorder symptoms in a clinic sample of children and adolescents with pervasive developmental disorders. J Child Adolesc Psychopharmacol. 2006;16:737-746.

51. Bourin M, Petit-Demoulière B, Dhonnchadha BN, Hascöet M. Animal models of anxiety in mice. Fundam Clin Pharmacol. 2007;21:567-74.

52. McKinnon CJ, Eggebrecht AT, Todorov A, Wolff JJ, Elison JT, Adams CM, et al. Restricted and repetitive behavior and brain functional connectivity in infants at risk for developing autism spectrum disorder. Biol Psychiatry Cogn Neurosci Neuroimaging. 2019;4(1):50-61.

53. Peça J, Feliciano C, Ting JT, Wang W, Wells MF, Venkatraman TN, et al. Shank3 mutant mice display autistic-like behaviours and striatal dysfunction. Nature. 2013;472:437-442.

54. Kwon CH, Luikart BW, Powell CM, Zhou J, Matheny SA, Zhang W, et al. Pten regulates neuronal arborization and social interaction in mice. Neuron. 2006;50:377-388.

55. Zhou M, Rebholz H, Brocia C, Warner-Schmidt JL, Fienberg AA, Nairn AC, et al. Forebrain overexpression of CK1delta leads to down-regulation of dopamine receptors and altered locomotor activity reminiscent of ADHD. Proc Natl Acad Sci U S A. 2010;107(9):4401-4406.

56. Hallmayer J, Cleveland S, Torres A, Philips J, Cohen B, Torigoe T, et al. Genetic heritability and shared environmental factors among twin pairs with autism. Arch Gen Psychiatry. 2011;68(11):1095-1102.

57. De Angelis M, Piccolo M, Vannini L, Siragusa S, De Giacomo A, Serrazzanetti DI, et al. Fecal microbiota and metabolome of children with autism and pervasive developmental disorder not otherwise specified. PLoS One. 2013;8(10):e76993.

58. Finegold SM, Dowd SE, Gontcharova V, Liu C, Henley KE, Wolcott RD, et al. Pyrosequencing study of fecal microflora of autistic and control children. Anaerobe. 2010;16(4):444-53.

59. Zhang M, Ma W, Zhang J, He Y, Wang J. Analysis of gut microbiota profiles and microbe-disease associations in children with autism spectrum disorders in China. Sci Rep. 2018;8:13981.

60. Rose DR, Yang H, Serena G, Sturgeon C, Ma B, Careaga M, et al. Differential immune responses and microbiota profiles in children with autism spectrum disorders and co-morbid gastrointestinal symptoms. Brain Behav Immun. 2018;70:354-368.

61. Kelly JR, Minuto C, Cryan JF, Clarke G, Dinan TG. Cross talk: The microbiota and neurodevelopmental disorders. Front Neurosci. 2017;11:490.

62. Sharon G, Sampson TR, Geschwind DH, Mazmanian SK. The central nervous system and the gut microbiome. Cell. 2016;167(4):915-932.

63. Matsuki T, Tajima S, Hara T, Yahagi K, Ogawa E, Kodama H. Infant formula with galacto-oligosaccharides (OM55N) stimulates the growth of indigenous bifidobacteria in healthy term infants. Benef Microbes. 2016;7(4):453-61.

64. Williams S, Chen L, Savignac HM, Tzortzis G, Anthony DC, Burnet PW. Neonatal prebiotic (BGOS) supplementation increases the levels of synaptophysin, GluN2A-subunits and BDNF proteins in the adult rat hippocampus. Synapse. 2016;70(3):121-124.

65. Wang Y, Li N, Yang J-J, Zhao D-M, Chen B, Zhang G-Q et al. Probiotics and fructo-oligosaccharide intervention modulate the microbiota-gut brain axis to improve autism spectrum reducing also the hyper-serotonergic state and the dopamine metabolism disorder. Pharmacol Res. 2020;157:104784.

66. MacFabe DF, Cain NE, Boon F, Ossenkopp KP, Cain DP. Effects of the enteric bacterial metabolic product propionic acid on object-directed behavior, social behavior, cognition, and neuroinflammation in adolescent rats: Relevance to autism spectrum disorder. Behav Brain Res. 2011;217(1):47-54.

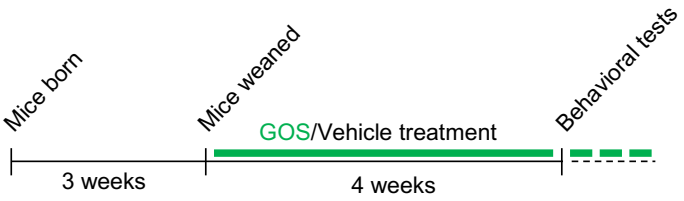

Figure 1. Scheme of the experimental design.

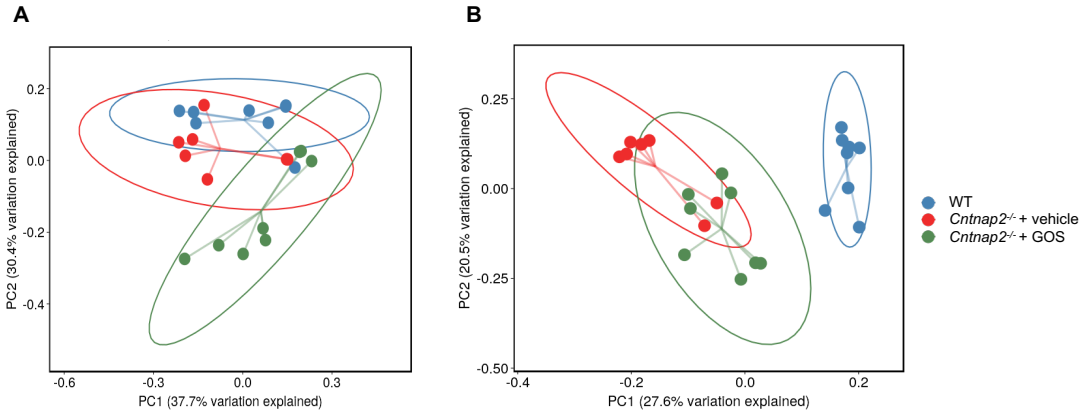

Figure 2. (**A**) PCoA of weighted UniFrac distances from the 16S rRNA gene sequencing dataset from feces of WT, *Cntnap2*$^{-/-}$ + vehicle and *Cntnap2*$^{-/-}$ + GOS mice (n = 7-8 per group, $p < 0.01$, $R^2 = 0.286$). (**B**) PCoA of unweighted UniFrac distances from the 16S rRNA gene sequencing dataset from feces of WT, *Cntnap2*$^{-/-}$ + vehicle and *Cntnap2*$^{-/-}$ + GOS samples (n = 7-8 per group, $p < 0.01$, $R^2 = 0.383$).

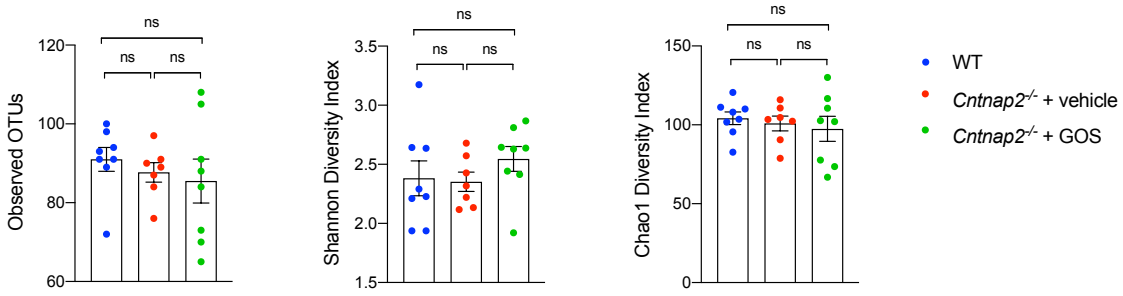

Figure 3. Alpha-diversity metrics. Number of operational taxonomical units - OTUs, Shannon diversity index and Chao1 diversity index from WT and *Cntnap2*$^{-/-}$ mice samples (n = 7-8 per group). Plots show mean ± SEM. ns, not significant.

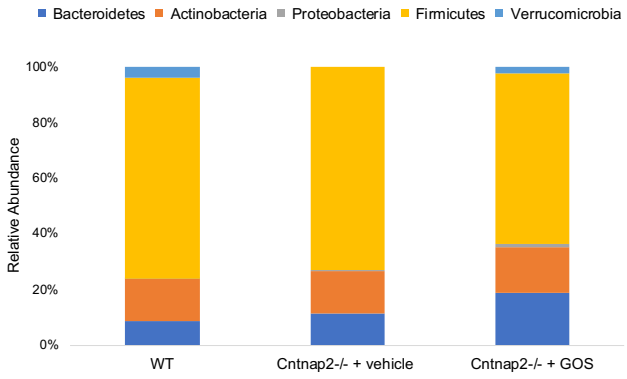

Figure 4. Relative abundance of the top 5 most abundant phyla from WT and *Cntnap2*$^{-/-}$ mice (n = 7-8 per group).

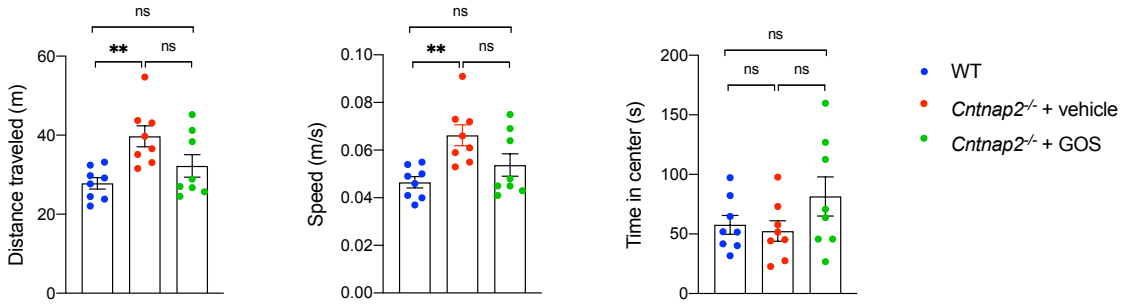

Figure 5. Anxiety-like and locomotor behavior in the open field test. Distance traveled (m), Speed (m/s) and Time in center (s) (n = 8 per group). Plots show mean ± SEM. The asterisks (**) represent significant difference among the treatments ($p < 0.01$). ns, not significant.

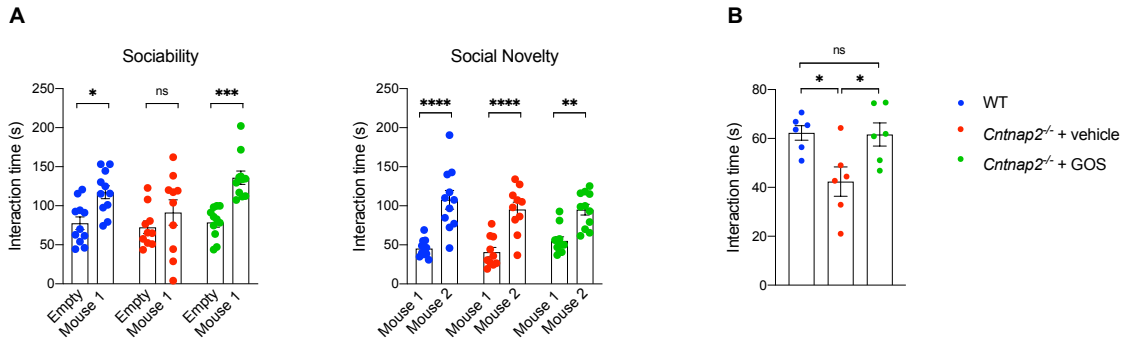

Figure 6. (**A**) Three-chamber social test for sociability and social novelty (n = 10-11 per group). (**B**) Reciprocal social interaction test (n = 6 pairs per group). Plots show mean ± SEM. The asterisk (*) represents significant difference among the treatments (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$). ns, not significant.

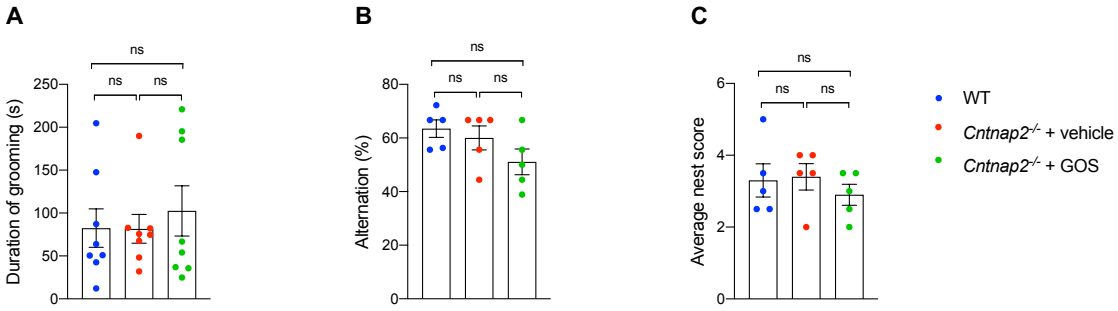

Figure 7. (**A**) Self-grooming behavior test (n = 8 per group). (**B**) T-maze spontaneous alternation task (n = 5 per group). (**C**) Nest-building test (n = 5 per group). Plots show mean ± SEM. ns, not significant.

Considerações Finais

O número crescente de crianças autistas vem encorajando os pesquisadores a desenvolverem novas estratégias para prevenir e tratar o TEA. Evidências do envolvimento da microbiota intestinal em distúrbios neurológicos fornecem novas possibilidades para o desenvolvimento de terapias promissoras no tratamento de sintomas comportamentais associados ao TEA. Nesse sentido, estudos com o objetivo de investigar os efeitos de pro- e prebióticos na composição e metabolismo da microbiota intestinal e sua relação com diversas doenças neurológicas, incluindo o TEA, têm se tornado mais frequentes.

Nosso estudo mostrou que os probióticos *L. reuteri* + *B. longum* combinados com GOS apresentaram elevada taxa de sobrevivência quando submetidos às condições gastrointestinais simuladas *in vitro*, evidenciando um efeito protetor do prebiótico GOS. Essa etapa do estudo foi de extrema importância, uma vez que a capacidade de sobrevivência às condições adversas do trato gastrointestinal é um dos principais requisitos para que os probióticos exerçam seus efeitos benéficos no cólon.

A combinação de *L. reuteri* + *B. longum* + GOS foi então selecionada para ser avaliada em estudos de curta e longa duração utilizando o SEMH®, um modelo colônico dinâmico *in vitro* que simula as diferentes partes do trato gastrointestinal humano. Os estudos no SEMH® permitiram avaliar os efeitos potenciais dos tratamentos com pro-, pre- e simbióticos sobre a composição e os metabólitos da microbiota intestinal de crianças com TEA. Os tratamentos avaliados mostraram resultados positivos e promissores na modulação da microbiota intestinal e metabólitos microbianos de crianças com TEA, estimulando a produção de AGCC e diminuindo os níveis de amônia. Os tratamentos pre- e simbiótico, especificamente, mostraram resultados similares e mais evidentes. Assim, hipotetizamos que o GOS, substrato utilizado seletivamente

pelos microrganismos hospedeiros que, por sua vez, produzem AGCC, poderia representar uma melhor estratégia para modular positivamente a microbiota, uma vez que ele foi o ponto em comum entre os tratamentos. Portanto, os resultados desta etapa do estudo foram a premissa para uma investigação mais aprofundada sobre a possível influência de prebióticos no TEA.

Além disso, visto que a administração de prebióticos, como uma possível estratégia no tratamento de distúrbios neurológicos, não tem sido amplamente explorada, investigamos se a administração de GOS afetou o comportamento e a composição da microbiota em um modelo animal de TEA. Observamos que o GOS melhorou significativamente os déficits sociais, no entanto, não teve efeito na modulação da microbiota.

Mais estudos são necessários para avaliar e compreender os efeitos de pro- e prebióticos, como potencial abordagem terapêutica, em diferentes modelos animais de distúrbios neurológicos levando em consideração a complexidade do metagenoma para entender a etiologia de diferentes comportamentos associados ao TEA. Além disso, futuros ensaios clínicos devem também investigar as hipóteses pelas quais a microbiota intestinal interfere nos comportamentos associados ao TEA, através da exploração dos mecanismos envolvidos na comunicação do eixo microbiota-intestino-cérebro. Tais investigações são necessárias não apenas para a reprodutibilidade das descobertas utilizando diferentes abordagens, como sistemas *in vitro*, modelos animais e ensaios clínicos, mas também para demonstrar o potencial terapêutico da modulação da microbiota intestinal nos distúrbios neurológicos como o TEA. Por fim, descobertas adicionais sobre o papel da microbiota intestinal no TEA podem auxiliar no desenvolvimento de novas terapias que beneficiam a saúde e o bem-estar geral de indivíduos autistas.

Referências

1. Thursby E, Juge N. Introduction to the human gut microbiota. Biochem J. 2017;474(11):1823-1836.

2. Derrien M, Alvarez AS, de Vos WM. The gut microbiota in the first decade of life. Trends Microbiol. 2019;27(12):997-1010.

3. Gilbert JA, Blaser MJ, Caporaso JG, Jansson JK, Lynch SV, Knight R. Current understanding of the human microbiome. Nat Med. 2018;24(4):392-400.

4. Long-Smith C, O'Riordan KJ, Clarke G, Stanton C, Dinan TG, Cryan JF. Microbiota-gut-brain axis: New therapeutic opportunities. Annu Rev Pharmacol Toxicol. 2020;60:477-502.

5. Rowland I, Gibson G, Heinken A, Scott K, Swann J, Thiele I, Tuohy K. Gut microbiota functions: Metabolism of nutrients and other food components. Eur J Nutr. 2018;57(1):1-24. 5

6. Zheng D, Liwinski T, Elinav E. Interaction between microbiota and immunity in health and disease. Cell Res. 2020;30:492-506.

7. Massier L, Blüher M, Kovacs P, Chakaroun RM. Impaired intestinal barrier and tissue bacteria: Pathomechanisms for metabolic diseases. Front in Endocrinol. 2021;12:1467.

8. Sampson TR, Mazmanian SK. Control of brain development, function, and behavior by the microbiome. Cell Host Microbe. 2015;17:565-576.

9. Vuong HE, Yano JM, Fung TC, Hsiao EY. The microbiome and host behavior. Annu Rev Neurosci. 2017;40:21-49.

10. Cryan JF, Dinan TG. Mind-altering microorganisms: The impact of the gut microbiota on brain and behaviour. Nat Rev Neurosc. 2012;13(10):701-712.

11. Carabotti M, Scirocco A, Maselli MA, Severi C. The gut-brain axis: Interactions between enteric microbiota, central and enteric nervous systems. Ann Gastroenterol. 2015;28:203-209.

12. Cryan JF, O'Riordan KJ, Cowan C, Sandhu KV, Bastiaanssen T, Boehme M, et al. The microbiota-gut-brain axis. Physiol Rev. 2019;99(4):1877-2013.

13. Mayer EA, Tillisch K, Gupta A. Gut/brain axis and the microbiota. J Clin Invest. 2015;125(3):926-938.

14. Grenham S, Clarke G, Cryan JF, Dinan TG. Brain-gut-microbe communication in health and disease. Front Physiol. 2011;2(94):1-15.

15. Sherwin E, Bordenstein SR, Quinn JL, Dinan TG, Cryan JF. Microbiota and the social brain. Science. 2019;366(6465):eaar2016.

16. Wang FB, Powley TL. Vagal innervation of intestines: Afferent pathways mapped with new en bloc horseradish peroxidase adaptation. Cell Tissue Res. 2007;329:221-230.

17. Bonaz B, Bazin T, Pellissier S. The vagus nerve at the interface of the microbiota-gut-brain axis. Front. Neurosci. 2018;12:49.

18. Powley TL, Spaulding RA, Haglof SA. Vagal afferent innervation of the proximal gastrointestinal tract mucosa: Chemoreceptor and mechanoreceptor architecture. J. Comp. Neurol. 2011;519:644-660.

19. Morais LH, Schreiber HL, Mazmanian SK. The gut microbiota–brain axis in behaviour and brain disorders. Nat Rev Microbiol. 2021;19:241-255.

20. Strandwitz P, Kim KH, Terekhova D, Liu JK, Sharma A, Levering J, et al. GABA-modulating bacteria of the human gut microbiota. Nat Microbiol. 2019;4:396-403.

21. Strandwitz P. Neurotransmitter modulation by the gut microbiota. Brain Res. 2018;1693:128-133.

22. Huang F, Wu X. Brain neurotransmitter modulation by gut microbiota in anxiety and depression. Front Cell Dev Biol. 2021;9:649103.

23. Clarke G, Grenham S, Scully P, Fitzgerald P, Moloney RD, Shanahan F, et al. The microbiome–gut–brain axis during early life regulates the hippocampal serotonergic system in a sex-dependent manner. Mol. Psychiatry. 2013;18:666-673.

24. Agus A, Planchais J, Sokol H. Gut microbiota regulation of tryptophan metabolism in health and disease. Cell Host Microbe. 2018;23(6):716-724.

25. Silva YP, Bernardi A, Frozza RL. The role of short-chain fatty acids from gut microbiota in gut-brain communication. Front Endocrinol. 2020;11:25.

26. Dalile B, Van Oudenhove L, Vervliet B, Verbeke K. The role of short-chain fatty acids in microbiota–gut–brain communication. Nat Rev Gastroenterol Hepatol. 2019;16:461-478.

27. Powell N, MacDonald TT. Recent advances in gut immunology. Parasite Immunol. 2017;39(6):e12430.

28. Wolf AJ, Underhill DM. Peptidoglycan recognition by the innate immune system. Nat Rev Immunol. 2018;18(4):243-254.

29. Gonzalez-Santana A, Heitz RD. Bacterial peptidoglycans from microbiota in neurodevelopment and behavior. Trends Mol Med. 2020;26(8):729-743.

30. De Theije CG, Wu J, Da Silva SL, Kamphuis PJ, Garssen J, Korte SM, et al. Pathways underlying the gut-to-brain connection in autism spectrum disorders as future targets for disease management. Eur J Pharmacol. 2011;668:70-80.

31. Mayer EA, Knight R, Mazmanian SK, Cryan JF, Tillisch K. Gut microbes and the brain: Paradigm shift in neuroscience. J Neurosci. 2014;34(46):15490-15496.

32. Lasheras I, Seral P, Latorre E, Barroso E, Gracia-García P, Santabárbara J. Microbiota and gut-brain axis dysfunction in autism spectrum disorder: Evidence for functional gastrointestinal disorders. Asian J Psychiatr. 2020;47:101874.

33. American Psychiatric Association – APA. Diagnostic and statistical manual of mental disorders (DSM-5). 5th ed. Washington DC: Am Psyc Assoc. 2013

34. Maenner MJ, Shaw KA, Baio J, Washington A, Patrick M, DiRienzo M, et al. Prevalence of autism spectrum disorder among children aged 8 years — autism and developmental disabilities monitoring network, 11 Sites, United States, 2016. MMWR Surveill Summ 2020;69(No. SS-4):1-12.

35. Kang D-W, Park JG, Ilhan ZE, Wallstrom G, Labaer J, Adams JB, et al. Reduced incidence of *Prevotella* and other fermenters in intestinal microflora of autistic children. PloS One. 2013;8:1-14.

36. Levy M, Kolodziejczyk A, Thaiss C, Elinav E. Dysbiosis and the immune system. Nat Rev Immunol. 2017;17: 219-232.

37. Srikantha P, Mohajeri MH. The possible role of the microbiota-gut-brain-axis in autism spectrum disorder. Int J Mol Sci. 2019;20(9):2115.

38. Tomova A, Husarova V, Lakatosova S, Bakos J, Vlkova B, Babinska K, Ostatnikova D. Gastrointestinal microbiota in children with autism in Slovakia. Physiol Behav. 2015;138:179-87.

39. Zhang M, Ma W, Zhang J, He Y, Wang J. Analysis of gut microbiota profiles and microbe-disease associations in children with autism spectrum disorders in China. Sci Rep. 2018;8:13981.

40. Finegold SM, Dowd SE, Gontcharova V, Liu C, Henley KE, Wolcott RD, et al. Pyrosequencing study of fecal microflora of autistic and control children. Anaerobe. 2010;16:444-453.

41. Song Y, Liu C, Finegold SM. Real-time PCR quantitation of clostridia in feces of autistic children. Appl Environ Microbiol. 2004;70(11):6459-6465.

42. De Angelis M, Piccolo M, Vannini L, Siragusa S, De Giacomo A, Serrazzanetti DI, et al. Fecal microbiota and metabolome of children with autism and pervasive developmental disorder not otherwise specified. PLoS One. 2013;8(10):e76993.

43. Wang L, Christophersen CT, Sorich MJ, Gerber JP, Angley MT, Conlon MA. Low relative abundances of the mucolytic bacterium *Akkermansia muciniphila* and *Bifidobacterium* spp. in feces of children with autism. Appl Environ Microbiol. 2011;77(18):6718-6721.

44. Strati F, Cavalieri D, Albanese D, De Felice C, Donati C, Hayek J, et al. New evidences on the altered gut microbiota in autism spectrum disorders. Microbiome. 2017;5(1):24.

45. McElhanon BO, McCracken C, Karpen S, Sharp WG. Gastrointestinal symptoms in autism spectrum disorder: A meta-analysis. Pediatrics. 2014;133:872-883.

46. Holingue C, Newill C, Lee LC, Pasricha PJ, Daniele Fallin M. Gastrointestinal symptoms in autism spectrum disorder: A review of the literature on ascertainment and prevalence. Autism Res. 2018;11(1):24-36.

47. Adams JB, Johansen LJ, Powell LD, Quig D, Rubin RA. Gastrointestinal flora and gastrointestinal status in children with autism - comparisons to typical children and correlation with autism severity. BMC Gastroenterol. 2011;11:22.

48. Hsiao EY. Gastrointestinal issues in autism spectrum disorder. Harv Rev Psychiatry. 2014;22:104-111.

49. Nikolov RN, Bearss KE, Lettinga J, Erickson C, Rodowski M, Aman MG, et al. Gastrointestinal symptoms in a sample of children with pervasive developmental disorders. J Autism Child Schizophr. 2009;39(3):405-413.

50. Sharon G, Sampson TR, Geschwind DH, Mazmanian SK. The central nervous system and the gut microbiome. Cell. 2016;167(4):915-932.

51. Shaaban SY, El Gendy YG, Mehanna NS, El-Senousy WM, El-Feki HSA, Saad K, et al. The role of probiotics in children with autism spectrum disorder: A prospective, open-label study. Nutr Neurosci. 2018;21:676-681.

52. Santocchi E, Guiducci L, Prosperi M, Calderoni S, Gaggini M, Apicella F, et al. Effects of probiotic supplementation on gastrointestinal, sensory and core symptoms in autism spectrum disorders: A randomized controlled trial. Front Psychiatry. 2020;11:550-593.

53. Grimaldi R, Gibson GR, Vulevic, J, Giallourou N, Castro-Mejía JL, Hansen LH, et al. A prebiotic intervention study in children with autism spectrum disorders (ASDs). Microbiome. 2018;6:133.

54. Dinan TG, Stanton C, Cryan JF. Psychobiotics: A novel class of psychotropic. Biol Psychiatry. 2013;74:720-726.

55. Sarkar A, Lehto SM, Harty S, Dinan TG, Cryan JF, Burnet PWJ. Psychobiotics and the manipulation of bacteria–gut–brain signals. Trends Neurosci. 2016;39(11):763-781.

56. Cheng LH, Liu YW, Wu CC, Wang S, Tsai YC. Psychobiotics in mental health, neurodegenerative and neurodevelopmental disorders. J Food Drug Anal. 2019;27(3):632-648.

57. Hill C, Guarner F, Reid G, Gibson GR, Merenstein DJ, Pot B, et al. The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. Nat Rev Gastroenterol Hepatol. 2014;11:506-514.

58. Yan F, Polk DB. Probiotics and probiotic-derived functional factors-mechanistic insights into applications for intestinal homeostasis. Front Immunol. 2020;11:1428.

59. Gareau MG, Sherman PM, Walker WA. Probiotics and the gut microbiota in intestinal health and disease. Nat Rev Gastroenterol Hepatol. 2010;7(9):503-14.

60. Bravo JA, Forsythe P, Chew MV, Escaravage E, Savignac HM, Dinan TG, et al. Ingestion of *Lactobacillus* strain regulates emotional behavior and central GABA receptor expression in a mouse via the vagus nerve. PNAS. 2011;108(38):16050-16055.

61. Bercik P, Park AJ, Sinclair D, Khoshdel A, Lu J, Huang X, et al. The anxiolytic effect of *Bifidobacterium longum* NCC3001 involves vagal pathways for gut–brain communication. Neurogastroenterol Motil. 2011;23(12):1132-1139.

62. Hsiao EY, McBride SW, Hsien S, Sharon G, Hyde ER, McCue T, et al. Microbiota modulate behavioral and physiological abnormalities associated with neurodevelopmental disorders. Cell. 2013;155(7):1451-63.

63. Buffington SA, Di Prisco GV, Auchtung TA, Ajami NJ, Petrosino JF, Costa-Mattioli M. Microbial reconstitution reverses maternal diet-induced social and synaptic deficits in offspring. Cell. 2016;165:1762-1775.

64. Gibson G, Hutkins R, Sanders ME, Prescott SL, Reimer RA, Salmien SJ, et al. Expert consensus document: The International Scientific Association for Probiotics and Prebiotics (ISAPP) consensus statement on the definition and scope of prebiotics. Nat Rev Gastroenterol Hepatol. 2017;14:491-502.

65. Rastall RA, Gibson GR. Recent developments in prebiotics to selectively impact beneficial microbes and promote intestinal health. Curr Opin Biotechnol. 2015;32:42-46.

66. Roberfroid M, Gibson GR, Hoyles L, McCartney AL, Rastall R, Rowland I, et al. Prebiotic effects: Metabolic and health benefits. Br J Nutr. 2010;104(2):61-63.

67. Burokas A, Arboleya S, Moloney RD, Peterson VL, Murphy K, Clarke G. Targeting the microbiota-gut-brain axis: Prebiotics have anxiolytic and antidepressant-like effects and reverse the impact of chronic stress in mice. Biol Psychiatry. 2017;82(7):472-487.

68. Schmidt K, Cowen PJ, Harmer CJ, Tzortzis G, Errington S, Burnet PW. Prebiotic intake reduces the waking cortisol response and alters emotional bias in healthy volunteers. Psychopharmacology. 2015;232(10):1793-1801.

69. Grimaldi R, Cela D, Swann JR, Vulevic J, Gibson GR, Tzortzis G, et al. In vitro fermentation of B-GOS: Impact on faecal bacterial populations and metabolic activity in autistic and non-autistic children. FEMS Microbiol Ecol. 2017;93(2):fiw233.

70. Swanson KS, Gibson GR, Hutkins R, Reimer RA, Reid G, Verbeke K, et al. The International Scientific Association for Probiotics and Prebiotics (ISAPP) consensus statement on the definition and scope of synbiotics. Nat Rev Gastroenterol Hepatol. 2020;17(11):687-701.

71. Wang Y, Li N, Yang J-J, Zhao D-M, Chen B, Zhang G-Q, et al. Probiotics and fructo-oligosaccharide intervention modulate the microbiota-gut brain axis to improve autism spectrum reducing also the hyper-serotonergic state and the dopamine metabolism disorder. Pharmacol Res. 2020;157:104784.

72. Kang DW, Adams JB, Gregory AC, Borody T, Chittick L, Fasano A, et al. Microbiota Transfer Therapy alters gut ecosystem and improves gastrointestinal and autism symptoms: an open-label study. Microbiome. 2017;5(1):10.

73. Kang DW, Adams JB, Coleman DM, Pollard EL, Maldonado J, McDonough-Means S, et al. Long-term benefit of Microbiota Transfer Therapy on autism symptoms and gut microbiota. Sci Reports. 2019;9(1):5821.

74. Venema K, Abbeele PVD. Experimental models of the gut microbiome. Best Pract Res Clin Gastroenterol. 2013;27:115-126.

75. Pham VT, Mohajeri MH. The application of in vitro human intestinal models on the screening and development of pre- and probiotics. Benefic Microbes. 2018;9(5):725-742.

76. Nissen L, Casciano F, Gianotti A. Intestinal fermentation in vitro models to study food-induced gut microbiota shift: An updated review. FEMS Microbiol Lett. 2020;367(12):fnaa097.

77. Sivieri K, Bianchi F, Tallarico MA, Rossi, EA. Fermentation by gut microbiota cultured in a simulator of the human intestinal microbial ecosystem is improved by probiotic *Enterococcus faecium* CRL 183. Funct Foods Health Dis. 2011;10:389-402.

78. Bianchi F, Larsen N, de Mello Tieghi T, Adorno M, Kot W, Saad S, et al. Modulation of gut microbiota from obese individuals by in vitro fermentation of citrus pectin in combination with *Bifidobacterium longum* BB-46. Appl Microbiol Biotechnol. 2018;102(20):8827-8840.

79. Sivieri K, Morales MLV, Saad SMI, Adorno MAT, Sakamoto IK, Rossi EA. Prebiotic effect of fructooligosaccharide in the simulator of the human intestinal microbial ecosystem (SHIME® Model). Journal of Medicinal Food. 2014;17(8):894-901.

80. Freire FC, Adorno MAT, Sakamoto IK, Antoniassi R, Chaves ACSD, Santos KMO, et al. Impact of multi-functional fermented goat milk beverage on gut microbiota in a dynamic colon model. Food Res Int. 2017;99:315-327.

81. Rodrigues VCDC, Duque ALRF, Fino LC, Simabuco FM, Sartoratto A, Cabral L, et al. Modulation of the intestinal microbiota and the metabolites produced by the administration of ice cream and a dietary supplement containing the same probiotics. Br J Nutr. 2020;6:1-12.

82. Gadad BS, Hewitson L, Young KA, German DC. Neuropathology and animal models of autism: Genetic and environmental factors. Autism Res Treat. 2013;2013:731935.

83. Tabouy L, Getselter D, Ziv O, Karpuj M, Tabouy T, Lukic I, et al. Dysbiosis of microbiome and probiotic treatment in a genetic model of autism spectrum disorders. Brain Behav Immun. 2018;73:310-319.

84. Chao OY, Yunger R, Yang YM. Behavioral assessments of BTBR T+Itpr3tf/J mice by tests of object attention and elevated open platform: Implications for an animal model of psychiatric comorbidity in autism. Behav Brain Res. 2018;347:140-147.

85. Sgritta M, Dooling SW, Buffington SA, Momin EN, Francis MB, Britton RA, et al. Mechanisms underlying microbial-mediated changes in social behavior in mouse models of autism spectrum disorder. Neuron. 2019;101(2):246-259.