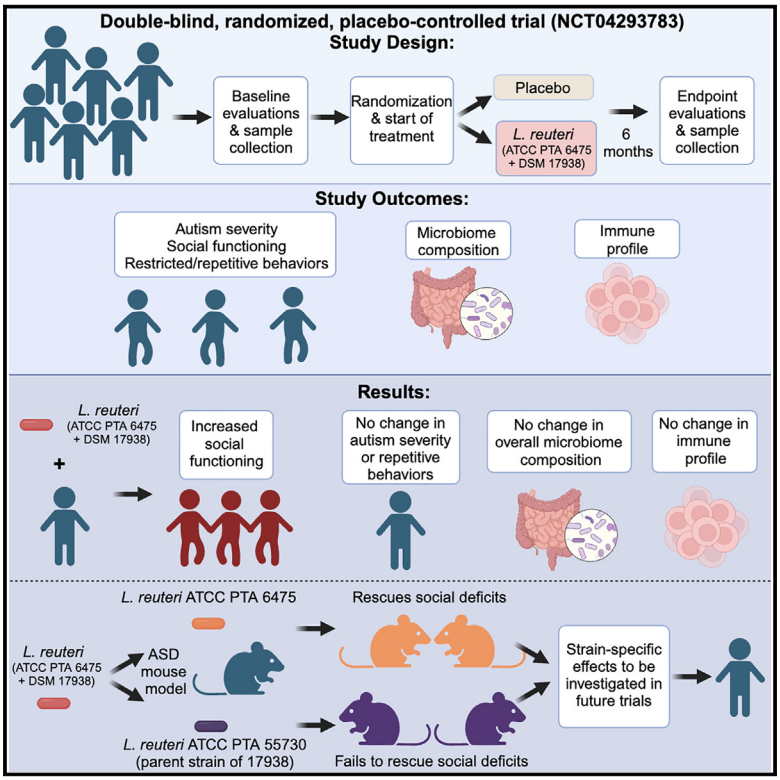
Clinical and Translational Report

Cell Host & Microbe

Precision microbial intervention improves social behavior but not autism severity: A pilot double-blind randomized placebo-controlled trial

Graphical abstract



Highlights

- *L. reuteri* (6475 + 17938) improves social functioning in children with autism
- *L. reuteri* does not improve overall autism severity or repetitive behaviors
- *L. reuteri* does not significantly alter microbiome composition or immune profile
- Only the 6475 strain reverses the social deficits in a mouse model for autism

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In brief

L. reuteri treatment (ATCC-PTA-6475 + DSM-17938) improves social functioning in children with autism, but not other symptoms. Interestingly, the ATCC-PTA-6475 strain, but not the parental strain of DSM-17938, improves social behavior in mice. The findings warrant larger trials in which the effect of specific strains is also tested.

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Precision microbial intervention improves social behavior but not autism severity: A pilot double-blind randomized placebo-controlled trial

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SUMMARY

Autism spectrum disorder (ASD) is characterized by the presence of restricted/repetitive behaviors and social communication deficits. Because effective treatments for ASD remain elusive, novel therapeutic strategies are necessary. Preclinical studies show that *L. reuteri* selectively reversed social deficits in several models for ASD. Here, in a double-blind, randomized, placebo-controlled trial, we tested the effect of *L. reuteri* (a product containing a combination of strains ATCC-PTA-6475 and DSM-17938) in children with ASD. The treatment does not alter overall autism severity, restricted/repetitive behaviors, the microbiome composition, or the immune profile. However, *L. reuteri* combination yields significant improvements in social functioning that generalized across different measures. Interestingly, ATCC-PTA-6475, but not the parental strain of DSM-17938, reverses the social deficits in a preclinical mouse model for ASD. Collectively, our findings show that *L. reuteri* enhances social behavior in children with ASD, thereby warranting larger trials in which strain-specific effects should also be investigated.

INTRODUCTION

Autism spectrum disorder (ASD) is a heterogeneous neurodevelopmental disorder characterized by impairments in social interaction and communication abilities in addition to the presence of restricted and repetitive patterns of behaviors and interests.[1] The worldwide prevalence of ASD has increased dramatically over the last three decades,[2–6] with the most recent reports in the United States (US) putting the prevalence at 1 in every 44 individuals.[4] The annual cost of caring for individuals with ASD in the US is projected to reach $461 billion by 2025.[7] Despite being a major public health concern, effective treatments for the core symptoms, including social communication deficits, remain elusive. Thus, the development of novel treatment strategies is an urgent priority.[8,9]

Traditional research on the biology of ASD has focused on the brain, aiming to identify key brain regions and circuits, relevant molecular mechanisms, and/or new genetic variants associated with ASD.[10] Consequently, current clinical studies using pharmacological therapeutics for ASD target the brain directly (e.g.,

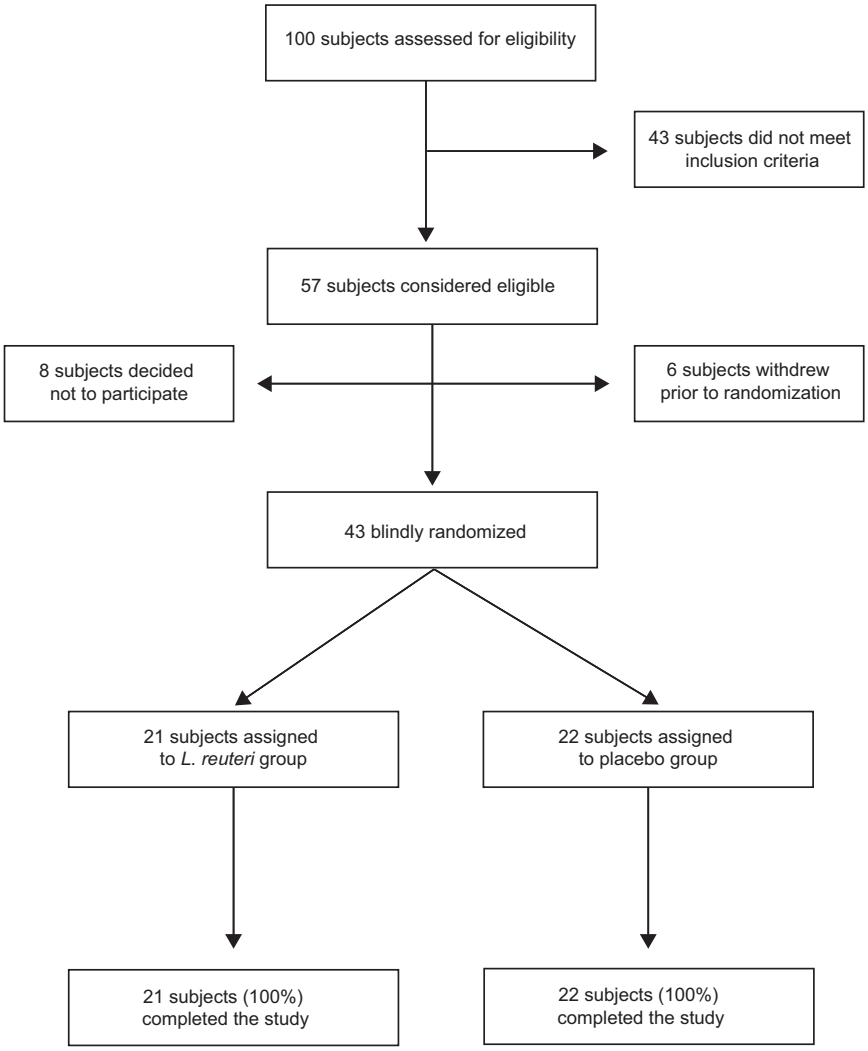

Figure 1. The CONSORT flow diagram for the clinical trial with *L. reuteri*

The CONSORT flow diagram details the progress, from screening through study completion, in the randomized, double-blind, placebo-controlled trial testing 6 months of precision bacterial intervention with *L. reuteri* versus placebo in children with ASD.

risperidone,[11] oxytocin,[12,13] vasopressin,[14] etc.). However, an expanding body of preclinical research shows that gut microbes are important modulators of host physiology,[15] including the endophenotypes associated with ASD.[16–20] More specifically, we serendipitously discovered that the commensal bacterial species *Limosilactobacillus* (*L.*) *reuteri* (formerly known as *Lactobacillus reuteri*[21]) selectively reversed the social deficits in a maternal high-fat-diet mouse model for neurodevelopmental disorders.[22]

Subsequent studies aimed at dissecting the mechanism(s) by which the bacteria modulates social behavior revealed that *L. reuteri* reverses the social deficits in ASD models via the vagus nerve and promotes social reward by targeting the oxytocin-dopaminergic reward circuit,[23,24] a critical pathway involved in social behaviors.[25,26] Furthermore, *L. reuteri* did not significantly alter the overall microbiome composition in mouse models for ASD,[23] and *L. reuteri* alone was sufficient to reverse social deficits in mice lacking microbiota (germ-free mice[23]), indicating that the effect of *L. reuteri* on social behavior is independent of other microbes in the gut. In addition, we recently found that *L. reuteri* reversed social deficits in the absence of a mature adaptive immune system, indicating that its prosocial effect is likely independent of B and T cells.[27] Importantly, these preclinical findings are generalizable to models for ASD with different underlying etiologies (e.g., genetic, idiopathic, and environmental[23,24]) and have been reproduced by several investigators.[28,29] In addition, the beneficial effects of *L. reuteri* appear to be selective for social behavior because the bacteria fail to reverse other behavioral deficits (e.g., repetitive behavior, changes in activity levels, anxiety-related behavior) in ASD mouse models.[22–24] Finally, *L. reuteri* has been certified as "generally recognized as safe" (GRAS) for use in humans by the United States Food and Drug Administration (FDA) and has already been safely administered to newborns within the first 3 months of life in a clinical trial for unrelated disorders.[30] Thus, we wondered whether *L. reuteri* would be a promising therapeutic option for improving symptoms in children with ASD, with a particular emphasis on social behavior.

To test this hypothesis, we performed a double-blind, randomized, placebo-controlled, parallel-design pilot trial in children with ASD. Importantly, we found that *L. reuteri*, compared with placebo, significantly improved social functioning, both in terms of reducing social deficits, as measured by the social responsiveness scale (SRS[31,32]), and increasing adaptive social functioning, as measured by the social adaptive composite score of the Adaptive Behavior Assessment System, Second Edition (ABAS-2[33]). *L. reuteri* did not improve overall autism severity, restricted and repetitive behaviors, and co-occurring psychiatric and behavioral problems, nor did it significantly modulate the microbiome or immune response. Thus, this safe microbial manipulation has the potential for improving social deficits associated with ASD in children.

RESULTS

Effects of *L. reuteri* treatment in a human trial: Participants

One hundred potential participants were assessed for eligibility (see Figure 1 for patient disposition throughout the study). Forty-three were excluded as they did not meet the inclusion/exclusion criteria. Of the 57 eligible participants, 8 declined to

Table 1. Baseline comparison between control and treatment groups

	Control	Treatment
N	22	21
Male/female sex, *n*	16/6	19/2
Age, mean (SD), years	5.94 (1.29)	6.23 (1.15)
NVIQ, mean (SD)	88.95 (21.32)	95.19 (25.05)
ADOS-2 total CSS, mean (SD)	7.13 (0.82)	6.75 (1.62)
SRS total T score, mean (SD)	84.59 (16.90)	88.50 (17.86)
CBCL internalizing score, mean (SD)	62.33 (8.21)	61.20 (9.88)
CBCL externalizing score, mean (SD)	56.62 (8.75)	59.45 (12.51)
ABAS-2 GAC score, mean (SD)	58.23 (15.44)	58.60 (12.45)
PSI/SF total score, mean (SD)	67.50 (22.27)	68.30 (28.10)
GSRS total score, mean (SD)	5.10 (3.39)	6.35 (4.89)

NVIQ, non-verbal intelligence quotient; ADOS-2, Autism Diagnostic Observation Schedule, Second Edition; CSS, calibrated severity score; SRS, social responsiveness scale; total T score, gender-normalized total score; CBCL, Child Behavior Checklist; ABAS-2, Adaptive Behavior Assessment System, second edition; GAC, general adaptive composite; PSI/SF, Parenting Stress Index Short Form; GSRS, Gastrointestinal Symptoms Rating Scale; no statistical difference observed between the two groups on any of the above variables.

participate, and 6 withdrew before baseline assessments were completed. Thus, a total of 43 children ultimately participated in the randomized trial to receive either *L. reuteri* (see STAR Methods; 21 participants: 19 males, 2 females; mean age 5.8 ± 1.3 years) or placebo (22 participants: 16 males, 6 females; mean age 5.5 ± 1.2 years [Table 1]). No differences were observed between the groups at baseline (Table 1). All the subjects in both the *L. reuteri* and placebo groups completed the study.

Effects of *L. reuteri* treatment in a human trial: Behavioral outcomes

We did not observe improvements on measures of the primary outcome measure of overall symptom severity as assessed by the Autism Diagnostic Observation Schedule, Second Edition (ADOS-2), total calibrated severity score (Table 2). Importantly, secondary efficacy analyses revealed that 6 months of *L. reuteri* treatment improved social behaviors as measured by the SRS total T score (Table 2). Social functioning is a complex and multifaceted domain, encompassing subdomains with at least partially distinct etiologies that may respond differently to different treatments. Therefore, we sought to determine whether *L. reuteri* had similar effects across different social subdomains that we identified in a recent large-scale factor analysis,[31] in line with goals of the National Institute of Mental Health's Research Domain Criteria initiative.[34] Accordingly, we observed improvement in the social communication subdomain but not in the social motivation or mental state understanding subdomains (Table 2). Importantly, and consistent with the results of the SRS, we observed a significant improvement in the adaptive social functioning subdomain (measured by the social adaptive composite score of the ABAS-2) in participants receiving *L. reuteri* combination in comparison with the placebo (Table 2). The positive signal was specific to social functioning, given that we did not observe improvements in other secondary outcome measures assessing restricted repetitive behaviors, general psychopathology, non-social aspects of adaptive functioning, parental stress, and gastrointestinal (GI) symptomatology, as assessed by the Restricted Repetitive Behaviors Scale (revised) (RBS-R) subscale scores, Child Behavior Checklist (CBCL) internalizing and externalizing problem scores, ABAS, Parenting Stress Index Short Form (PSI/SF) total score, and Gastrointestinal Symptoms Rating Scale (GSRS) total score (Table 2). Thus, *L. reuteri* treatment combination selectively improves social functioning in children with ASD.

Effects of *L. reuteri* treatment in a human trial: Safety evaluation

Minimal or no adverse effects (AEs; Table S1) were observed, with the exception of one subject in the active group who experienced worsening of baseline GI agitation and irritability requiring temporary interruption of treatment, which was followed by symptom resolution. Although most reported AEs were GI, no differences between the *L. reuteri* and placebo groups were detected from chi-squared tests for individual side effects ($X^2 = 0.42$, $p = 0.49$, *Phi* = 0.12) or all GI AEs combined ($X^2 = 0.73$, $p = 0.99$, *Phi* =.05).

Effects of *L. reuteri* treatment in a human trial: Microbiome profile

Given that *L. reuteri* can produce anti-microbial peptides,[35,36] we examined whether introduction of *L. reuteri* alters the human gut microbial ecology, a primary outcome for the study. To this end, metagenomic analyses were performed in feces from the participants (see STAR Methods). To assess microbiome composition, we analyzed several metrics for alpha diversity and beta diversity. There was no significant time-by-treatment effect across several metrics for alpha diversity (i.e., observed operational taxonomic units [OTUs], Shannon diversity index, Chao1 index, and Faith's phylogenetic diversity; Figures 2A and S1A–S1C) or beta diversity (i.e., Brays-Curtis, unweighted UniFrac, weighted UniFrac, and Jaccard distances; Figures 2B and S1D–S1F), indicating that *L. reuteri* did not induce widespread changes in the microbiome composition. In addition, when we performed differentially abundant taxa analyses, we only identified 4 out of 1,424 taxa (0.28%) as differentially abundant as a function of time and treatment. As expected, *L. reuteri* was detected in all the individuals in the treatment group—but none of the placebo group—at the end of the study (Figure 2C). In contrast, the other 3 differentially abundant species showed changes that were inconsistent among individuals and occurred in both the control and treatment group (Figures S1H–S1J). In conclusion, *L. reuteri* treatment did not considerably alter the overall microbiome composition, consistent with the preclinical findings in mouse models for ASD.[23]

Effects of *L. reuteri* treatment in a human trial: Immunological profile

L. reuteri has been shown to interact with the host's immune system.[37,38] Thus, we sought to determine whether *L. reuteri* treatment would affect different components of the immune system—a secondary outcome for the study. Examination of comprehensive immunological panels revealed that *L. reuteri*

Table 2. Summary of behavioral outcomes

	Mean (SD)						
	Baseline		End of treatment		Group × time		
	Control	Treatment	Control	Treatment	*F*	p	η_p^2
Autism Diagnostic Observation Schedule, Second Edition (ADOS-2)							
Total calibrated severity score	7.13 (0.82)	6.75 (1.62)	7.13 (1.39)	6.75 (1.58)	0.001	0.99	0.000
Social responsiveness scale (SRS)							
Total T-score[a]	84.59 (16.90)[a]	88.50 (17.86)[a]	85.09 (17.14)[a]	80.70 (16.75)[a]	3.78[a]	0.031[a]	0.163[a]
Social communication[a]	11.75 (3.6)[a]	12.3 (5.1)[a]	13.5 (4.1)[a]	10.3 (3.5)	6.513[a]	0.005[a]	0.325[a]
Social motivation	5.81 (4.29)	4.71 (3.29)	5.69 (3.30)	3.29 (3.34)	1.962	0.160	0.127
Mental state understanding	20.88 (7.43)	20.57 (5.80)	22.19 (6.31)	19.86 (4.94)	1.010	0.378	0.070
Repetitive Behavior Scale-Revised (RBS-R)							
Repetitive motor behaviors	7.10 (5.17)	7.68 (5.89)	7.10 (5.60)	6.95 (6.51)	1.26	0.294	0.064
Compulsions	2.90 (2.98)	3.06 (3.67)	2.52 (2.87)	3.00 (4.58)	0.128	0.881	0.007
Ritualistic/sameness	7.14 (6.09)	8.00 (8.16)	6.05 (6.72)	5.63 (8.41)	0.328	0.723	0.017
Restricted behaviors	2.19 (1.81)	3.26 (2.28)	2.00 (2.26)	2.84 (2.63)	0.296	0.746	0.016
Child Behavior Checklist (CBCL)							
Internalizing problems	62.33 (8.21)	61.20 (9.88)	60.38 (8.79)	59.00 (8.16)	1.038	0.364	0.052
Externalizing problems	56.62 (8.75)	59.45 (12.51)	56.48 (9.44)	57.40 (12.98)	2.378	0.106	0.111
Adaptive Behavior Assessment System, Second Edition (ABAS-2)							
General adaptive composite score	58.23 (15.44)	58.60 (12.45)	58.95 (17.11)	60.80 (17.69)	1.265	0.293	0.061
Conceptual adaptive composite score	61.00 (12.32)	65.35 (14.40)	61.82 (13.51)	67.95 (17.76)	1.032	0.366	0.050
Social adaptive composite score[a]	65.86 (12.32)[a]	65.60 (12.73)[a]	63.86 (13.50)[a]	69.70 (14.45)[a]	4.45[a]	0.018[a]	0.186[a]
Practical adaptive composite score	58.64 (16.34)	57.70 (13.77)	60.86 (20.69)	58.30 (18.25)	0.279	0.624	0.024
Parenting Stress Index Short Form (PSI/SF)							
Total score	67.50 (22.27)	68.30 (28.10)	63.50 (29.96)	66.25 (32.40)	0.479	0.623	0.025
Gastrointestinal Symptoms Rating Scale (GSRS)							
Total score	5.10 (3.39)	6.35 (4.89)	4.14 (3.86)	4.20 (3.00)	0.823	0.447	0.042

n = 21–22 per group. See also Table S1.
[a]Highlights statistically significant outcomes.

treatment did not change the overall innate or adaptive immune profile (Tables 3 and S2). More specifically, we did not identify significant changes in the frequency of several types of innate immune cells (i.e., dendritic cells [DCs], monocytes, natural killer [NK] cells, and innate lymphoid cells [ILCs]) or adaptive immune cells (i.e., T helper cells [T_h1, T_h2, T_h17, $T_h1/17$], T regulatory cells [T_{reg}], $CD8^+$ T cells, mucosal-associated invariant T [MAIT] cells, and T cell receptor $[TCR]_{\gamma\delta}^+$ cells) (Table 3). Of note, the frequency of a subtype of ILCs (i.e., ILC1) was increased in the control group but not in the treatment group.

Moreover, no significant time-by-treatment changes were identified in most of the soluble immune factors (e.g., cytokines and chemokines, Table S2). Although there were a few changes, most of them were driven by either changes specific to or concomitant with the control group and not by specific changes in the treatment group (Table S2). Of potential interest, the only significant and specific change driven by *L. reuteri* treatment was a reduction in the levels of soluble CD40L. However, whether this is involved in the changes in social behavior and how these levels compare to typically developing children remain to be determined. In conclusion, *L. reuteri* treatment did not dramatically alter the host's overall immune profile.

Effects of different strains of *L. reuteri* on social behavior in a mouse model for ASD

The *L. reuteri* tablet used as the treatment in the human trial is a product that contains two strains of *L. reuteri* (see STAR Methods, *L. reuteri* ATCC PTA 6475 and DSM 17938). However, it is unclear whether both strains can improve social behavior or whether only one strain is mediating the prosocial effect. Our previous preclinical studies in mice with social deficits showed that *L. reuteri* ATCC PTA 6475 is sufficient to improve social behavior.[22–24]

To determine whether the prosocial effect was strain-specific, we first sought to determine whether the combination of strains would improve social behavior in mice as it did in children with ASD. To answer this question, we treated BTBR mice, an idiopathic ASD mouse model,[39] with a combination of *L. reuteri* ATCC PTA 6475 and *L. reuteri* ATCC 55730 (the parent strain of DSM 17938, which has been shown to be genetically [apart from two antibiotic-resistance-containing plasmids cured from ATCC 55730] and phenotypically equivalent across several metrics [e.g., survival through the gut, adherence to mucus, blood safety profile, and efficacy in improving acute gastroenteritis in children] to its daughter strain[40,41]). Briefly, mice were tested for social behavior using the 3-chamber sociability and social

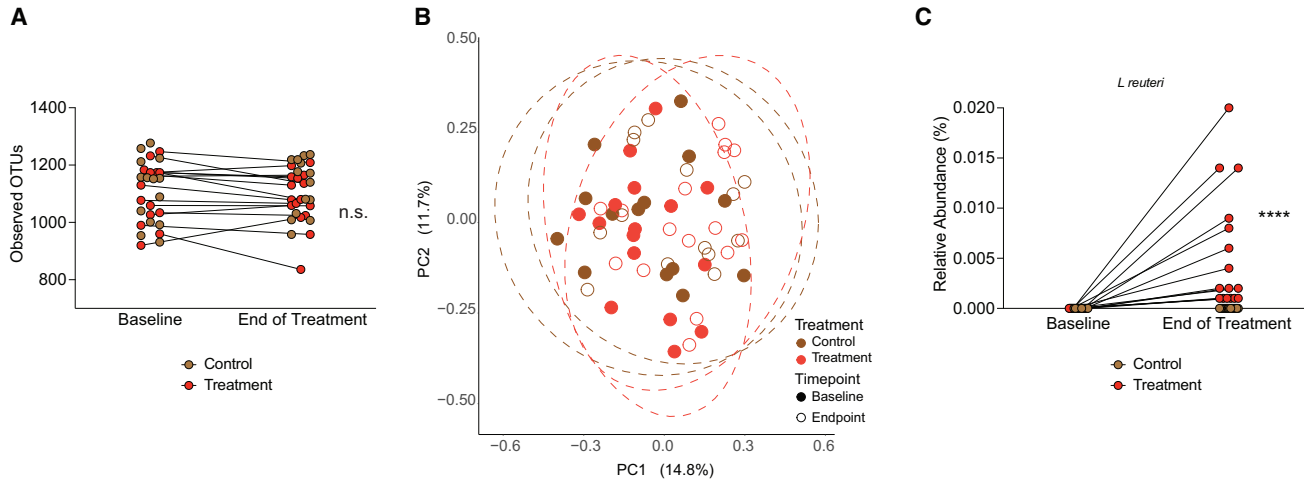

Figure 2. Metagenomics profile of control and treatment groups

(A) Alpha diversity of metagenomic sequencing of control and treatment groups (n = 14–16 per group per time point) as measured by the number of observed operational taxonomic units (observed OTUs; two-way repeated measures ANOVA—time × treatment: $F_{(1, 28)} = 2.282$, p = 0.142).

(B) Beta diversity of metagenomic sequencing of control and treatment groups (n = 14–16 per group per time point) as measured by principal coordinates analysis (PcoA) of Bray-Curtis distance (PERMANOVA: $R^2 = 0.00743$, p = 0.9911).

(C) Relative abundance of *L. reuteri* levels in the metagenomic sequencing of control and treatment groups (n = 14–16 per group per time point; DESeq2 time × treatment: $p_{adj} = 1.02 \times 10^{-5}$).

See also Figure S1.

novelty tests (Figures 3A and 3B), as previously described.[22–24] In the sociability test, we compared the amount of time that the experimental mouse spent interacting with either a stranger mouse or an empty wired cup; whereas, in the social novelty test, we measured the amount of time that the experimental mouse spent interacting with either a familiar mouse or a novel mouse (Figure 3A). Unlike the social C57BL/6J mice, BTBR mice displayed impaired sociability and social novelty, as reflected by the lack of preference for the stranger mouse in the sociability test (Figure 3C) and for the novel mouse in the social novelty test (Figure 3D), consistent with previous results.[23] 4 weeks of treatment with this *L. reuteri* combination reversed the social deficits in BTBR mice (Figures 3C and 3D) but did not affect locomotor activity or increased repetitive behaviors (Figure S5).

Given that different *L. reuteri* strains have distinct effects on host phenotypes (e.g., immunomodulation, metabolism[36,38]), we next sought to determine whether the prosocial effect in the ASD mouse model was driven by both strains or only one of the two strains. Treatment with *L. reuteri* ATCC PTA 6475 improves social behavior in BTBR mice (Figures 3E and 3F), consistent with our previous findings.[23] Remarkably, however, treatment with *L. reuteri* ATCC 55730 fails to improve social behavior in BTBR mice (Figures 3E and 3F). Given these results, it would be interesting to determine whether the prosocial effect of the *L. reuteri* treatment (ATCC PTA 6475 + DSM 17938) in children with ASD is mediated by the *L. reuteri* ATCC PTA 6475 strain alone.

DISCUSSION

The gut-microbiota-brain axis is emerging as a potential new therapeutic target for the treatment of central nervous system disorders.[17,42] Yet, evidence in humans that microbial-based therapeutics improve core symptoms of neurological disorders is missing. For instance, in a preliminary open-label trial, a small-molecule drug that reduces a microbial metabolite has recently shown some improvement in anxiety and irritability in children with ASD, but whether this intervention improves some of the core ASD symptoms (i.e., social interaction, communication, and repetitive behaviors) is unknown.[43] Notably, several studies have found that children with ASD possess distinct gut microbiota profiles compared with neurotypical children.[44–46] However, it is unclear whether the changes in the microbiota cause the symptoms of ASD or are simply correlated with restricted dietary preferences.[47,48] Regardless, recent clinical studies showed that direct modulation of the gut microbiome, via fecal microbiota transplants (FMTs), improves some behavioral symptoms in children with ASD.[49–51] Unfortunately, FMTs present several important challenges, including an increased risk of adverse events,[52] the need to source donor stool, a lack of standardized protocols for preparation and administration, and the need to screen the stool for pathogens.[53,54] Thus, we believe that the use of a probiotic (single or combination of strains) could be a better and safer therapeutic option.

In this regard, in this double-blinded, randomized, placebo-controlled pilot clinical trial, despite the lack of significant improvements in the overall severity of ASD symptoms, we provide evidence that precision targeting of the gut-microbiota-brain axis with *L. reuteri* combination improves a defining behavioral symptom of ASD—the deficits in social behavior. More specifically, *L. reuteri* combination treatment had significant positive effects in both reducing severity of social impairments and improving adaptive social skills (Table 2).

Table 3. Summary of blood immune cell frequency profiling

	Mean (SD)						
	Baseline		End of treatment		Treatment × time		
	Control	Treatment	Control	Treatment	*F*	p	η_p^2
Innate immune response (% within CD45$^+$ cells)							
Plasmacytoid dendritic cell (pDC)	0.34 (0.24)	0.41 (0.19)	0.55 (0.33)	0.39 (0.20)	2.044	0.1604	0.048
Conventional dendritic cell (cDC)	0.18 (0.18)	0.23 (0.16)	0.38 (0.24)	0.34 (0.19)	1.718	0.1973	0.040
Classical monocytes	7.25 (3.36)	10.18 (5.01)	11.77 (5.56)	14.17 (6.27)	0.084	0.773	0.002
Intermediate monocytes	0.22 (0.13)	0.31 (0.17)	0.41 (0.20)	0.63 (0.54)	1.071	0.307	0.025
Non-classical monocytes	1.15 (0.56)	1.59 (0.83)	1.31 (0.88)	1.93 (1.13)	0.389	0.537	0.009
NK CD56^{bright}	0.62 (0.30)	0.66 (0.27)	0.46 (0.25)	0.43 (0.21)	0.728	0.399	0.016
NK CD56^{med}	6.08 (4.35)	9.20 (5.14)	5.82 (3.38)	7.10 (3.73)	1.675	0.203	0.039
Total ILC	0.22 (0.12)	0.19 (0.09)	0.57 (0.45)	0.37 (0.28)	2.155	0.150	0.050
ILC1[a]	0.015 (0.01)[a]	0.014 (0.01)[a]	0.035 (0.04)[a]	0.016 (0.01)[a]	4.839[a]	0.034[a]	0.106[a]
ILC2	0.043 (0.03)	0.045 (0.02)	0.026 (0.02)	0.029 (0.01)	0.006	0.940	0.0001
ILC3	0.039 (0.04)	0.030 (0.02)	0.069 (0.05)	0.042 (0.02)	2.030	0.162	0.05
Adaptive immune response (% within CD45$^+$ cells)							
Th1	3.57 (0.90)	3.44 (0.73)	2.95 (0.71)	2.57 (0.90)	0.948	0.336	0.023
Th2	0.23 (0.10)	0.28 (0.17)	0.16 (0.09)	0.26 (0.27)	1.083	0.304	0.026
Th17	0.94 (0.34)	1.07 (0.54)	0.84 (0.28)	0.87 (0.55)	1.548	0.221	0.036
Th1/17	0.77 (0.34)	0.82 (0.47)	0.63 (0.26)	0.56 (0.28)	2.161	0.149	0.050
T_{reg}	0.30 (0.12)	0.37 (0.22)	0.27 (0.13)	0.25 (0.08)	2.222	0.144	0.051
CD8	19.49 (5.12)	18.8 (3.48)	18.45 (4.81)	17.99 (4.11)	0.098	0.756	0.002
MAIT	1.25 (0.90)	1.22 (0.97)	1.01 (0.71)	0.81 (0.49)	0.945	0.337	0.023
TCR$\gamma\delta$	5.33 (3.62)	6.22 (3.99)	4.00 (1.69)	4.85 (2.91)	0.002	0.967	<0.0001
Lymph B	8.45 (2.17)	7.72 (2.12)	9.96 (2.47)	9.82 (3.01)	0.535	0.469	0.013

n = 21–22 per group. See results and STAR Methods sections for full names of abbreviated immune cell types. See also Figure S2 and Table S2.
[a]Highlights statistically significant outcomes.

The *L. reuteri*-mediated improvements in individuals with ASD were selective to the social domain, consistent with the preclinical findings.[22–24] It is important to note that social functioning is complex and multifaceted, encompassing several subdomains underpinned by at least partially distinct neural networks. For instance, while the social motivation subdomain is subserved by mesolimbic-cortical-striatal circuits,[55,56] the mental state understanding subdomain is mediated by circuits in the dorsal and ventral medial prefrontal cortex, temporo-parietal junction, and the posterior cingulate cortex/precuneus.[57] Therefore, focusing on specific social subdomains may be a more effective approach for identifying promising therapeutics because it is unlikely that one drug will engage multiple neural systems. Accordingly, *L. reuteri* treatment led to improvements in specific aspects of social functioning. Thus, in future studies, it will be important to focus on specific, well-defined clinical subdomains rather than overly broad global functioning composite scores.

Although *L. reuteri* treatment improved components of social functioning, it did not significantly alter the overall gut microbiome composition (Figures 2 and S1) or the host immune response (Table 3; Figure S2). These findings are consistent with our previous preclinical results in mouse models for ASD, which show that *L. reuteri* works independently of other microbes[23] and independently of the host's adaptive immune response.[27]

Of likely future clinical relevance, we found that the ATCC PTA 6475 strain reversed social deficits in a mouse model for ASD (Figure 3), but not ATCC PTA 55730 (the parental strain of the DSM 17938[40]), suggesting that the prosocial effects of *L. reuteri* may be strain-specific. Interestingly, a report that was published during the revision of this manuscript showed that a formulation containing a single strain of *L. reuteri* (*L. reuteri* ATCC 23272 with dextran microparticles and maltose[58]) yielded a positive effect on social functioning in children with ASD.[59] Taken together, these results indicate that only specific strains of *L. reuteri* may improve social functioning. Therefore, future clinical trials should investigate whether administration of a single strain of *L. reuteri* (e.g., ATCC PTA 6475 or ATCC 23272) yields a similar or greater improvement in social behavior than a combination of strains (e.g., ATCC PTA 6475 + ATCC 55730, ATCC PTA 6475 + ATCC 23272, or ATCC 55730 + ATCC 23272).

Limitations of the study

As with other pilot studies, our promising findings need to be considered in light of certain limitations. First, despite the use of a randomized controlled trial design, this study is somewhat limited by the sample size, in part due to the recruitment period occurring during the height of the COVID-19 pandemic in Italy. Thus, given the limited sample size, it will be important to replicate the findings with a larger sample size. However, as

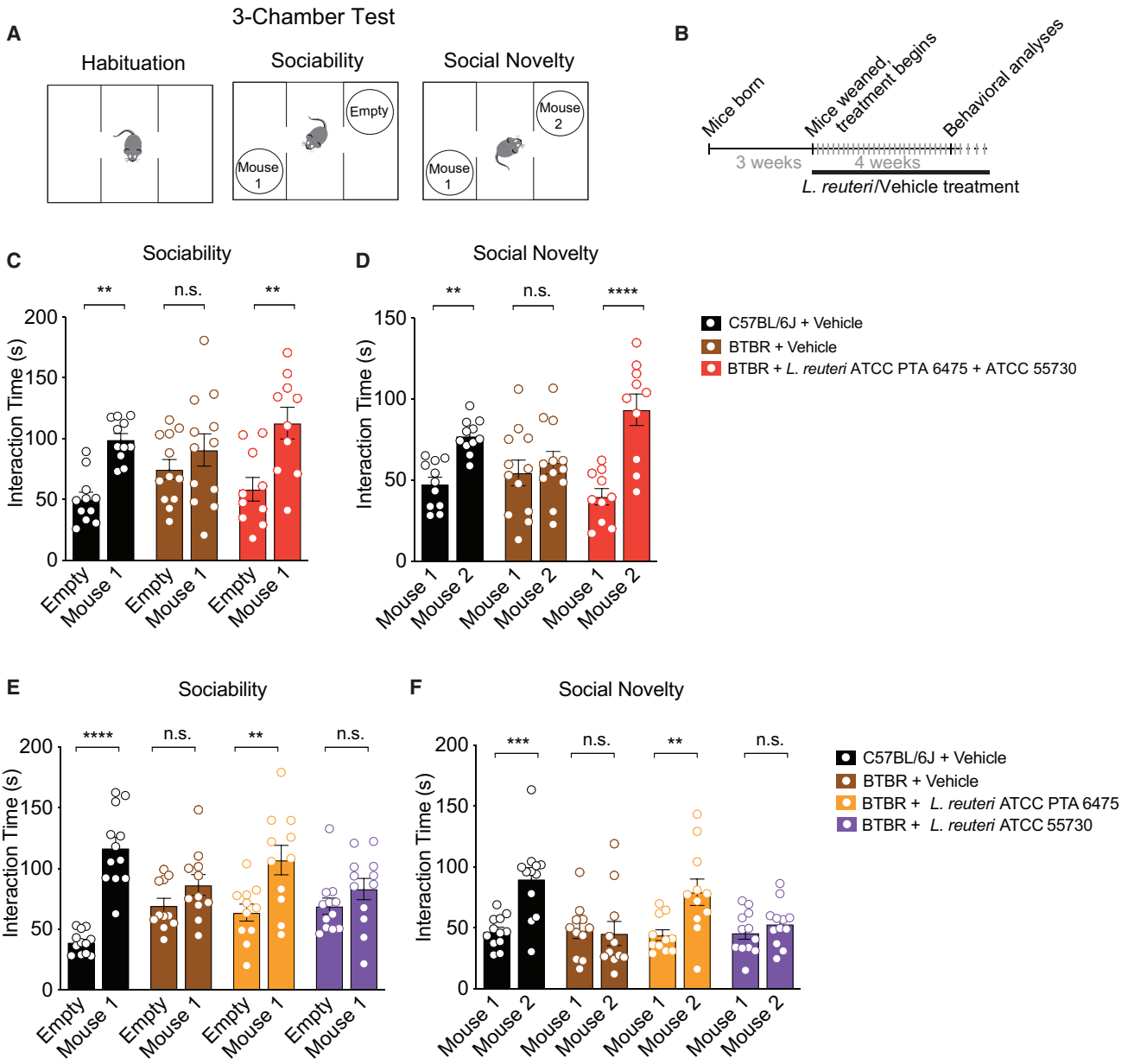

Figure 3. Strain-specific effects of *L. reuteri* on social behavior

(A) Schematic of the 3-chamber test for social behavior.

(B) Schematic of experimental design for the treatment with *L. reuteri*.

(C and D) Social behavior in *L. reuteri* ATCC PTA 6475- + ATCC 55730-treated mice in the 3-chamber test (n = 10–12 per group; C, sociability: C57BL/6J + vehicle: $t = 3.553$, p = 0.002, BTBR + vehicle: $t = 1.214$, p = 0.6890, BTBR + *L. reuteri* ATCC PTA 6475 + ATCC 55730: $t = 3.758$, p = 0.0012, two-way ANOVA with Bonferroni correction $F_{(2,60)} = 2.340$, p = 0.1051; D, social novelty: C57BL/6J + vehicle: $t = 3.202$, p = 0.0066, BTBR + vehicle: $t = 0.7210$, p > 0.9999, BTBR + *L. reuteri* ATCC PTA 6475 + ATCC 55730: $t = 5.519$, p < 0.0001, two-way ANOVA with Bonferroni correction $F_{(2,60)} = 6.455$, p = 0.0029).

(E and F) Social behavior in *L. reuteri* ATCC PTA 6475- or ATCC 55730-treated mice in the 3-chamber test (n = 11–12 per group; E, sociability: C57BL/6J + vehicle: $t = 7.018$, p < 0.0001, BTBR + vehicle: $t = 0.5789$, p = 0.5789, BTBR + *L. reuteri* ATCC PTA 6475: $t = 3.745$, p = 0.0013, BTBR + *L. reuteri* ATCC 55730: $t = 1.278$, p = 0.8188, two-way ANOVA with Bonferroni correction $F_{(3,84)} = 6.997$, p = 0.0003; F, social novelty: C57BL/6J + vehicle: $t = 4.272$, p = 0.0002, BTBR + vehicle: $t = 0.2392$, p > 0.9999, BTBR + *L. reuteri* ATCC PTA 6475: $t = 3.351$, p = 0.0048, BTBR + *L. reuteri* ATCC 55730: $t = 0.7258$, p > 0.9999, two-way ANOVA with Bonferroni correction $F_{(3,84)} = 4.488$, p = 0.0057).

Bar graphs are represented as mean ± SEM with individual data points.

See also Figure S3.

indicated above, during the revision of our manuscript, an independent, small randomized-controlled cross-over clinical trial performed in the US showed a potential positive effect for *L. reuteri* on social functioning in children with ASD.[59] Taken together, these data support the notion that the prosocial effect of *L. reuteri* in humans is reproducible.

Second, although there was a positive and specific signal for improvements in social functioning, the current study was not designed nor sufficiently powered to identify predictors of positive treatment response. For example, there were very few female participants in this study. Although there is a well-established male bias in ASD diagnoses, future studies should make efforts to recruit more females. It is also important to acknowledge that, although the difference was not statistically significant, the treatment group had higher IQ scores than the placebo group at baseline. Given the well-established heterogeneity in treatment response among individuals with ASD, it will be crucial for future studies to characterize profiles of responders and non-responders to *L. reuteri* treatment in large sample sizes.

Third, ADOS-2 was chosen as a primary outcome measure, given that it is a clinician-administered observational assessment and thus might be less prone to bias compared with questionnaire measures. However, given the narrow range of scores in the ADOS-2 for each item, which limits its ability to detect subtle changes,[60] it is not surprising that ADOS-2 was not modified by treatment with *L. reuteri*. Indeed, a number of studies have found that, although ADOS-2 can detect changes in overall ASD severity over a period of several years,[61,62] it is not effective in capturing changes in shorter periods of time, which restricts its utility as a treatment outcome measure.[63]

Finally, there is currently a lack of instruments designed to capture different aspects of social functioning that are generalizable across species (e.g., subdomains of social behavior in mice and humans). Therefore, it will be important to continue to develop such measures and then to further characterize translational continuities and discontinuities in treatment effects.

Nonetheless, these positive findings warrant follow-up studies in larger cohorts, focusing on different aspects of social functioning as a primary outcome and utilizing multi-modal measurements to further assess the efficacy of *L. reuteri* treatment to improve social functioning in children with ASD. More importantly, if the findings of this pilot study are validated by a larger clinical trial, the clinical impact could be substantial given that there are currently no FDA-approved treatments to improve social functioning abilities. In conclusion, we believe that our work signifcantly contributes to the transformative idea that the core symptoms of ASD (and likely other brain disorders) can be treated with specific gut microbes via the gut-microbiome-brain axis.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.chom.2023.11.021.

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AUTHOR CONTRIBUTIONS

Conceptualization and design of clinical trial, L.M., E.V., L.A., R.A., A.R., M.S., F.C., and R.F.; acquisition of clinical trial data, L.M., E.V., L.A., R.A., A.R., M.S., and A.S.; analysis of clinical trial behavioral data, L.M., M.U., and A.Y.H.; analysis of clinical trial metagenomic data, J.L.W., M.P., D.A., and L.E.; analysis of clinical trial immunological data, S.W.D., E.V., and A.S.; conceptualization and design of preclinical mouse experiments, S.W.D. and M.C.-M.; acquisition and analysis of preclinical mouse data, S.W.D.; writing, reviewing, and editing, L.M., S.W.D., M.U., J.L.W., M.S., E.V., A.Y.H., M.C.-M., and R.F.

DECLARATION OF INTERESTS

M.C.-M., S.W.D., and J.L.W. are employees of Altos Labs, Inc. M.C.-M. is a shareholder of Altos Labs, Inc. and Mikrovia, Inc.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial strains		
L. reuteri and placebo tablets	BioGaia	Gastrus and placebo
L. reuteri ATCC PTA 6475	Laboratory of Dr. Rob Britton	N/A
L. reuteri ATCC PTA 55730	Laboratory of Dr. Jens Walter	N/A
Critical commercial assays		
MGI FS DNA library prep kit	MGI	1000017572
Human Cytokine/Chemokine/Growth Factor Panel A	Millipore	HCYTA-60K-PXBK38
LIVE/DEAD™ Fixable Aqua Dead Cell Stain Kit	Thermo Fisher Scientific	I34957
Experimental models: Organisms/strains		
C57BL/6J mice	The Jackson Laboratory	#000667
BTBR mice	The Jackson Laboratory	#002282
Deposited Data		
Metagenomic sequencing data	European Nucleotide Archive	PRJEB60702
Software and algorithms		
AnyMaze	Stoelting Co.	https://www.any-maze.com/
FlowJo v10.8	BD Biosciences	https://www.flowjo.com/solutions/flowjo
xPONENT 4.2 for MAGPIX	Luminex	https://us.diasorin.com/en/licensed-technologies/reagents-accessories/software
Prism v9.0	GraphPad	https://www.graphpad.com/updates/prism-900-release-notes
SPSS Statistics v24.0	IBM	https://www.ibm.com/support/pages/downloading-ibm-spss-statistics-24
mPlus v8.0	StatModel	http://www.statmodel.com/verhistory.shtml
R v4.2.2	The R Foundation	https://cran.r-project.org/bin/windows/base/old/4.2.2/
R Studio	R Studio	https://posit.co/download/rstudio-desktop/
Trimmomatic v0.39	Bogler et al.[64]	http://www.usadellab.org/cms/?page=trimmomatic
Bowtie v2.5.0	Langmead and Salzberg[65]	https://bowtie-bio.sourceforge.net/bowtie2/index.shtml
Kraken2 v2.1.2	Wood et al.[66]	https://github.com/DerrickWood/kraken2
Braken v2.8	Lu et al.[67]	https://github.com/jenniferlu717/Bracken/releases/tag/v2.8
Phyloseq v1.42.0	McMurdie and Holmes[68]	https://bioconductor.org/packages/release/bioc/html/phyloseq.html
Struo2	Youngblut and Ley[69]	https://github.com/leylabmpi/Struo2
Vegan v2.6-4	Oksanen et al.[70]	https://cran.r-project.org/web/packages/vegan/index.html
DESeq2 v1.38.3	Love et al.[71]	https://bioconductor.org/packages/release/bioc/html/DESeq2.html
Antibodies		
Mouse monoclonal anti human CD45 (clone ALB12) Conjugate FITC	Beckman Coulter	#: IM0647; RRID: AB_3073537

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Recombinant monoclonal anti human CRTh2 (clone REA598) Conjugate PE	Miltenyi Biotec	#: 130-113-602; RRID:AB_2733801
Mouse monoclonal anti human CD161 (clone HP-3G10) Conjugate PE-Dazzle 594	BioLegend	#: 339939; RRID:AB_2565867
Mouse monoclonal anti human CD56 (clone QA17A16) Conjugate PE-Cy7	BioLegend	#: 985912; RRID:AB_2922663
Mouse monoclonal anti human CXCR3 (clone G025HT) Conjugate APC-Alexa Fluor 647	BioLegend	#: 353711; RRID:AB_10962946
Mouse monoclonal anti human CD8 (clone B9.11) Conjugate APC-Alexa 700	Beckman Coulter	#: A66332; RRID:AB_2750854
Mouse monoclonal anti human TCR-γδ (clone 11F2) Conjugate APC-Vio770	Miltenyi Biotec	#: 130-113-501; RRID:AB_2751120
Mouse monoclonal anti human CCR6 (clone G034E3) Conjugate BV421	BioLegend	#: 353407; RRID:AB_10916530
Mouse monoclonal anti human CD3 (clone SP34-2) Conjugate BV605	BD Biosciences	#: 562994; RRID:AB_2737938
Mouse monoclonal anti human CD4 (clone M-T477) Conjugate BV650	BD Biosciences	#: 742735; RRID:AB_2741009
Mouse monoclonal anti human CD25 (clone M-A251) Conjugate PE-CF594	BD Biosciences	#: 562403; RRID:AB_11151919
Mouse monoclonal anti human CD4 (clone OKT4) Conjugate BB700	BD Biosciences	#: 566829; RRID:AB_2869889
Mouse monoclonal anti human CD11c (clone BU15) Conjugate PE-Cy7	Beckman Coulter	#: B96763; RRID: AB_3073556
Mouse monoclonal anti human CD127 (clone R34.34) Conjugate APC-Alexa Fluor 700	Beckman Coulter	#: A71116; RRID:AB_2889979
Recombinant monoclonal anti human CD14 (clone REA599) Conjugate APC-Vio770	Miltenyi Biotec	#: 130-110-522; RRID:AB_2655063
Mouse monoclonal anti human CD3 (clone OKT3) Conjugate eFluor 450	Thermo Fisher Scientific	#: 48-0037-42; RRID:AB_1272055
Mouse monoclonal anti human CD19 (clone SJ25-C1) Conjugate Pacific Blue	Thermo Fisher Scientific	#: MHCD1928; RRID:AB_10373689
Mouse monoclonal anti human CD56 (clone NCAM16.2) Conjugate BV421	BD Biosciences	#: 562751; RRID:AB_2732054
Mouse monoclonal anti human CD123 (clone 7G3) Conjugate BV605	BD Biosciences	#: 564197; RRID:AB_2732049
Mouse monoclonal anti human CD16 (clone 3G8) Conjugate BV786	BD Biosciences	#: 563689; RRID:AB_2744299
Mouse monoclonal anti human CD117 (clone 2B8) Conjugate FITC	Thermo Fisher Scientific	#: 11-1171-82; RRID:AB_465186
Mouse monoclonal anti human CD127 (clone MB15-18C9) Conjugate PE-Vio770	Miltenyi Biotec	#: 130-113-412; RRID:AB_2726160
Mouse monoclonal anti human NKp44 (clone 2.29) Conjugate APC	Miltenyi Biotec	#: 130-120-484; RRID:AB_2811336
Mouse monoclonal anti human CD45 (clone 5B1) Conjugate APC-Vio770	Miltenyi Biotec	#: 130-113-115; RRID:AB_2725943
Mouse monoclonal anti human CD14 (clone RMO52) Conjugate APC-Alexa Fluor 700	Beckman Coulter	#: A99020; RRID:AB_3073559
Mouse monoclonal anti human CD16 (clone 3G8) Conjugate Pacific Blue	Beckman Coulter	#: B36292; RRID: AB_3073560
Mouse monoclonal anti human CD19 (clone HIB19) Conjugate BV650	BioLegend	#:302238; RRID:AB_11126981
Other		
Human Clinical Trial	ClinicalTrials.gov	NCT04293783

RESOURCE AVAILABILITY

Lead Contact

Further information and reasonable requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Mauro Costa-Mattioli (mcostamattioli@altoslabs.com).

Materials Availability

This study did not generate new unique reagents. Information on reagents used in this study is available in the key resources table.

Data and Code Availability

Raw metagenomic sequencing reads are available from the European Nucleotide Archive using accession number PRJEB60702 (ENA: PRJEB60702). Other deidentified data from the human clinical trial and preclinical mouse studies will be made available upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Clinical Trial – Participant Enrollment

This trial was a single-center randomized double-blind, parallel-group, placebo-controlled study to test the effect of *L. reuteri* supplementation on the behavioral profiles of children with autism spectrum disorder (ASD). The study was approved by Policlinico Tor Vergata Foundation Hospital (PTV) Institutional Review Board (#244/19) and prospectively registered on ClinicalTrials.gov (NCT04293783). Parents or legal guardians of the study participants were provided written consent before study enrollment and the initiation of experimental procedures. Recruitment began in April 2020 and ended in May 2020.

Participants were screened for eligibility and recruited from the Child-Adolescence Psychiatry Unit of the University of Rome Tor Vergata (Italy), PTV Foundation Hospital among children followed by the clinical unit. Male and female children between the ages of 2 and 8 years with a diagnosis of ASD based on the criteria in the Diagnostic and Statistical Manual of Mental Disorders, 5th edition[1] and confirmed by the administration of the gold standard measure Autism Diagnostic Observation Schedule, Second Edition (ADOS-2)[72] – performed by a licensed multidisciplinary team of child psychiatrists or clinical psychologists – were included in this study. Children with neurological and genetic syndromes, coeliac disease, other organic gastrointestinal disorders, or on a special diet were excluded from this study. Any modification in daily diet and pharmacological treatment had to be reported on a weekly diary. Participants were allowed to continue the concomitant treatment such as behavioral and speech therapy, as long as no modification in terms frequency and type of intervention occurred during the 6-month trial period. Detailed information concerning concomitant rehabilitative treatment were collected at the baseline and final evaluations. A total of 43 children (35 males, 8 females) between the ages of 2-8 years were ultimately enrolled in the study. Given that this recruitment occurred during the height of the COVID-19 pandemic, we did not meet the estimated enrollment of approximately 80 individuals.

Preclinical study - Animal Husbandry

Wild-type C57BL/6J (stock #000664) and BTBR (stock #002282) mice were obtained from Jackson Laboratories (Bar Harbor, Maine). Mice were housed under standard husbandry condition with 2-4 mice per cage in filter-top caging with corn cob bedding and *ad libitum* access to food and water. Only male mice were used given that more males experience ASD, and that the majority of the human participants in our study were males. Animals were randomly assigned to treatment groups. Treatment began when the mice were approximately 4 weeks of age and behavioral experiments began when the mice were approximately 8 weeks of age. 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.

Preclinical Study - Culture and Treatment with *L. reuteri* for Animal Experiments

L. reuteri ATCC PTA 6475 and ATCC PTA 55730 were cultured anaerobically in MRS broth at 37°C in a 90% N2, 5% CO2, 5% H2 environment as previously described.[22] 55730 was chosen in this study as it is nearly genetically identical to its daughter strain, DSM 17938, except for two antibiotic-resistance plasmids which were removed from ATCC PTA 55730 and otherwise possess similar phenotypic and probiotic properties.[40] Importantly, no difference has been found between 17938 and 55730 in clinical trials.[40,41]

Briefly, cultures were centrifuged, washed, resuspended in PBS, and frozen at -80°C until use. For the combination experiments, each strain was grown, washed, and resuspended separately and then combined in equal proportions prior to freezing. PBS (vehicle) or *L. reuteri* was added to the drinking water daily to minimize dosage variability. The experimental group received live bacteria (~1 x 10^8 organisms/mouse/day), while the control group received equal volume of PBS. Mice drank the treated water *ad libitum* during the treatment period.

METHOD DETAILS

Clinical Trial – Assessment and Treatment Plans

After enrollment, a baseline assessment including standardized measures of cognitive skills, autism symptoms and behavioral profile was performed (Table 1). Cognitive abilities were assessed using the non-verbal Intellectual Quotient (IQ) through the Leiter-R scale.[73] The ADOS-2[72] was also used to assess overall autism severity. Social abilities were assessed using (i) the SRS total T-score,[32] the Social Communication, Social Motivation and Mental State Understanding subdomains as derived from SRS items[31] and (ii) the social adaptive composite score of the ABAS-2.[33] Adaptive abilities were assessed using the specific subscales of the ABAS-2. Finally, restricted and repetitive behaviors were measured using the RBS-R[74,75] and psychiatric and behavioral problems were assessed using the CBCL.[76]

After baseline assessments, children were randomly assigned to either the treatment (BioGaia Gastrus, $\geq$ 2x10^8 CFU *L. reuteri* DSM 17938 and ATCC PTA 6475/tablet) or placebo group. Block randomization by age and gender was performed using www.randomization.com, by the probiotic company BioGaia. Packaging, appearance, odor, and taste were the same for both products. Participants were instructed to take two tablets by mouth per day for 6 months. A weekly food diary, reporting concomitant medications necessary for acute condition (e.g., antibiotics, anti-inflammatories) and detailing modification in the experimental treatment (e.g., suspension) was completed by parents. A follow-up assessment was performed at 6-months (end-of-study) with all the instruments (Table 2).

The Autism Diagnostic Observation Schedule, Second Edition (ADOS-2[72]): The ADOS-2 is a semi-structured diagnostic instrument appropriate for children 12 months of age to adulthood, that allows the assessment of individuals through behavioral observations during specific play, social, and language tasks. The ADOS-2 provides empirically-derived thresholds for the diagnoses of ASD that show good sensitivity and specificity. In this study, we used the total calibrated severity score (CSS), a metric developed to standardize ADOS-2 severity scores across age and language levels and enable comparison across different ADOS-2 modules.[77] A higher score indicates more severe symptoms.

The Social Responsiveness Scale (SRS[32]): The SRS is a 65-item, norm-referenced, parent report questionnaire originally designed for use with children aged 4 through 18, and more recently extended to younger and older age brackets. Each item is rated on a four-point Likert scale (from 1 = "not true" to 4 = "almost always true") with higher scores indicating higher trait severity/atypicality. Raw scores can be transformed into T-Scores based on age and gender. The total T-Scores are considered in addition to the scores of specific subscales. SRS social items were originally organized into the following subscales: social awareness, social cognition, social communication, social motivation and autistic mannerism,[32] however, subsequent factor analyses were not able to support these conceptually based factors.[31,78] The largest factor analysis of the SRS to date (N= 27,953 children and adolescents) showed that three-factor solution encompassing social motivation, social communication and mental state understanding factors provided the best fit to the data (comparative fit index = 0.952, Tucker Lewis Index = 0.937, root mean square error of approximation = 0.054). Thus, in this study, we used this three-factor solution.

The Repetitive Behavior Scale-Revised (RBS-R[74,75]): The RBS-R is a rating scale for measuring the presence and severity of a variety of forms of restricted, repetitive behavior that are characteristic of individuals with autism. RBS-R was originally designed to capture the following 6 distinct subscales: stereotypies, self-injurious behaviors, compulsions, rituals, insistence on sameness, and restricted, however, subsequent studies have supported five-factor solution with stereotypies, self-injurious behaviors, compulsions, ritualistic/sameness, and restricted behaviors subscales.[75,79,80] Given that self-injurious behaviors are not classically considered as repetitive behaviors, we did not include this subscale but instead utilized the other empirically-derived subscales (i.e., stereotypies, compulsions, ritualistic/sameness, and restricted behaviors). We did not consider total RBS-R scores given the complexity and heterogeneity of this domain and recommendations that clinical trials should focus on discrete RBS-R subdomains rather than overall index of severity.[81] A higher score indicate more severe behavioral symptoms.

The Child Behavior Checklist (CBCL[76]): The CBCL is a norm-referenced, parent report questionnaire measure designed to assess behavioral, emotional, and social problems in children and adolescents. In addition to the total score, the CBCL also provides internalizing and externalizing scores that can be combined across the two age-specific versions (2.5-5 years and 6-18 years of age). Thus, in this study we focused on internalizing and externalizing behaviors. A higher score indicates more severe problems.

The Adaptive Behaviors Assessment System, Second Edition (ABAS-2[33]): ABAS-2 evaluates the adaptive skills needed to interact, care for oneself, respond to others, and meet environmental demands at home, school, work, and in the community. The ABAS-2 assessed different domains including General Adaptive Composite (GAC) and three adaptive domains (conceptual, social, and practical). A higher score indicates better adaptive functioning or less severe symptoms.

The Parenting Stress Index Short Form (PSI/SF[82]): The PSI/SF is a 36-item parent report instrument designed to evaluate stress within the parent-child relationship. In this study, we focused on the overall parental stress indexed by the total PSI/SF score. A higher score indicates a higher level of parental stress.

Gastrointestinal Symptoms Rating Scale (GSRS[83]): The GSRS is a 15 items questionnaire that evaluates different gastrointestinal symptoms including abdominal pain, reflux syndrome, diarrhea syndrome, indigestion syndrome (borborygmus, abdominal distension, eructation and increased flatus) and constipation syndrome (constipation, hard stools and feeling of incomplete evacuation[84]). In this study, we focused on the total GSRS score as an index of overall severity of gastrointestinal symptoms. A higher score indicates higher gastrointestinal discomfort.

Clinical Trial – Metagenomic Profiling

The sequencing library was prepared using the MGI FS DNA library prep kit as outlined by the manufacturer, with the exception that 50 ng of input DNA was used. Additionally, since a smaller amount of input DNA was used, a single bead clean-up step was employed. Samples were sequenced using a DNBSEQ-G400 sequencer (MGI, Shenzhen, China) as described previously.[85]

Clinical Trial – Immunological Profiling

Peripheral blood mononuclear cells (PBMC) were isolated as previously described[86] by Ficoll gradient centrifugation (GE Healthcare) from 5 mL of whole blood from study participants, and stained with fluorochrome-conjugated antibodies for specific markers of each immune subpopulation. In particular: panel 1: CD45, CRTH2, CD161, CD19, CD56, CXCR3, CD8, TCR-γδ, CCR6, Live Dead, CD3, CD4 for T helper (Th)1, 2, 17, 1/17 lymphocytes, cytotoxic lymphocytes (CD8 T cells), γδT cells, mucosal invariant T (MAIT) cells, natural killer (NK) cells, B lymphocytes; panel 2: CD45, CD25, CD16, CD11c, CD127, CD14, CD3, CD19, CD56, Live Dead, CD123, CD4 for monocytes, plasmacytoid and conventional dendritic cells (pDC and cDC, respectively), T regulatory cells; panel 3: CD117, CD127, CRTH2, CD161, CD117, NKP44, Live Dead, CD3, CD14, CD16, CD19 for innate lymphoid cells (ILC) 1,2,3. After staining, cells were acquired on a Cytoflex (Beckman Coulter) flow cytometer. Data were analyzed with FlowJo v 10.8. The gating strategies are shown in Figures S2–S4.

Plasma cytokine and chemokine profiles were analyzed using Millipore multiplex magnetic bead-based antibody detection kits, following the manufacturer's instructions. Specifically, Human Cytokine/Chemokine/Growth Factor Panel A (Millipore Cat. No. HCYTA-60K-PXBK38) was used for detection of the following analytes: interleukin (IL)-1α, IL-1β, IL-1Ra, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12p70, IL-12p40, IL-13, IL-15, IL-17A, granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon-gamma (IFN)-γ, IFN-α2, interferon gamma-induced protein 10 (IP-10), monocyte chemoattractant protein (MCP) 1, MCP3, macrophage inflammatory protein (MIP)-1α, MIP-1β, macrophage-derived chemokine (MDC), tumor necrosis factor-alpha (TNF)-α, fraktalkine, soluble CD40-Ligand (sCD40L), and transforming growth factor (TGF)-α. Plasma aliquots (50 μL) were used for analysis, with a minimum of 50 beads per analyte acquired. Each sample was analyzed in duplicate. Median fluorescence intensities were measured using a Luminex 200 analyzer. Standard curves and values were calculated using xPONENT 4.2 software for MAGPIX®. Data were analyzed and reported as concentration readings (pg/mL). IL-2, IL-3, IL-5, IL-7, IL-15, IL-17A, and IL-12p70 were undetected in all samples. Subjects were removed from the analysis if both timepoints not present (e.g., not sufficient sample remaining).

Preclinical Study - Animal Behavior Tests

Social behavior, locomotor activity, and repetitive behaviors were measured using the 3-Chamber Test was performed as previously described.[22–24,87] Briefly, behavior tests were conducted on mice aged 8-12 weeks. All experiments were conducted during the light cycle. Mice were habituated to the experimenter for 3 days prior to the start of the behavioral experiment. The experimenter was blind to the treatment group.

On the day of the test, mice were placed in an empty 60 x 40 x 23 cm Plexiglas arena divided into three equally-sized interconnected chambers for a 10-minute habituation period. During habituation, the subject's position was continuously tracked using the automated tracking software, AnyMaze (Stoelting Co., Wood Dale, USA) to measure locomotor activity. In addition, spontaneous self-grooming was measured during habituation by a trained observer using the AnyMaze software.

Sociability was evaluated during a second 10-minute period in which the subject could interact either with an empty wire cup (Empty) or a wire cup containing a stranger mouse (Mouse 1). Stranger mice were age- and sex-matched to the subject mouse. The interaction time was scored by measuring the time the subject mouse spent sniffing or climbing upon either the empty cup or the cup containing the stranger mouse. The position of the empty cup/stranger mouse in the left or right chamber during the sociability period was counterbalanced between trials, to avoid bias. Preference for social novelty was evaluated, in a third 10-minute 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.

QUANTIFICATION AND STATISTICAL ANALYSIS

Clinical Trial – Behavioral Data Analysis

To examine the primary the effects of the *L. reuteri* on primary and secondary outcomes in humans we used general linear modelling approach. Reported results were based on the linear mixed effect random intercept model with an unstructured variance–covariance structure, which enabled reliable estimation of the variance parameters. Type 1 error rate of 0.05 was used for analyses of the efficacy. All analyses were supplemented with the partial eta squared effect size, with values of 0.01 indicating small, 0.06 medium and 0.14 large effect sizes. Analyses were conducted using Mplus 8.0 (Los Angeles, CA) and IBM SPSS Statistics Version 24.0 (Armonk, NY).

Clinical Trial – Metagenomic Profiling

Adapter trimming and initial quality control were performed using Trimmomatic v0.39[64] and human reads were removed in silico after mapping to the GRCh38 reference using Bowtie2 v2.5.0.[65] Filtered reads were then taxonomically profiled using Kraken2 v2.1.2 (with confidence parameter set to 0.5)[66] setting the confidence parameter to 0.5 and Bracken v2.8[67] with the Struo2 generated GTDB-r207

Kraken2 and Bracken databases.[69] Subjects were removed from the analysis if both timepoints not present (e.g., DNA extraction or sequencing failed) which resulted in a final dataset for microbiome analysis that contained 30 subjects (control n = 14, treatment n = 16). Results were imported into R v4.2.2 and filtering, diversity analyses, and plotting were performed using Phyloseq v1.42.0.[68] The final table was filtered to include taxa that had > 10 reads in 25% or more samples and the coefficient of variation was < 3.0. For alpha-diversity analyses, samples were rarefied to 3860940 total counts and a two-way repeated measures ANOVA was performed after normality testing. For beta-diversity analyses, a two-way PERMANOVA was performed using vegan v2.6-4.[70] Taxa barplots were generated using QIIME 2 2022.8.[88] For differential abundance analyses, a likelihood ratio test comparing the full model (Time + Treatment + Time*Treatment) with the reduced model (Time + Treatment) was performed using DESeq2 v1.38.3.[71] An adjusted p-value (P_{adj}) < 0.05 was considered significant for all analyses. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, n.s. = not significant.

Clinical Trial – Immune Profile Data Analysis

Statistical analyses for the immunological profiles were performed using GraphPad's Prism 9 (La Jolla, CA). A two-way repeated measures ANOVA with a type 1 error rate of 0.05 was used for analyses of the efficacy. All analyses were supplemented with the partial eta squared effect size, with values of 0.01 indicating small, 0.06 medium and 0.14 large effect sizes.

Preclinical Study – Behavioral Data Analysis

Statistical analyses for the preclinical mouse studies were performed using GraphPad's Prism 9 (La Jolla, CA). One- or two-way ANOVA with either Tukey's or Bonferroni test to correct for multiple comparisons as indicated in the figure legends were performed. Bar graphs on figures represent mean +/- S.E.M. with individual data points shown as well. P, t, q, and F 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, n.s. = not significant.

ADDITIONAL RESOURCES

The clinical trial was preregistered on ClinicalTrials.gov with study ID: NCT04293783 (https://clinicaltrials.gov/study/NCT04293783).