Full-length Article

The live biotherapeutic *Blautia stercoris* MRx0006 attenuates social deficits, repetitive behaviour, and anxiety-like behaviour in a mouse model relevant to autism

Paromita Sen, Eoin Sherwin, Kiran Sandhu, Thomaz F.S. Bastiaansen, Gerard M. Moloney, Anna Golubeva, Patrick Fitzgerald, Ana Paula Ventura Da Silva, Barbara Chruścicka-Smaga, Loreto Olavarria-Ramírez, Clementine Druelle, David Campos, Pooja Jayaprakash, Kieran Rea, Ian B. Jeffery, Helene Savignac, Sasha Chetal, Imke Mulder, Harriet Schellekens, Timothy G. Dinan, John F. Cryan

APC Microbiome Ireland, University College Cork, Cork, Ireland
Department of Psychiatry & Neurobehavioural Science, University College Cork, Cork, Ireland
Department of Anatomy and Neuroscience, University College Cork, Cork, Ireland
4D Pharma Research Ltd, Aberdeen, UK
4D Pharma Cork Ltd, Cork, Ireland

ARTICLE INFO

Keywords: Microbiota Gut-brain axis BTBR Live biotherapeutic Autism

ABSTRACT

Autism spectrum disorder (ASD) is a neurodevelopmental disorder characterised by deficits in social behaviour, increased repetitive behaviour, anxiety and gastrointestinal symptoms. The aetiology of ASD is complex and involves an interplay of genetic and environmental factors. Emerging pre-clinical and clinical studies have documented a potential role for the gut microbiome in ASD, and consequently, the microbiota represents a potential target in the development of novel therapeutics for this neurodevelopmental disorder. In this study, we investigate the efficacy of the live biotherapeutic strain, *Blautia stercoris* MRx0006, in attenuating some of the behavioural deficits in the autism-relevant, genetic mouse model, BTBR T<sup>+</sup> Itpr3tf/J (BTBR). We demonstrate that daily oral administration with MRx0006 attenuates social deficits while also decreasing repetitive and anxiety-like behaviour. MRx0006 administration increases the gene expression of oxytocin and its receptor in hypothalamic cells *in vitro* and increases the expression of hypothalamic arginine vasopressin and oxytocin mRNA in BTBR mice. Additionally at the microbiome level, we observed that MRx0006 administration decreases the abundance of *Alistipes putredinis*, and modulates the faecal microbial metabolite profile. This alteration in the metabolite profile possibly underlies the observed increase in expression of oxytocin, arginine vasopressin and its receptors, and the consequent improvements in behavioural outcomes. Taken together, these findings suggest that the live biotherapeutic MRx0006 may represent a viable and efficacious treatment option for the management of physiological and behavioural deficits associated with ASD.

1. Introduction

The aetiology of autism spectrum disorders (ASD) is complex and involves a multifaceted interplay of genetic and environmental factors (Muhle et al., 2018). According to the Diagnostic and Statistical Manual of Mental Disorders, 5th Edition (DSM-5), ASD is characterized by deficits in social behaviour/communication and restricted, repetitive and/or sensory patterns of behaviour or interests (2017). Individuals with ASD often present comorbid behavioural pathologies like anxiety, depression, and cognitive impairments (Arnett et al., 2018; DeFilippis, 2018; Zaboski and Storch, 2018). Additionally, growing evidence suggests that gastrointestinal dysfunction is concurrent with ASD (Saurman et al., 2020). Several recent studies have documented alterations in the gastrointestinal microbiota in individuals with ASD, including an
increased abundance of potentially pathogenic genera, such as Clostridium and Desulfovibrio, along with a reduction in the presence of beneficial genera such as Bifidobacterium (Finegold et al., 2017; Parra-cho et al., 2005; Song et al., 2004). Furthermore, altering the gut microbiota with probiotics and prebiotics can improve gastrointestinal and behavioural outcomes in individuals with ASD (Grimaldi et al., 2017). These preliminary studies suggest alterations in the microbiota-gut-brain axis might underlie certain behavioural aspects of ASD and present new opportunities for treatment. Indeed, alterations in microbiota-gut-brain axis communication are thought to underlie symptoms of ASD in individuals (Kraneveld et al., 2016; Li and Zhou, 2016; Srikantha and Mohajeri, 2019). This alteration in the microbiota-gut-brain axis communication is reflected by observed changes in circulating microbial metabolites (Srikantha and Mohajeri, 2019).

Pre-clinical studies have also provided a considerable understanding of the role of the microbiota-gut-brain axis in neurodevelopmental disorders. The BTBR T+Ipr c/l/J (BTBR) mouse is a well-established genetic model of the behavioural and physiological deficits relevant to autism. These mice display deficits in sociability, heightened anxiety, and stereotyped behaviours such as increased self-grooming (Amodeo et al., 2021; Guo and Commons, 2017; Meyza and Blanchard, 2017; O’Connor et al., 2021). Additionally, BTBR mice show agenesis of the corpus callosum, decreased cortical thickness and have a lack of hippocampal commissure (Dodero et al., 2013; Fenlon et al., 2015). Similar atypical anatomical morphology is also observed in patients with ASD (Bethlehem et al., 2020; Lefebvre et al., 2015; Wegiel et al., 2017). Of note, the relative abundance of Bifidobacteria and Blautia genera were found to be dramatically reduced in BTBR mice (Coretti et al., 2017; Golubeva et al., 2017). Thus, it is plausible to target the microbiota as a potential therapeutic strategy for the treatment of ASD. Indeed, we have recently shown that dietary intervention with the fermented drink, Kefir, altered the microbiota profile and reduced stereotyped behaviours (Amodeo et al., 2021; de Wouw et al., 2021). Moreover, treatment with probiotic strains like Lactobacillus reuteri has been shown to alleviate inflammatory (Kalyana Chakravarthy et al., 2018) and metabolic (Liu et al., 2021) diseases. Since inflammation is often observed in individuals with ASD (Siniscalco et al., 2018), Blautia was identified as a potential candidate for investigation given its anti-inflammatory effects. Indeed, this was consistent with our findings which report an altered abundance of Blautia in BTBR mice (Golubeva et al., 2017). Thus, in the current study, we investigated the effect of daily oral administration of Blautia stercoris MRx0006 as a potential treatment approach in addressing the core ASD-associated behavioural deficits in the BTBR mouse model.

2. Methodology

2.1. Ethical approval

Procedures on live animals were conducted under licence from the Government of Ireland Department of Health (B100/3774) in accordance with National and European directive 2010/63/EU. Ethical approval was obtained from University College Cork and procedures were carried out in accordance with guidelines laid down by University College Cork’s Animal Welfare Body.

2.2. Experimental animals

Animals were bred and housed in the animal facility under a 12-hour light/12-hour dark cycle, at 21°C and 50% humidity. Standard rodent chow and water were available ad libitum. All efforts were made to reduce the number of animals used and to minimise the suffering of the animals. Animals were weighed daily to ensure they maintained a stable body weight. Additionally, they were visually inspected regularly for diseases or injuries. BTBR T+Ipr c/l/J mice (The Jackson Laboratory, USA) were bred in-house with brother-sister mating. Male pups were weaned at postnatal (P) day 21 and housed in groups of 3-4 mice per cage. At eight weeks, mice were randomly allocated to receive either vehicle (phosphate-buffered saline; PBS) or MRx0006 treatment: BTBR Vehicle and BTBR MRx0006 groups, respectively. An appropriate sample size was computed when the study was being designed. A total of 13 BTBR Vehicle and 12 BTBR MRx0006 animals were used.

2.3. Preparation of bacteria for in vivo administration

100 μL of MRx0006 glycerol stock (4D Pharma Research Ltd., Aberdeen, UK) was inoculated in 10 mL of sterile yeast extract casein fatty acid (YCF) broth and grown overnight at 37°C under anaerobic conditions. From the overnight culture, the optical density (OD) of bacterial suspension was measured and adjusted to an OD600nm of 1.0 in fresh YCF broth. 100 μL of the adjusted bacteria suspension (OD600nm of 1.0) was further inoculated into 10 mL of fresh sterile YCF broth and grown under anaerobic conditions until it reached late log phase based on the growth curve (8h). The bacterial culture was centrifuged at 4000 × g at 4°C for 10 minutes. The supernatant was removed and the bacterial pellet was resuspended in sterile maximum recovery diluent (MRD) and gently vortexed. The bacterial suspension was centrifuged again at 4000 × g at 4°C for 10 minutes. The supernatant was removed under anaerobic conditions, and the bacterial pellet was resuspended in sterile PBS (pH 7.4) and gently vortexed.

2.4. MRx0006 administration

Treatment commenced when mice reached eight weeks of age. Animals received either 1.0 × 10⁹ colony forming units(CFU)/200μL of MRx0006 or 200μL of sterile PBS via oral gavage once daily. Mice were given a three-week lead-in period of dosing prior to the commencement of the behavioural testing; treatment continued until day 77.

2.5. Behavioural testing

Animals were treated with MRx0006 daily for three weeks prior to the start of the behavioural experiments and the administration of the biotherapeutic was maintained throughout the behavioural testing phase. Following three weeks of treatment, all animals were subjected to a battery of behavioural tests (Fig. 1). The 3-chamber test (3CT) was used to assess social interaction. The female urine sniffing test (FUST) was used to measure recognition of social cues, as well as anhedonic-like behaviour. Both the marble-burying (MB) and self-grooming (SG) tests were used to assess stereotype-like behaviour. The elevated plus maze (EPM) and open-field (OF) tests were used to measure anxiety-related behaviour. The novel object recognition (NOR) test was used to measure cognitive function, i.e., learning and memory. The forced swim test (FST) was used to measure stress coping behaviour. Sufficient time was allowed between tests to avoid cumulative stress carry-over. To reduce potential anxiogenic factors, all mice were habituated to the testing room by placing home-cages there for 1 hour prior to testing. Unless specified, light intensity was set at 60 lux. All arenas were cleaned with 70% ethanol between test mice. A researcher who was blinded to the group designations remained in the testing room during each behavioural test. All outputs were measured by an experimenter blind to the experimental groups.
The three-chamber sociability test (3CT) was used to assess social preference and recognition and was conducted as previously described (Desbonnet et al., 2014). The testing apparatus was a three-chambered, rectangular box. The dividing walls between each chamber (20 cm × 40 cm × 22 cm, L × W × H) had small circular openings (5 cm diameter), allowing for access to all chambers. The two outer chambers contained wire cup-like cages (10 cm bottom diameter, 13 cm height), allowing for auditory, olfactory, and visual, but no physical contact. The test consisted of three 10-minute phases: (1) Habituation, (2) Social Preference, and (3) Social Recognition. In the first phase (Habituation), the test mouse was allowed to explore the entire box with both wire cup-like cages left empty to allow for habituation to the novel environment. In the second phase (Social Preference), one wire cup-like cage contained a novel, age-matched, conspecific, male mouse, whereas the other cage contained an inanimate object. In the third phase (Social Recognition), the mouse of the previous trial was left in the wire cup-like cage (familiar mouse), while the object was replaced with a conspecific mouse (novel mouse). The test mouse was held in the middle chamber while the conspecific mouse and object were placed in the cup wire-like cages. The location of the conspecific mice and object were systematically altered in-between test mice. All animals were age- and sex-matched. The three-chamber test apparatus and wire cup-like cages were cleaned with 70% ethanol after each test mouse and left to dry for a few minutes. The discrimination index was calculated as follows: Time spent interacting with object or mouse/Total time spent interacting × 100%.

2.7. Female urine sniffing test

The female urine sniffing test is used primarily to assess anhedonic-like behaviour (Malkesman et al. 2010). This behavioural assay can also be interpreted as a measure of social cue recognition. Experimental mice were singly housed one week prior to the test in order to remove the effect of single housing on the day of the experiment. Mice were habituated to the presence of a cotton-tipped applicator for 5 minutes prior to the start of the experiment. A cotton-tipped applicator dipped in 60 µl sterile water was placed into the home cage, and the test mouse was allowed to investigate the applicator for three minutes. The cotton-tipped applicator was then removed. This was followed by an inter-trial interval of 45 minutes, during which no cotton tip was in the cage. Lastly, a new cotton-tipped applicator was dipped into 60 µl female urine, freshly collected from a cohort of female mice of the same strain in the oestrus stage of their cycle, and placed into the cage. The test mouse was allowed to investigate this applicator for three minutes. The total duration spent interacting with the water and urine cotton-tipped applicators was recorded.

2.8. Marble burying test

The marble-burying test can be used to assess compulsive repetitive behaviour (Hsiao et al., 2013). Clean plexiglass cages (35 cm × 28 cm × 18.5 cm, L × W × H) were filled with a 5 cm layer of standard rodent bedding. Twenty glass marbles (15 mm diameter) were laid on top of the bedding, equidistant from each other in a 4 rows × 5 marbles arrangement. The test mouse was placed in the cage and allowed to explore for 30 minutes under a light intensity of 1000 lux. The number of buried marbles (defined as > 2/3 of the marble not being visible anymore) were counted.

2.9. Self-grooming test

Mice were tested for repetitive and anxiety-like behaviour by assessing self-grooming. Mice were scored for spontaneous grooming behaviour as previously described (Yang et al., 2007). The test mouse was individually placed in an inverted 500 mL glass beaker for 20 minutes. The total time spent grooming all body regions was recorded during the last 15 minutes.

2.10. Open field test

The open-field test is used to assess locomotor activity, as well as the response to a novel stressful environment (O’Leary et al., 2014). The open field consists of a black board, surrounded by black walls. The test mouse was placed into the open-field arena (43 cm × 35 cm × 25 cm, L × W × H) and allowed to explore it for 10 minutes. The total distance travelled and the time spent in the inner zone (centre) of the open-field arena were recorded and scored using Ethovision videotracking system (Noldus Information Technology, UK).

2.11. Elevated plus maze test

The elevated plus maze (EPM) test is broadly used to measure anxiety (Walf and Frye, 2007). The plus-shaped arena with two open and two closed arms (50 cm × 5 cm × 15 cm walls, L × W × H) was elevated 1 metre above the ground. The test mouse was placed in the centre of the EPM arena facing an open arm and allowed to explore the maze for 5 minutes. Time spent in open and closed arms was recorded and presented as % of (time in open arms + time in closed arms).

2.12. Forced swim test

To assess stress-coping behaviour, mice were subjected to the forced swim test (FST) as previously described (Savignac et al., 2014). The test mouse was placed into a transparent glass cylinder (50 cm high × 20 cm internal diameter) containing 15-cm-depth water (24–25 °C) for 6
minutes. The animals were gently dried after the test and water was renewed after each animal. Time spent immobile was scored during the last 4 minutes of the test. The animals were considered immobile when floating in the water without struggling and making only those movements necessary to keep its head above the water.

2.13. Novel object recognition test

The novel object recognition (NOR) test is commonly used to assess hippocampal-dependent memory (discrimination between familiar and novel objects) and takes place over three 10-minute trials on three consecutive days: (1) Habituation, (2) Acquisition, and (3) Retention (Burokas et al., 2014; Desbonnet et al., 2014). In the first trial (Habituation), the test mouse was allowed to explore the open-topped, rectangular NOR arena (40 cm × 32 cm × 23 cm, L × W × H). In the second trial (Acquisition), the test mouse was exposed to two identical objects placed in the corners of the arena. In the third trial (Retention), one of the familiar objects was substituted with a novel one. The time spent exploring novel and familiar objects was manually recorded by an experimenter blind to the treatment groups. Object exploration was defined when the animal’s nose came within a 2 cm radius of the object.

2.14. Cell culture

Mouse adult hypothalamic immortalised cell lines (mHypoA 2.28) were purchased from CELLtutions Biosystems Inc. (Ontario, Canada). Cells were maintained in Dulbecco’s Modified Eagle Medium (DMEM; D5796, Sigma-Aldrich), supplemented with 10% heat-inactivated foetal bovine serum (FBS; F7524, Sigma-Aldrich) and 1% penicillin/streptomycin (15140148, Thermo Fisher Scientific) at 37°C in a humidified atmosphere with 5% CO₂.

2.15. Treatment of mHypo cells with bacterial supernatants

mHypoA 2.28 cells were seeded in 12-well plates at a density of 0.1 × 10⁵ cells per well. Cells were incubated for 24 hours in standard culture conditions. 2 hours before the experiment, growth media was replaced with serum-free DMEM (# D5796, Sigma-Aldrich) containing 1% Non-Essential Amino Acids (MEM, # 11140050, Thermo Fisher). Following this step, cells were exposed to MRx0006 bacterial cell-free supernatant (BCFS) for 2 hours. After the incubation step, the BCFS was removed, cells were washed in PBS then lysed in 350 µL lysis/binding buffer and stored at -80°C until RNA extraction.

2.16. Quantitative real-time polymerase chain reaction (qRT-PCR)

Oxytocin (OXT) and oxytocin receptor (OXT-R) expression was evaluated in immortalized hypothalamic cells (mHypoA 2.28) by quantitative PCR (qPCR) with the use of TaqMan® Universal PCR Master Mix (Applied Biosystems, Warrington, UK), 6-carboxyfluorescein (FAM™) dye-labelled TaqMan® MGB probes (Applied Biosystems™) specific to Oxt (Mm Pt 58. 26998679.g) and OxtR (Mm Pt 58. 10711295) and analysed on the LightCycler 480 instrument (Roche, Warrington, UK). Samples were heated to 95°C for 10 minutes, and then subjected to 50 cycles of amplification by melting at 95°C and annealing at 60°C for 1 minute. Cycle threshold (Ct) values were normalised to Actb as a housekeeping gene, and analysed using the 2−ΔΔt method and then expressed as a fold change (Livak and Schmittgen, 2001).

2.17. Intestinal motility assay

The assay is based on the propulsion of non-absorbable coloured dye through the intestinal tract (Nagakura et al., 1996). Briefly, mice were singly housed and habituated to new cages for three hours for acclimatization (8:00 – 11:00 am). Animals had access to food and drinking water ad libitum throughout the experiment. Following the habituation period, mice received 200 µL of 6% carmine dye (C1022, Sigma-Aldrich) in 0.5% methylcellulose (Sigma-Aldrich) dissolved in sterile PBS administered by oral gavage. The latency for the excretion of the first red-coloured faecal pellet was timed and recorded as a measure of gastrointestinal motility.

2.18. Gene expression analysis in the hypothalamus

Animals were sacrificed through decapitation; whole brain was promptly excised and gross-dissected on wet ice separating frontal cortex, striatum, hippocampus, hypothalamus, amygdala, and cerebellum. The tissue was snap-frozen on dry ice and stored at −80°C. Total RNA from the hypothalamus was extracted using the MirVana RNA Extraction Kit (# AM1561, Thermo Fisher Scientific). RNA concentration and quality were assessed with the NanoDrop™ spectrophotometer. RNA was reverse transcribed to cDNA using the High Capacity cDNA Kit (# 4368813, Applied Biosystem) according to the manufacturer’s protocol. Oxt, OxtR, Arginine Vasopressin (Avp) and Arginine Vasopressin Receptor 1b (Agrp1b) expression was evaluated using the RNA from hypothalamic tissues with real-time RT-PCR using TaqMan® Universal PCR Master Mix and FAM™ dye-labelled TaqMan® MGB probes specific to target genes (all from Applied Biosystems™). PCR was run in triplicate on the LightCycler 480 instrument (Roche, Warrington, UK). Samples were heated to 95°C for 10 minutes, and then subjected to 50 cycles of amplification by melting at 95°C and annealing at 60°C for 1 minute. Cycle threshold (Ct) values were normalised to Actb as a housekeeping gene, and analysed using the 2−ΔΔt method and then expressed as a fold change BTBR MRx0006 vs BTBR Vehicle (Livak and Schmittgen, 2001).

2.19. Plasma collection and corticosterone assay

Using lithium-heparin-coated capillary blood collection tubes, trunk blood was collected on day 77, the terminal time point of the study. Blood was centrifuged at 3,500g at 4°C for 15 minutes. Plasma was aspirated and stored at −80°C. Plasma corticosterone was measured by ELISA as previously described by (Bastaiaasen et al., 2021), following vendor instructions (ADI-900-097, ENZO Corticosterone ELISA). Concentration was expressed in ng ml⁻¹.

2.20. 16S rRNA sequence-based microbiota composition and diversity analysis in caecal content

2.20.1. DNA extraction from caecal matter

Caecal contents were snap frozen on dry ice and stored at −80°C. DNA was extracted using the QIAmp Fast DNA Stool Mini Kit (Qiagen, UK) according to manufacturer’s instructions with the addition of a 3-minute vortex step using 2 ML screw-cap tubes (Sarstedt, Wexford, Ireland) containing 0.25 g of a 1:1 mix of 0.1 mm and 1.5 mm sterile zirconia beads plus a single 2.5 mm bead (BioSpec Products, Bartlesville, USA). Briefly, 200 mg of sample was added to a tube with beads with 1 mL of Qiagen InhibitEx® Buffer and vortexed for 3 minutes. Samples were then incubated at 70°C for 5 minutes to lyse cells. Samples were centrifuged, and DNA was pelleted and treated with proteinase K. Next, DNA was washed with buffers AW1 and AW2 and eluted in 200 µL of buffer ATE. DNA was quantified with high sensitivity DNA quantification assay kit on the Qubit™ 3.0 Flurometer (Bio-Sciences, Dublin, Ireland).

2.20.2. 16S rRNA gene sequence-based microbiota analysis

The V3-V4 regions of the 16S rRNA gene were amplified and prepared for sequencing according to the 16S Metageneic Sequencing Library Protocol http://www.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf. The protocol involved two PCR reactions on the extracted DNA. The DNA was first
amplified using primers specific to the V3-V4 regions of the 16S rRNA gene: (Forward primer 5'TGTGAGCAAGCAGTCACTTGAATAGAGAGACAGCCTACGAGGCGWGCAG; reverse primer 5'GTCGCTCGTGCT GCGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC). Each reaction contained 2.5 μl genomic DNA, 5 μl forward primer (1 μM), 5 μl reverse primer (1 μM) and 12.5 μl 2X Kapa HiFi Hotstart ReadyMix (Kapa Biosystems Ltd., UK). PCR amplification was carried out using the following program: 95°C for 5 minutes, 25 cycles of 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds, 72°C for 5 minutes and held at 4°C. PCR products were visualised using gel electrophoresis and then purified using AMPure XP beads (Labplan, Kildare, Ireland). Following this, a second PCR reaction was carried out on the purified DNA using two indexing primers per sample (Illumina Nextera XT Indexing Primers, Illumina, Netherlands). Each reaction contained 5 μl purified DNA, 5 μl index 1 primer (N7xx), 5 μl index 2 primer (S5xx), 25 μl 2X Kapa HiFi Hot Start Ready mix and 10 μl PCR grade water. PCR amplification was completed using the previous program but with only 6 amplification cycles instead of 25. PCR products were visualized and purified as described above. Samples were quantified using the Qubit™ 3.0 Fluorometer (Bio-Sciences, Dublin, Ireland) along with the high sensitivity DNA quantification assay kit and then pooled in an equimolar fashion (20 nM). The sample pool was prepared following Illumina guidelines and sequenced on the MiSeq sequencing platform in Clinical Microbiomics, Denmark using standard Illumina sequencing protocols.

2.20.3. Bioinformatic sequence analysis

Paired-end reads were assembled using FLASH. Raw sequence reads were quality trimmed using the QIME suite of tools (Version 1.8.0). This included the filtering of reads which failed to reach a quality score of >25 and the removal of mismatched barcodes and sequences below length thresholds. Denoising, chimera detection and operational taxonomic unit (OTU) grouping at 97% similarity were performed in QIIME using USEARCH v7. OTU sequences were aligned using PyNAST and the SILVA SSURef Database Release 111 and using SPINGO classifier (Allard et al., 2015) was used to determine taxonomy.

2.21. Microbiota analysis

Further data-handling and analysis was done in R (Version 4.1.1) with the Rstudio GUI (Version 1.4.1717). Species were excluded if they only detected as non-zero in 20% or fewer of total samples from the count table as the ratios are invariant to subsetting and this study employs compositional data analysis techniques (Gloor et al., 2017). The clr(lite) function in the Tjazi library was used to compute the centred log-ratio transformed values of the remaining taxa as is appropriate for handling compositional data. Custom scripts and the Tjazi library can be found online at https://github.com/thomazbastiaanssen/Tjazi (Bas Bastiaanssen, 2018). Zeroes were replaced using the runif method (Lubbe et al., 2021). Differential abundance was assessed using a linear model, followed by Storey’s Q-value False Discovery Rate (FDR) correction. In all cases, a q-value < 0.2 was considered significant.

2.22. Caecal content metabolomics

2.22.1. Sample extraction

50-100 mg of each caecum sample were weighed into a 1 ml Eppendorf tube and four times the weight in MeOH was added to the sample. All samples were vortexed at the highest speed (until the sample was detached from the bottom of the tube). Samples were vortexed for 2 minutes and left to rest for 5 minutes. Samples were centrifuged at 14,000 g for 15 minutes and 100 μl of supernatant was transferred into a high recovery vial. For the quality control (QC): 20 μl aliquots from each vial were collected and then prepared as the samples. Samples were then dried under nitrogen and reconstituted in 100 μl MeOH and 100 μl of mobile phase A1.

2.22.2. Liquid chromatography-mass spectrometry (LC-MS)

The analysis was carried out using a ultra-performance liquid chromatography (UPLC) system (Vanquish, Thermo Fisher Scientific) coupled with a high-resolution quadrupole-orbitrap mass spectrometer (Q Exactive™ HF Hybrid Quadrupole-Orbitrap, Thermo Fisher Scientific). An electrospray ionization interface was used as an ionization source. Analysis was performed in negative ionization mode. Each sample was analysed two times, one time in Parallel Reaction Monitoring (PRM) mode which targeted bile acids and another time in Full Scan mode which was a non-targeted method. In the Full-Scan analysis, a QC sample was analysed in MS/MS mode for the identification of compounds. The UPLC was performed using a slightly modified version of the protocol described by the Phenomenex application TNI1217. Data was processed using Compound Discoverer 3.0 (Thermo Fisher Scientific). The metabolites were clustered according to the pathway through which they function.

2.22.3. Compound extraction

Bile acid quantification was carried out using TraceFinder software. The analytical method selects the exact mass of each of the bile acids and then fragments them using MS/MS. Data was processed using Compound Discoverer 3.0 (Thermo Fisher Scientific). LC-MS/MS data was analysed using compounds annotated at Level 1 and Level 2.

2.23. Statistical analysis

Data were assessed for normal distribution and equality of variances using Shapiro-Wilk test and Levene’s test, respectively. For data sets with confirmed normal distribution, a series of unpaired t-tests were performed to estimate the mean differences between two groups. Outliers that were more than 2 standard deviations apart were removed. For assessment of data from 3CT, FUST and NOR, a 2-way ANOVA was used after checking for equality of variance. For data sets where normal distribution was violated, non-parametric Mann-Whitney U test was used to test for statistical significance. Statistical significance was assumed at p < 0.05. All statistical analysis was done on the Rstudio statistical software (Version 3.6.2). All graphs were generated either on Rstudio or on GraphPad Prism (Version 8.3.0).

3. Results

3.1. Treatment with MRx0006 improves social behaviour in the BTBR mouse model

Previous work has shown that BTBR mice show decreased social preference and recognition compared to C57BL/6 mice (Arakawa, 2020; Golubeva et al., 2017; Peleh et al., 2020; Zilka et al., 2017). The efficacy of MRx0006 in influencing social behaviour and cognition was assessed in the 3 Chamber Test (3CT) paradigm. MRx0006 administration significantly increased the amount of time spent interacting with the novel conspecific mouse over an inanimate object (Fig. 2A). The 2-way ANOVA revealed a significant effect of treatment (F(1,46) = 7.089; p = 0.01), while this preference was significantly higher in MRx0006 mice compared to water (Tukey’s post-hoc: p < 0.001), while this preference was not observed in BTBR Vehicle animals (Tukey’s test; p = 0.52) (Fig. 2B).
3.2. Treatment with MRx0006 attenuates stereotypic behaviour in the BTBR mouse model

Previous work has shown that BTBR mice have enhanced engagement in repetitive behaviours in the marble burying (MB) test as well as repetitive self-grooming compared to C57BL/6 mice (Golubeva et al., 2017; Silverman et al., 2010).

To determine the effect of MRx0006 on stereotyped behaviours, we investigated its efficacy in the repetitive self-grooming and MB tests. MRx0006 treatment reduced the time spent in repetitive self-grooming duration as compared to the BTBR Vehicle group (Mann-Whitney U test; p = 0.003) (Fig. 2C). Similarly, BTBR MRx0006 mice significantly decreased the number of buried marbles compared to the BTBR Vehicle group (Student’s t-test; p = 0.03) (Fig. 2D).

3.3. Treatment with MRx0006 attenuates anxiety-like behaviour in the BTBR mouse model

Based on recent evidence, BTBR mice are a stress-reactive strain, and this heightened reactivity might be partially responsible for the ASD behavioural phenotypes observed in this mouse strain (Benno et al., 2009).

To investigate whether administration of MRx0006 was capable of modulating anxiety-related behaviour in BTBR animals, we investigated its efficacy in the open field (OF) and elevated plus maze (EPM) tests. Treatment with MRx0006 resulted in reduced anxiety-like behaviour as indexed by a greater duration of time in the centre of the open field (Student’s t-test; p = 0.03) (Fig. 2E), while there was no significant effect of the treatment on the overall locomotor activity (Mann-Whitney U test; p = 0.51) (Supplementary Fig. 1C). There was no observed effect of MRx0006 treatment on the time spent in open arms (Student’s t-test; p = 0.74) or open arm entries in the EPM (Student’s t-test; p = 0.69) (Supplementary Fig. 1A-B).

3.4. Treatment with MRx0006 does not change worsen stress- coping behaviours in BTBR mice

Depression is not one of the core symptoms of ASD and BTBR mice do not display a depressive phenotype. Stress-coping behaviours can be evaluated using the forced swim test (FST) (Commons et al., 2017; Molendijk and de Kloet, 2019). In this test, the physical immobility displayed by the animal is thought to be an indication of passive stress coping behaviour. BTBR mice have been shown to spend significantly less time immobile than C57BL/6 mice in this test, which confirms a more active stress coping behaviour in BTBR mice.

No changes were observed in BTBR animals treated with MRx0006 in the FST (Mann-Whitney U test; p > 0.05) (Supplementary Fig. 1D). BTBR mice have increased corticosterone in response to acute stress (Golubeva et al., 2017). Therefore, we investigated the corticosterone response to an acute stressor in the form of the FST. No changes in corticosterone levels were observed in BTBR animals treated with MRx0006, following exposure to the acute stress of the FST (2-way ANOVA repeated measures, F(1,15) = 1.626, p = 0.205) (Supplementary Fig. 1E).

3.5. Treatment with MRx0006 did not alter cognition in BTBR mice

The novel object recognition (NOR) test is a commonly used trial to assess hippocampal-dependent memory (O’Connor et al., 2021). Treatment with MRx0006 did not significantly alter the mice’s preference for the novel object as confirmed by the 2-way ANOVA (F(1,13) = 0.142; p = 0.71) (Supplementary Fig. 1F).
3.6. Treatment with MRx0006 significantly reduced intestinal motility time in BTBR mice

Previous studies have shown altered intestinal motility in the BTBR mice compared to C57BL/6 mice (Golubeva et al., 2017) and in ASD patients (Wasilewska and Klukowski, 2015). Administration of MRx0006 to BTBR mouse showed significant reduction in intestinal motility, as determined by carmine red administration (Student’s t-test; \( p = 0.03 \)) (Supplementary Fig. 1G).

3.7. Treatment with MRx0006 increases oxytocin and oxytocin receptor expression in hypothalamic cell lines

To delineate the central neurochemical pathways underlying the behavioural properties of MRx0006, we investigated whether the live biotherapeutic could influence the expression of the neuropeptide hormones oxytocin and arginine vasopressin mRNA, in addition to their cognate receptor mRNAs, in vitro in murine hypothalamic cells.

In vitro administration of the MRx0006 bacterial cell-free supernatant to the murine hypothalamic cell line (mHypoA 2.28) resulted in significantly increased expression of oxytocin mRNA compared to the vehicle-control group, where the supernatant medium (YCFA) was administered (Mann-Whitney U test, \( p = 0.00007 \)) (Supplementary Fig. 1H). Furthermore, there was also a significant increase in oxytocin receptor expression following treatment with MRx0006 bacterial cell-free supernatant compared to the vehicle-control group (Mann-Whitney U test, \( p = 0.0003 \)) (Supplementary Fig. 1I).

3.8. Treatment with MRx0006 influences hypothalamic expression of neuropeptide mRNA

In order to investigate whether the live biotherapeutic also influences the expression of oxytocin, arginine vasopressin and their receptors in vivo, their expression in the BTBR mice hypothalamus was quantified. BTBR mice treated with MRx0006 had a trend towards a greater amounts of oxytocin mRNA in the hypothalamus (Student’s t-test, \( p = 0.06 \)) (Fig. 3A) but no change was observed in oxytocin receptor (Student’s t-test, \( p = 0.12 \)) (Fig. 3B) mRNA expression. Moreover, treatment with MRx0006 increased the mRNA expression of arginine vasopressin in the hypothalamus (Student’s t-test, \( p = 0.02 \)) (Fig. 3C) but resulted in no changes in arginine vasopressin receptor 1b mRNA expression (Student’s t-test, \( p = 0.82 \)) (Fig. 3D) between the two groups.

3.9. Treatment with MRx0006 did not significantly alter levels of bile acids

Bile acids are known to play an important role in the digestion of dietary lipids and lipid metabolism (de Aguiar Vallim et al., 2013). Furthermore, they are also involved in the maintenance of intestinal permeability (Gadaleta et al., 2011; Hegyi et al., 2018; Raimondi et al., Fig. 3. MRx0006 treatment alters neuropeptide expression. BTBR mice treated with MRx0006 displayed (A) a trend towards increased hypothalamic oxytocin neuropeptide mRNA expression (Student’s t-test, \( p = 0.06 \)), (B) no difference in hypothalamic oxytocin receptor mRNA expression (Student’s t-test, \( p = 0.12 \)), (C) increased hypothalamic arginine vasopressin mRNA expression (Student’s t-test, \( p = 0.02 \)) and (D) no difference in arginine vasopressin receptor 1b expression (Student’s t-test, \( p = 0.82 \)). Data are presented as mean ± SEM; dots on each graph represent individual animals; \( n = 9 \) for vehicle treatment group and \( n = 9 \) for MRx0006 treatment group for (B) and (D); \( n = 8 \) for vehicle treatment group and \( n = 9 \) for MRx0006 treatment group for (A) and (C). * represents \( p<0.05 \), ** represents \( p<0.01 \), *** represents \( p<0.001 \).
It has been shown previously that BTBR mice have reduced plasma bile acid levels and deficient intestinal bile acid signalling (Golubeva et al., 2017). Treatment with MRx0006 did not significantly alter levels of primary or secondary bile acids in BTBR mice (Supplementary Fig. 2).

### 3.10. Treatment with MRx0006 increases the abundance of a specific gut microbiota species

We have previously demonstrated that dietary intervention with Kefir does not alter gut microbiota alpha or beta diversity but increases the abundance of specific beneficial microbiota species in the BTBR mouse and improves behavioural outcomes (van de Wouw et al. 2021). In the current study, linear model analysis, followed by Storey’s Q-value FDR correction demonstrated some, albeit minor differences, in caecal microbiome composition following administration of MRx0006. Additionally, treatment with MRx0006 decreased the abundance of *Alistipes putredinis* (linear model with Storey’s Q-value FDR correction, q = 0.00017) (Fig. 4A).

#### 3.11. Treatment with MRx0006 altered levels of several caecal microbial metabolites

Caecal metabolites were grouped according to the pathways they act on and interrogated for differences between BTBR Vehicle and BTBR MRx0006 groups. In BTBR mice receiving MRx0006 there was a significant reduction in the tryptophan metabolite: 4-indolecarbaldehyde (Fig. 4B). Additionally, alterations in carbohydrates and fatty acid metabolites were observed in mice treated with MRx0006 (Fig. 4C). The levels of one antioxidant, 2,4-quinolinediol, was increased and several other unclassified metabolites were reduced (Fig. 4D). The concentration of several amino acids were also altered by treatment with MRx0006 (Fig. 4E). Furthermore, several nucleic acid metabolites were reduced due to MRx0006 treatment (Fig. 4F) (Student’s t-test, 4-indolecarbaldehyde: p = 0.025; 1-palmitoyl-sn-glycerol 3-phosphate: p = 0.039, ethyl lactate: p = 0.0004; (7R,8S)-7,8-dihydroxy-3,7-dimethyl-6-oxo-7,8-dihydro-6H-isochromene-5-carbaldehyde: p = 0.003; monoethyl succinate: p = 0.0004; docosahexaenoic acid ethyl ester: p = 0.00003, biacetyl; 5-(2-Nitroprop-1-enyl)-1,3-benzodioxole: p = 0.0002; 1,2,3,4-tetrahydro-3-isoquinolinecarboxylic acid: p = 0.027; 1-methyladenine; p = 0.00017). ** represents p < 0.05, *** represents q < 0.001. (A) BTBR mice treated with MRx0006 showed species level alteration of *Alistipes putredinis* (linear model with Storey’s Q-value FDR correction, q = 0.00017) (Fig. 4A).

![Fig. 4. MRx0006 treatment alters specific gut microbiota species and caecal metabolites. (A) BTBR mice treated with MRx0006 showed species level alteration of *Alistipes putredinis* (linear model with Storey’s Q-value FDR correction, q = 0.0001659664) * represents q < 0.05, ** represents q < 0.01, *** represents q < 0.001. BTBR mice treated with MRx0006 had (B) a reduction in the tryptophan metabolite 4-indolecarbaldehyde (Student’s t-test; p = 0.025)(C) an increase in (7R,8S)-7,8-dihydroxy-3,7-dimethyl-6-oxo-7,8-dihydro-6H-isochromene-5-carbaldehyde (Student’s t-test; p = 0.003), and a reduction in ethyl lactate (Student’s t-test; p = 0.0004) and 1-palmitoyl-sn-glycerol 3-phosphate (Student’s t-test; p = 0.039). Among the fatty acid metabolites monoethyl succinate (Student’s t-test; p = 0.0004), docosahexaenoic acid ethyl ester (Student’s t-test; p = 0.00003), biacetyl, 5-(2-Nitroprop-1-enyl)-1,3-benzodioxole (Student’s t-test; p = 0.0002) were decreased, while 1,2,3,4-tetrahydro-3-isoquinolinecarboxylic acid (Student’s t-test; p = 0.027) was increased; (D) 2,4-quinolinediol (Student’s t-test; p = 0.04) was significantly increased and a decrease in 2908 (Student’s t-test; p = 0.033), (±)-12(13)-DHHOME (Student’s t-test; p = 0.0001), 1,5-isooquinolinediol (Student’s t-test; p = 0.002) and 4-ethynylaniline (Student’s t-test; p = 0.015) (E) a decrease in 1,5-naphthalenediamine (Student’s t-test; p = 0.02), 3,5-dihydroxybenzoic acid (Student’s t-test; p = 0.028), indole-3-acrylic acid (Student’s t-test; p = 0.023), ketoleucine (Student’s t-test; p = 0.04), phenylalanine (Student’s t-test; p = 0.030), porphobilinogen (Student’s t-test; p = 0.007), prolyleucine (Student’s t-test; p = 0.012), tryptophan (Student’s t-test; p = 0.021), tyrosine (Student’s t-test; p = 0.023) and trisopropanolamine (Student’s t-test; p = 0.001). (F) The nucleic acid metabolites 1-methyladenine (Student’s t-test; p = 0.042) and 5-methylcytosine (Student’s t-test; p = 0.019) were reduced with MRx0006 treatment along with skatole (Student’s t-test; p = 0.038), 3-indoleacetonitrile (Student’s t-test; p = 0.003) and acetyl-β-methylcoline (Student’s t-test; p = 0.026). Statistical analysis: n = 13 for vehicle treatment group and n = 12 for MRx0006 treatment group. * represents p < 0.05, ** represents p < 0.01, *** represents q < 0.001.***
Additionally, we also observed an amelioration of anxiety-like behaviour and anxiety in the BTBR mouse model of ASD. These data point the way for the potential development of psychobiotic-based approaches targeting the microbiota-gut-brain axis for managing symptoms associated with deficits in these behaviours in developmental disorders such as autism. In the present study, we demonstrate that oral MRx0006 treatment was well-tolerated with no adverse non-specific clinical signs observed, as suggested by regular inspection of fur status and changes in body weight.

Consistent with previous studies (Arakawa, 2020; Golubeva et al., 2017; Peleh et al., 2020; Zilka et al., 2017), BTBR mice displayed reduced sociability, which was ameliorated with MRx0006 treatment. BTBR mice also lack a preference for female urine. While the female urine sniffing test was originally designed to assess anhedonia-like behaviour in rodents (Malkesman et al., 2010), the test is based upon the ability of male mice to sense chemo-attractant pheromones in female urine that results in social interaction. Consequently, this test was used to infer changes in the recognition of social cues. MRx0006 increased the amount of time BTBR mice spent smelling female urine, which suggests that the live biotherapeutic was capable of improving the recognition of ‘social pheromones’. Taken together, these tests demonstrate that the live biotherapeutic MRx0006 alleviates social deficits in the BTBR mouse model.

Other core atypical ASD-associated symptoms like stereotypic behaviour and anxiety-like behaviour were also assessed in this study. Indeed, previous studies have demonstrated that BTBR mice also display compulsive repetitive behaviours in self-grooming (Golubeva et al., 2017) and marble burying tests (Amodeo et al., 2012). We demonstrate that treatment with MRx0006 reduced repetitive behaviours, as was assessed by both marble burying and self-grooming tests in BTBR mice. Additionally, we also observed an amelioration of anxiety-like behaviour in the open field test with MRx0006 treatment. However, MRx0006 did not reduce anxiety-like behaviour in the elevated plus maze test. This could have been due to differences in sensitivity of the two anxiety tests. Furthermore, this difference can also arise from the fact that the two tests might measure different aspects of anxiety and MRx0006 only addresses certain facets of anxiety, and not others (Anchan et al., 2014).

While previous probiotic, prebiotic and biotherapeutic studies have demonstrated improvement of certain ASD-associated behaviours, few of these treatments have led to an improvement of all core ASD-associated behavioural deficits. Bacteroides fragilis, for instance, demonstrated efficacy in improving the repetitive and anxiety-like behaviours observed in the maternal immune activation (MIA) model of ASD (Hsiao et al., 2013) but did little in terms of ameliorating deficits in social behaviour. Similarly, the probiotic Lactobacillus reuteri improved social behaviour, but could not improve repetitive or anxiety-like behaviours in mice born from mothers on a high-fat diet (Buffington et al., 2016). These studies suggest that these bacterial strains are selective towards targeting certain facets of ASD-associated behaviours but are not capable of improving all three core symptoms of the disorder. Thus, the ability of the live biotherapeutic MRx0006 to rescue all three behavioural deficits in BTBR mice highlights its efficacy and potential therapeutic utility. Unlike previous therapeutic treatments, which have primarily focused on altering specific neurotransmitters in the brain (Amodeo et al., 2021; Sgritta et al., 2019; Witt et al., 2019), MRx0006 targets chronic inflammation which accompanies ASD, which administration of Blautia is capable of reducing (Kalayana Chakravarty et al., 2018). It is possible that MRx0006 addresses all core symptoms of ASD through reduction in inflammation. Indeed, previous literature shows that inflammation possibly underlies all core symptoms of ASD (Erbas et al., 2015; Jang et al., 2018; Moieni and Eisenberger, 2018). Future work should investigate the influence of MRx0006 on inflammation that leads to the rescue of all three core symptoms of ASD.

Previous studies have indicated that the gut microbiota provides a potential therapeutic target for ASD. For instance, the gut microbiota is known to influence social behaviour via the oxytocin system, a key hormone known to regulate social and anxiety behaviour (Erdman and Poutahidis, 2016; Varian et al., 2017). Previous studies have demonstrated that treatments with certain probiotics such as Lactobacillus reuteri improve social behavioural deficits by increasing the expression and release of this neuropeptide in the paraventricular nucleus of the hypothalamus (Buffington et al., 2016; Tabouy et al., 2018). Furthermore, pharmacological interventions that alleviate ASD associated social behaviour often do so by increasing levels of hypothalamic oxytocin (Guastella and Hickie, 2016; Huang et al., 2021). Thus, in the current study, we wanted to investigate whether MRx0006 also rescues ASD-associated behaviour partly by influencing hypothalamic oxytocin levels. We observed a trend towards an increase in hypothalamic oxytocin in animals administered MRx0006 with observed improvements in sociability. Furthermore, we also observed an increase in oxytocin and its receptor in immortalised mouse hypothalamic cells following treatment with MRx0006 bacterial cell-free supernatant, suggesting that circulating bacterial metabolites from MRx0006 might play a role in increasing hypothalamic oxytocin levels. Recent studies have demonstrated that arginine vasopressin plays a role in modulating social behaviour (Taylor et al., 2022). Additionally, these studies also suggest that oxytocin and arginine vasopressin might also signal via each other’s receptors to influence social behaviour (Smith et al., 2019).

Indeed, oxytocin and arginine vasopressin systems have emerged as the key regulators of social behaviour and are often used as biomarkers for disorders of social function (Boender and Young, 2020). Increasing numbers of pharmacological interventions aim to also alleviate ASD-associated behavioural deficits by increasing the levels of hypothalamic arginine vasopressin (Parker et al., 2019; Schneider et al., 2020). Thus, in the current study, we also investigated whether MRx0006 influences levels of hypothalamic arginine vasopressin. Interestingly we also observed an increase in the hypothalamic expression of arginine vasopressin in BTBR mice. Reduced arginine vasopressin is known to also result in impairments in social behaviour and an increase in anxiety-like behaviour (Caldwell, 2017; Egashira et al., 2007). Furthermore, a recent placebo-controlled trial demonstrated that intranasal vasopressin application can improve social behaviour in children with ASD (Parker et al., 2019). Thus, it is possible that the social deficits and anxiety-like behaviours in BTBR mice treated with MRx0006 are alleviated due to this increase in hypothalamic oxytocin and arginine vasopressin levels. Future experiments, blocking hypothalamic oxytocin and arginine vasopressin after MRx0006 treatment are needed to confirm if this is indeed the case.

Previous studies have demonstrated that probiotic administration can alter the abundance of various other gut microbiota species (Li et al., 2020; Wang et al., 2015). Demonstration of alterations in the microbiome composition supports the hypothesis that the microbiota is important in the maintenance of host homeostasis and that corrective intervention through probiotics and live biotherapeutics may play a role in re-establishing the balance of the bidirectional signalling of the microbiome.
microbiota-gut-brain axis (Emge et al., 2016). In this study, we observed a decrease in the abundance of *Alistipes putredinis* after the administration of the live biotherapeutic, MRx0006. The genus *Alistipes* is commonly associated with chronic inflammation (Luna et al., 2017; Parker et al., 2020). Interestingly, chronic inflammation is also observed in patients with ASD (Prata et al., 2019), and also in the BTBR mice (Careaga et al., 2015). Since a reduction in inflammation in BTBR mice reduces stereotyped behaviour and improves sociability (Zhang et al., 2019), it is possible that the observed decrease in *Alistipes* might mitigate inflammation, and thus alleviate the observed autism-related behaviours.

Furthermore, patients with ASD often have co-morbid gastrointestinal symptoms. These gastrointestinal symptoms possibly result from compositional alterations of gut microbial communities. Such changes might contribute to behavioural pathologies observed in ASD, via the microbiota-gut-brain axis (Arnold et al., 2019; Strati et al., 2017). The administration of the live biotherapeutic decreased the relative abundance of *Alistipes putredinis*. Indeed, a previous patient study reported increased abundance of *Alistipes* in individuals with ASD (De Angelis et al., 2013). Therefore, it is possible that the decreased abundance of *Alistipes putredinis* due to the administration of MRx0006, might mitigate gut microbiota dysbiosis and thus underlie some of the observed improvements in autism-associated behavioural deficits (Holingue et al., 2018).

To further explore the possible mechanism of action of MRx0006 via the microbiota-gut-brain axis, we examined whether there were any alterations in caecal metabolites. Recent studies have demonstrated that administration of the probiotic *Lactobacillus reuteri* rescues behavioural deficits in mouse models of autism by alterations of circulating bacterial metabolites (Buffington et al., 2021). Indeed, several different microbial metabolites were altered in the caecum of BTBR mice treated with MRx0006. Interestingly, MRx0006 administration led to a reduction in the levels of tryptophan. Interestingly, increased levels of serotonin have been repeatedly reported to be present in the blood of individuals with ASD (Janusonis, 2008). It has also been demonstrated that reducing dietary tryptophan, a precursor to serotonin, can improve behavioural outcomes in another mouse model relevant to autism (Tanaka et al., 2018). Indeed, increased serotonergic neuron densities were reported in certain brain regions in the BTBR mice (Gou and Commons, 2017) and serotonin receptor blockade is known to decrease repetitive behaviour in BTBR mice (Amodeo et al., 2021). Thus, it is plausible that the live biotherapeutic MRx0006 improved autism-associated behavioural deficits in BTBR mice by reducing the levels of circulating tryptophan and serotonin.

Previous studies associated an increase in oxidative stress to autism (Pangrazzi et al., 2020). Furthermore, a previous study has demonstrated that an increase in oxidative stress in BTBR mice increases autism-associated repetitive behaviour (Nadeem et al., 2019). Interestingly, in this study we show that the levels of 2,4-quinolinediol were increased in BTBR mice treated with the live biotherapeutic, MRx0006. Since quinolinediols are known to have anti-inflammatory properties (Zhang et al., 2021), it is possible that an increase in 2,4-quinolinediol lowers inflammation and alleviates the repetitive behaviour in BTBR mouse.

Live biotherapeutics interact with human biological systems through a wide range of mechanisms. Host-microbe interactions are typically initiated in the gut, and live biotherapeutics do not exert their biological effects directly on the host, but instead by inhibiting pathogens, producing active metabolites or other molecules absorbed systemically, modulating the mucosal immune system activity, activating cellular pathways within the epithelial cells, or modulating the activity of the nervous system. Moreover, all or some of the above effects may occur simultaneously, initiated by a single bacterial strain, which in turn, will mediate different types of signals, activating diverse physiological pathways within the host with effects in organs and tissues distal from the gastrointestinal tract, such as in the central nervous system.

Finally, given that toxicity is a significant obstacle in the development of new drugs, live biotherapeutics are non-engineered strains of human commensal bacteria, originally isolated from healthy donors, and hence are expected to have an attractive safety profile. Given that ASD is a neurodevelopmental disorder, and live biotherapeutics comprised of non-engineered strains of human commensal bacteria are expected to have favourable safety and tolerability, it may be appropriate to start treatment with MRx0006 at an earlier stage of life which could lead to a safe treatment option with the potential to prevent as well as treat symptoms of ASD.

5. Conclusions

The data demonstrates that the single strain live biotherapeutic, *Blautia stercoris* MRx0006, from the *Blautia* genus possesses exhibits the ability to improve the core behavioural (sociability, stereotypy, and anxiety) symptoms of ASD in the BTBR mouse model. Previous studies have demonstrated how probiotics, prebiotics and biotherapeutics can improve some but not all three of the core behavioural deficits in animal models relevant to autism. To our knowledge, this is the first live biotherapeutic to have demonstrated the ability to target all three core behavioural deficits, and consequently, it represents a potential novel therapeutic strategy in the management of ASD. These improvements are likely mediated by alterations of levels of key metabolites and neuropeptides.

Moreover, these results further confirm the potential of microbiota-derived therapeutics in supporting mental health by modulating the microbiota-gut-brain axis (Berding et al., 2021). In the future, studies on the validation of this live biotherapeutic to improve ASD symptomology in a clinical setting are now warranted.

Funding

This work was funded by 4D Pharma Research Ltd., Aberdeen, UK. APC Microbiome Ireland is a research centre funded by Science Foundation Ireland (SFI), through the Irish Governments’ national development plan (grant no. 12/RC/2273_P2). JFC and TGD have received research funding from IFF, 4D pharma plc., Cremo SA, Nutricia Danone. HS, LJ, SC and IM were or continue to be employees of 4D pharma plc. All other authors declare no-no competing interests.

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.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at [https://doi.org/10.1016/j.bbi.2022.08.007](https://doi.org/10.1016/j.bbi.2022.08.007).

References

Allard, G., Ryan, F.J., Jeffery, L.B., Claesson, M.J., 2015. SPINGO: a rapid species-classifier for microbial amplicon sequences. BMC Bioinf. 16, 324.

Amodeo, D.A., Jones, J.H., Sweeney, J.A., Ragozzino, M.E., 2012. Differences in BTBR T + tf/J and C57BL/6J mice on probabilistic reversal learning and stereotyped behaviors. Behav. Brain Res. 227 (1), 64–72.

Amodeo, D.A., Oliver, B., Pahua, A., Hitchcock, K., Bykowski, A., Tice, D., Mustel, A., Ryan, B.C., 2021. Serotonin 6 receptor blockade reduces repetitive behavior in the BTBR mouse model of autism spectrum disorder. Pharmacol. Biochem. Behav. 200, 173076.
of antioxidant response in BTBR T+tf/J mice. Prog. Neuro-Psychopharmacol. Biol. Psychiatry 89, 245–253.

Nagakura, Y., Kamato, T., Nishida, A., Ito, H., Yamano, M., Miyata, K., 1996. Characterization of 5-hydroxytryptamine (5-HT) receptor subtype influencing colonic motility in conscious dogs. Naunyn Schmiedebergs Arch. Pharmacol. 355, 489–498.

O’Connor, R., van De Wouw, M., Moloney, G.M., Ventura-Silva, A.P., O Riordan, K., Golubeva, A.V., Dinh, T.G., Scheltekens, H., Cryan, J.F., 2021. Strain differences in behaviour and immunity in aged mice: Relevance to Autism. Behav. Brain Res. 399, 113020.

O’Leary, C., Deshommet, L., Clarke, N., Petit, E., Tigue, O., Lai, D., Harvey, R., Waddington, J.L., O’Tuathail, C., 2014. Phenotypic effects of maternal immune activation and early postnatal milieu in mice mutant for the schizophrenia risk gene neuregulin-1. Neuroscience 277, 294–305.

Pangrazi, L., Balasco, L., Bozzi, Y., 2020. Oxidative Stress and Immune System Dysfunction in Autism Spectrum Disorders. Int. J. Mol. Sci. 21 (9), 3299.

Parker, K.J., Oztan, O., Libove, R.A., Momin, E.N., Francis, M.B., Britton, R.A., and Savignac, H.M., Kiely, B., Dinan, T.G., Cryan, J.F., 2014. Bifidobacteria exert strain-specific effects on stress-related behavior and physiology in BALB/c mice. ISME J. 9 (1), 1–15.

Prata, J., Machado, A.S., von Doellinger, O., Almeida, M.I., Barbosa, M.A., Coelho, R., Peleh, T., Ike, K.G.O., Frentz, I., Buwalda, B., de Boer, S.F., Hengerer, B., Kas, M.J.H., Parker, K.J., Oztan, O., Libove, R.A., Mohsin, N., Karhson, D.S., Sumiyoshi, R.D., Yang, M.u., Zhodzishsky, V., Crawley, J.N., 2007. Social deficits in BTBR T+tf/J mice are not related to autism spectrum disorders. Brain Behav. Immun. 73, 310–319.

Saurman, V., Margolis, K.G., Luna, R.A., 2020. Autism Spectrum Disorder as a Brain-Gut-Related Derivatives. Mini Rev. Med. Chem. 21 (16), 2261–2275.

Sengupta, P., Sen et al. 2022. CRISPR-Cas9 editing of the arginine-vasopressin V1a receptor produces paradoxical changes in social behavior in Syrian hamsters. Proc Natl Acad Sci U S A 119, e2121037119.

Sen, P., Kuchna, I., Wegiel, J., Murthy, S., and Sung, Y., 2014. Phenotypic effects of maternal immune activation and early postnatal milieu in mice mutant for the schizophrenia risk gene neuregulin-1. Neuroscience 277, 294–305.

Soupion, B.J., Poutahidis, T., DiBenedictis, B.T., Levkovich, T., Ibrahim, Y., Didyk, E., Shlikman, L., Cheung, H.K., Hardan, A., Ricciardi, C.E., Rolandaviku, V., Veenna, A.H., Aro, E.J., Ermdan, S.E., 2017. Microbial lysate upregulates host oxytocin. Brain Behav. Immun. 61, 36–49.

Stenman, O., Pihl, E., Pihl, S., Kaire, J., Kivelä, P., and Aho, J., 2014. New evidences on the altered gut microbiota in autism spectrum disorders. Microbiome 5 (1).

Stratt, F., Cavalieri, D., Albanese, D., De Felice, C., Donati, C., Hayek, J., Jouson, 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).

Tabouy, L., Getzelter, D., Ziv, O., Karpuj, M., Tabouy, T., Lukic, I., Maayouf, R., Werbner, N., Ben-Amram, H., Nuriel-Ohayon, M., Koren, O., Elliott, E., 2018. Dysbiosis of microbiome and probiotic treatment in a genetic model of autism spectrum disorders. Brain Behav. Immun. 73, 310–319.

Taylor, J.H., Walton, J.C., McConnell, K.E., Norvelle, A., Liu, Q., Vander Velden, J.W., Borland, J.M., Hart, M., Jin, C., Hufman, K.L., et al. (2022). CRISPR-Cas9 editing of the arginine-vasopressin V1a receptor produces paradoxical changes in social behavior in Syrian hamsters. Proc Natl Acad Sci U S A 119, e2121037119.

Toothaker, C., Cazamanti, R., Brown, W.T., Chadman, K., Winiweter, T., Nowicki, K., Kuchna, I., Ma, S.Y., Wegiel, J., 2017. Partial Agenesis and Hypoplasia of the Corpus Callosum in Idiopathic Autism. J. Neuropathol. Exp. Neurol. 76, 225–237.

Walf, A.A., Frye, C.A., 2007. The use of the elevated plus maze as an assay of anxiety-related behavior in rodents. Nat. Protoc. 2 (2), 322–328.

Wang, J., Tang, H., Zhang, C., Yao, Y., Derrien, M., Roher, E., van-Hylckama Vlieg, J. E., Strisell, K., Zhao, L., Obin, M., Shen, J., 2015. Modulation of gut microbiota during probiotic-mediated attenuation of metabolic syndrome in high fat diet-fed mice. ISME J. 9 (1), 1–15.

Wasielowska, J., Kukowsk, M., 2015. Gastrointestinal symptoms and autism spectrum disorder: links and risks - a possible new overlap syndrome. Pediatric Health Med Ther 6, 153–166.

Way, L., Florey, M., Kaczmarski, W., Brown, W.T., Chadman, K., Winiweter, T., Nowicki, K., Kuchna, I., Ma, S.Y., Wegiel, J., 2017. Partial Agenesis and Hypoplasia of the Corpus Callosum in Idiopathic Autism. J. Neuropathol. Exp. Neurol. 76, 225–237.

Witt, N.A., Lee, B., Gherzi, K., Zhang, W.Q., Pehson, A.L., Sánchez, C., Gould, G.G., 2017. Vortioxetine Reduces Marble Burying but Only Temporarily Enhances Social Interaction Preference in Adult Male BTBR T+tf/J Mice. ACS Chem. Neurosci. 10 (10), 4319–4327.

Yang, M.u., Zhodzishsky, V., Crawley, J.J., 2007. Social deficits in BTBR T+tf/J mice are not related to autism spectrum disorders. Brain Behav. Immun. 73, 310–319.

Zaboski, B.A., Storch, E.A., 2018. Comorbid autism spectrum disorder and anxiety disorders: a brief review. Future Neuro 13 (5), 51–59.

Zhang, S.-S., Tan, Q.-W., Guan, L.-P., 2021. Antioxidant, Anti-inflammatory, Antibacterial, and Analgesic Activities and Mechanisms of Quinolines, Indoles and Related Derivatives. Mini Rev. Med. Chem. 21 (4), 379–395.

Zilkha, N., Kuperman, Y., Kimchi, T., 2017. High-fat diet exacerbates cognitive rigidity and social deficiency in the BTBR mouse model of autism. Neuroscience 345, 142–154.