Maternal gut microbiota mediate intergenerational effects of high-fat diet on descendant social behavior

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SUMMARY

Dysbiosis of the maternal gut microbiome during pregnancy is associated with adverse neurodevelopmental outcomes. We previously showed that maternal high-fat diet (MHFD) in mice induces gut dysbiosis, social dysfunction, and underlying synaptic plasticity deficits in male offspring (F_1). Here, we reason that, if HFD-mediated changes in maternal gut microbiota drive offspring social deficits, then MHFD-induced dysbiosis in F_1 female MHFD offspring would likewise impair F_2 social behavior. Metataxonomic sequencing reveals reduced microbial

richness among female F_1 MHFD offspring. Despite recovery of microbial richness among MHFD-descendant F_2 mice, they display social dysfunction. Post-weaning *Limosilactobacillus reuteri* treatment increases the abundance of short-chain fatty acid-producing taxa and rescues MHFD-descendant F_2 social deficits. *L. reuteri* exerts a sexually dimorphic impact on gut microbiota configuration, increasing discriminant taxa between female cohorts. Collectively, these results show multigenerational impacts of HFD-induced dysbiosis in the maternal lineage and highlight the potential of maternal microbiome-targeted interventions for neurodevelopmental disorders.

In brief

Di Gesù, Matz et al. find that maternal high-fat diet results in intergenerational (F_1, F_2), but not transgenerational (F_3), descendant social dysfunction. Metataxonomic sequencing and fecal microbiota transplant data implicate dysbiosis of the maternal gut microbiome in intergenerational social deficits, which can be restored by post-weaning *Limosilactobacillus reuteri*-6475 supplementation.

Graphical Abstract
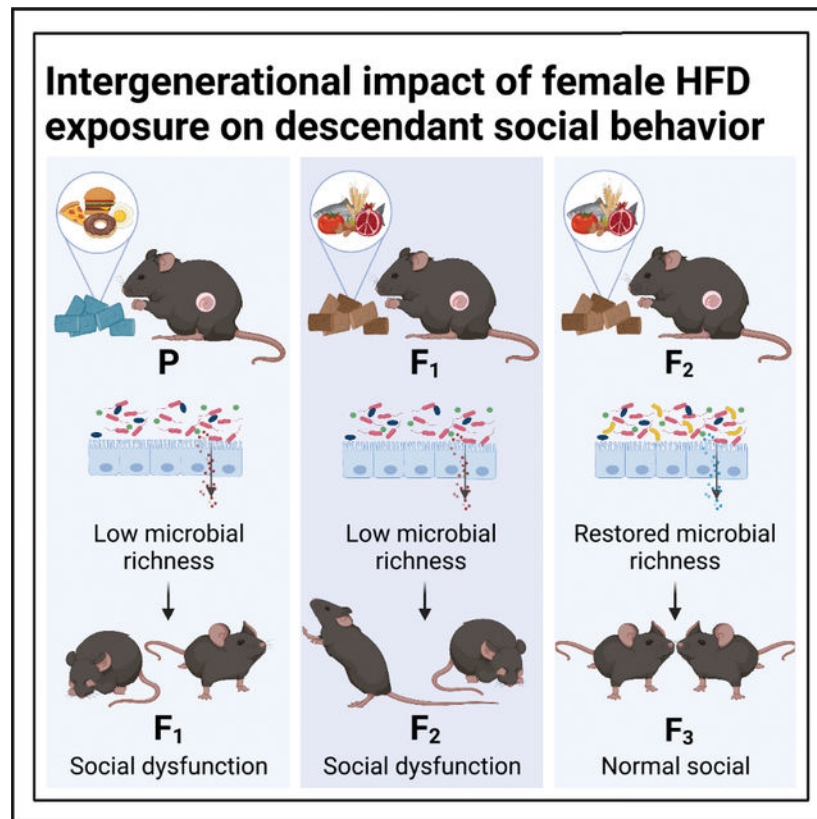
INTRODUCTION

Gut microbiota are emerging as significant contributors to healthy brain function, as well as neurodysfunction and neurological disease (Buffington et al., 2021; Hertz-Picciotto et al.,

2018; Lopez-Aranda et al., 2021; Morais et al., 2021; Nagpal and Cryan, 2021; Parenti et al., 2020; Rosenberg et al., 2007). Early-life neurodevelopment is particularly vulnerable to the adverse effects of disruption of the gut microbial community, or "dysbiosis" (Champagne-Jorgensen et al., 2020; Coley and Hsiao, 2021; Oliphant et al., 2021), as demonstrated by recent findings that dysbiosis of the maternal gut microbiome during pregnancy caused by infection or antibiotic-mediated microbial depletion alters fetal neurodevelopment, contributing to abnormal brain structure and function underlying maladaptive, autism-like behaviors in offspring (Di Gesu et al., 2021; Kim et al., 2017, 2022; Vuong et al., 2020). Notably, gut microbiome composition is driven primarily by diet (Bisanz et al., 2019; Carmody et al., 2015; David et al., 2014; Gacesa et al., 2022), and Western diet (Kopp, 2019), with high refined carbohydrate and fat but low fermentable fiber content, is increasingly implicated in the growing burden of non-communicable chronic disorders, including neuropsychiatric diseases (Blaser, 2017; Sonnenburg and Sonnenburg, 2019). Western diet is likewise implicated in the growing prevalence of metabolic disorders and obesity (Franks and McCarthy, 2016; Klurfeld and Kritchevsky, 1986; Kopp, 2019), and epidemiological studies identify maternal obesity as a significant risk factor for neurodevelopmental disorders, including autism spectrum disorder (ASD) (Kong et al., 2018; Li et al., 2016a, 2016b; Panjwani et al., 2019). Whether Western diet-induced dysbiosis of the maternal gut microbiome and the corresponding disruption of its remodeling during pregnancy (Gohir et al., 2015, 2019) are causally related to behavioral phenotypes and the underlying neuropathology in offspring remains unclear, as does the potential for diet-induced dysbiosis in a single generation to affect neurodevelopment and behavior across multiple descendant generations.

Working with both human populations and preclinical animal models, we and others have shown that high-fat, low-fiber diets drive functional, disease-associated changes in mammalian gut microbial ecology—generally characterized by an overall decrease in microbial (alpha) diversity and accompanying loss of beneficial functionalities afforded to the host by a complex microbial ecosystem (Afroz et al., 2021; Carmody et al., 2015; Di Gesu et al., 2021; Turnbaugh et al., 2008). Yet, the precise mechanisms mediating the relationship between westernized maternal diet and offspring neurobehavioral outcomes remains unestablished. Intriguingly, a recent study including 213 mother-child dyads found that alpha diversity of the prenatal maternal gut microbiome was predictive of child behavioral health outcomes at age 2 years (Dawson et al., 2021). Specifically, the abundance of short-chain fatty acid (SCFA)-producing bacterial taxa from families *Lachnospiraceae* and *Ruminococcaceae* in the maternal prenatal microbiome positively correlated with normative behavior in children and healthy maternal diet. SCFAs are essential microbially-derived metabolites that are synthesized through degradation of dietary plant fiber (i.e., microbiota-accessible carbohydrates [MACs]). SCFAs modulate activity of the host nervous and immune systems (Erny et al., 2021) and regulate histone deacetylase activity (Dupraz et al., 2021), while antibiotic- and diet-driven reduction in SCFA production is associated with disease pathology (reviewed in Dalile et al., 2019; O'Riordan et al., 2022). Notably, low-fiber diet-induced loss of microbial richness has been shown to compound over generations. Successive generations of low MAC diet consumption led to the extinction of community members that could not be restored by reintroduction of dietary MACs alone, instead

requiring active restoration of negatively selected taxa (Sonnenburg et al., 2016). Western diet-induced dysbiosis of the maternal gut microbiome during pregnancy and lactation in a single generation could thereby drive a sustained negative impact on neurodevelopment and behavior across multiple descendant generations.

Existing epidemiological studies on the multigenerational effects of maternal nutritional status during pregnancy primarily focus on populations affected by famine. These studies have identified specific epigenetic modifications that confer heightened transgenerational risk for neuropsychiatric disorders, including schizophrenia (Hoek et al., 1998; St Clair et al., 2005). Multigenerational clinical studies of maternal overnutrition mostly focus on descendant metabolic metrics (Campisano et al., 2019; Chang et al., 2019; Cirillo et al., 2021; Dudley et al., 2011; Friedman, 2018), while preclinical studies of parental (P) overnutrition largely follow the paternal lineage, investigating the role of germ-line reprogramming (Rodgers et al., 2015; Sarker et al., 2018), leaving complexities presented by the mother-offspring dyad relatively unexplored. However, given that the pathology underlying neurodevelopmental disorders begins *in utero* (Courchesne et al., 2020; de la Torre-Ubieta et al., 2016; Usui et al., 2021; Willsey et al., 2013), it is critical to elucidate the complex mechanisms by which maternal diet impacts fetal neurodevelopment (reviewed in Di Gesu et al., 2021). Working in a preclinical mouse model for maternal diet-induced obesity (Buffington et al., 2016), we recently demonstrated that maternal high-fat diet (MHFD) induced functional changes in gut microbiome composition as well as autism-like social deficits and underlying changes in synaptic plasticity in dopaminergic neurons within the ventral tegmental area—a key locus in the social reward circuit (Bariselli et al., 2018; Gunaydin et al., 2014; Hung et al., 2017) —of male offspring. Yet, the impact of MHFD on female offspring gut microbiome composition and, relatedly, the influence of ancestral HFD-induced dysbiosis in the maternal lineage on descendant brain function and behavior beyond the first generation (F_1) remain completely unexplored.

Given that MHFD-induced dysbiosis of the gut microbiome is vertically transmitted to F_1 offspring (Buffington et al., 2016; Chu et al., 2016; Ma et al., 2014), we hypothesized that social deficits would be observed in F_2 offspring, even in the absence of direct exposure to HFD. To address this hypothesis, we took an inter-disciplinary approach combining 16S ribosomal RNA (rRNA) gene amplicon (metataxonomic) sequencing, behavioral neuroscience, fecal microbiota transplants (FMTs) in gnotobiotic recipients, and anaerobic microbiology. As predicted, we observed dysbiosis of the gut microbiome in F_1 MHFD-descendant females and social dysfunction in their offspring (F_2), despite recovery of F_2 gut microbial richness. FMTs from MHFD-descendant F_2 donors were sufficient to rescue baseline social deficits in gnotobiotic mice, and we observed normal social behavior in F_3 MHFD descendants. Consistent with our finding in MHFD F_1 male offspring, F_2 social deficits were rescued by treatment with the probiotic *Limosilactobacillus reuteri*-6475. Surprisingly, we observed a sexually dimorphic impact of *L. reuteri* on host microbiota configuration, reflected by an increased number of discriminant taxa between F_2 female cohorts, revealing a relative malleability of the female gut microbiome in response to probiotic intervention, which may inform future therapeutic development.

RESULTS

MHFD-descendant F_2 mice display social dysfunction despite no direct exposure to MHFD

We and others previously reported detrimental effects of MHFD exposure during gestation and lactation on social behavior in first-generation (F_1) offspring and identified underlying deficits in the mesocorticolimbic dopaminergic social reward circuit of MHFD F_1 males (Bordeleau et al., 2021; Buffington et al., 2016; Kang et al., 2014). Here, we sought to determine whether social deficits observed in the F_1 generation were recapitulated in F_2, even in the absence of direct exposure to MHFD. To generate F_2 (Figure 1A), we fed C57Bl/6N females a regular diet (RD) or HFD for 6 weeks prior to mating to produce the F_1 generation. Total weight and lean mass of P generation females did not differ between diet cohorts at time of mating (Figures S1A and S1B), but fat mass was significantly increased in the HFD-fed group as early as 1 week on diet (Figure S1C). Shifts in gut community structure were apparent within 1 week on HFD and sustained at 4 weeks (Figure S1D), while alpha diversity metrics, including observed operational taxonomic units (OTUs) (Figure S1E) and the Shannon index (Figure S1F), and the ratio of the two most abundant phyla occupying the mammalian gut microbiome, *Firmicutes:Bacteroidota* (Figure S1G), differed significantly between RD- versus HFD-fed females by 4 weeks on diet. We maintained the P generation females on their assigned prepregnancy diet throughout pregnancy and lactation, sires had minimal exposure to HFD prior to F_1 conception, and all F_1 mice were weaned onto RD chow, regardless of maternal diet. We collected stool samples from MRD and MHFD F_1 females to characterize their gut microbial ecology using 16S rRNA gene amplicon (metataxonomic) sequencing, then bred MRD control (N = 5 pairs) and MHFD (N = 4 pairs) F_1 offspring to produce MRD- and MHFD-descendant F_2 generation mice, respectively (Figure 1A). We caged the F_2 mice according to sex and maternal lineage at weaning, then assessed social and anxiety-like behavior in 7- to 10-week-old male and female MRD and MHFD F_2 descendants. We collected stool samples from the respective F_2 cohorts at the time of behavior (Figure 1B).

We assessed MRD- versus MHFD-descendant F_2 cohort social behavior using Crawley's three chamber (3C) test for sociability and preference for social novelty (Figure 1C). We analyzed male and female behavior separately to allow for identification of sexually dimorphic phenotypes, given the disproportionate prevalence of ASD among human males (Constantino and Charman, 2012) and the need to deepen our understanding of how biological sex contributes to ASD risk. Consistent with our hypothesis, while MRD-descendant F_2 males showed strong sociability and preference for social novelty, MHFD-descendant F_2 males were significantly impaired (Figures 1D, 1E, 1H, S2A, S2B, S2E, and S2F). Conversely, while MHFD-descendant F_2 females displayed a mild reduction in sociability compared with their F_2 MRD counterparts (Figures 1F, 1I, S2C, and S2G), neither MRD nor MHFD F_2 females showed a statistically significant preference for interaction with a novel over a familiar mouse in the social novelty phase (Figures 1G, 1I, S2D, and S2H).

We next measured reciprocal social interaction between sex-matched stranger pairs (Figure 1J). Consistent with our 3C results, MHFD-descendant F_2 male pairs spent significantly less

time interacting than MRD-descendant F_2 male pairs, while no difference in interaction time was observed between pairs of MRD- versus MHFD-descendant F_2 females (Figure 1K). MHFD female F_2 offspring did show a reduction in contact duration, while no differences in number of contacts were observed for either sex (Figures S2I and S2J). Taken together, these data identify a strong social dysfunction phenotype among MHFD-descendent F_2 males, despite no direct exposure to MHFD. In contrast, we detected only a mild social dysfunction phenotype in F_2 females and carefully consider this finding in the discussion and the limitations of the study.

While social dysfunction is a core behavioral symptom of ASD, a proportion of individuals with ASD have comorbid anxiety disorders and/or attention-deficit/hyperactivity disorder (ADHD) (Avni et al., 2018). We thus used the open-field test to assess hyperactivity and anxiety-like behavior in the F_2 generation (Figure S3A). Neither cohort demonstrated changes in locomotor, as measured by distance traveled (Figure S3B) and speed (Figure S3C), or anxiety-like behavior, interpreted from time spent in the center of the open-field arena (Figure S3D), regardless of sex. Females displayed increased distance and speed compared with males, independent of diet. These results are consistent with data obtained during the habituation phase of the 3C task (Figure S3E), which showed no differences in locomotor activity driven by either sex or diet (Figures S3F and S3G). Thus, the social deficits in MHFD-descendant F_2 males and females (Figures 1 and S3) are not due to hyperactivity or increased anxiety-like behavior.

MHFD-descendant F_1 and F_2 mice harbor a distinct gut microbiome composition, with recovery of richness in the F_2 generation

Maternal gut microbiota play a critical role in modulating fetal brain development in ways that impact the function of behaviorally relevant neural circuits in adolescent and adult offspring (Di Gesu et al., 2021; Kim et al., 2017, 2022; Vuong et al., 2020). We previously showed that alterations in alpha and beta diversity of the HFD-fed adult female mouse microbiome are reflected in long-term gut dysbiosis in their male MHFD offspring (F_1) (Buffington et al., 2016). Notably, however, we did not sequence the MHFD-descendant F_1 female gut microbiome in prior work. Given that gut microbiota are vertically transmitted from mammalian mothers to their offspring (Ferretti et al., 2018), we hypothesized that (1) MHFD would similarly affect the gut microbiome composition of MHFD-descendant F_1 females and, consequently, (2) changes observed in MHFD F_1 females would be likewise reflected in the MHFD F_2 generation. We thus performed 16S rRNA gene amplicon (metataxonomic) sequencing to characterize the microbial gut composition and community structure of MRD- versus MHFD-descendant F_1 mothers and their offspring (F_2).

We first assessed beta diversity—the variation of microbial communities between samples —of averaged rarefied 16S rRNA gene sequences detected in F_1 and F_2 fecal samples. Principal-coordinate analysis (PCoA) using unweighted UniFrac, which incorporates phylogenetic distances but does not account for the abundance of operational taxonomic units (OTUs), revealed statistically significant clusters (Figure 2A). Specifically, fecal microbial communities of F_1 MHFD females clustered together and were distinct from both F_1 and F_2 MRD cohorts. F_2 MHFD offspring communities likewise clustered to

cohort, but the MHFD-descendant F_2 gut microbiota shifted away from that of the F_1 MHFD cohort toward the F_2 MRD cohort, demonstrated by partially overlapping 95% confidence intervals between the two groups. As expected, F_1 and F_2 MRD communities show low degrees of variation, appearing more similar to each other than the MHFD groups. Distinct clustering was not observed by weighted UniFrac, which accounts for the relative taxa abundance, but did reach statistical significance (Figure S4A).

Next, we assessed within-sample diversity (alpha diversity) by comparing observed OTU counts, Chao1 index, and Shannon index in F_1 females (Figures 2B–2D). Consistent with our observation in the P generation HFD-fed dams, the average number of OTUs identified per sample, which reflects the richness of each microbial community, was significantly reduced in MHFD-descendant F_1 females(Figure 2B). The Chao1 index, a richness estimator based on low-abundance OTUs, was likewise decreased in MHFD-descendant compared with MRD-descendant F_1 females (Figure 2C), while the Shannon index, which accounts for both abundance and evenness, did not indicate a statistically significant difference between cohorts (Figure 2D). Surprisingly, when we analyzed the MRD versus MHFD F_2 generation 16S sequencing dataset, however, no statistically significant differences were identified across any alpha diversity metric (Figures 2E–2G), revealing a recovery of microbial richness in the F_2 generation. Further assessment of beta diversity by linear discriminant analysis (LDA) effect size (LEfSe) identified 19 differentially abundant genus-level taxa between maternal diets in the F_1 generation (Figure 3A). Likewise, comparison of F_2 offspring cohorts revealed statistically significant differences in the abundance of 20 taxa (Figure 3B), including enrichment in genera *Faecalibacterium* and *Parabacteroides,* with diminished abundance of genera *Alistipes* and family *Prevotellaceae* and Erysipelotrichaceae, between the MHFD- versus MRD-descendant F_2 generation. Taken together, these results demonstrate a comparable effect of MHFD on the male and female F_1 offspring gut microbiome that is only partially recapitulated in F_2, and a potential role for low abundance species in driving phenotypic differences observed between F_2 descendant groups.

FMT from either MRD- or MHFD-descendant F_2 donors rescues social dysfunction in formerly germ-free recipients

Gut microbiota metabolize dietary nutrients into bioactive metabolites that modulate host physiology, including the function of neural circuits underlying behavior (Buffington et al., 2021; Dodd et al., 2017; Williams et al., 2014). To establish the relationship between the functional composition of the F_2 MHFD-descendant microbiome and their social deficits, we performed FMT from either MRD- or MHFD-descendent F_2 donors into germ-free (GF) recipient mice. We chose to perform FMTs into GF recipients because they are the current gold standard for establishing the causality of microbiota-led effects on host behavioral outcomes (Nagpal and Cryan, 2021). Furthermore, we previously found that FMT from only MRD, but not MHFD, F_1 male donors was sufficient to rescue social deficits in GF-recipient males (Buffington et al., 2016). Here, we transplanted juvenile male GF mice with anaerobically prepared fecal donor slurry prepared from stool isolated from MRD- or MHFD-descendant F_2 males via oral gavage. We performed this study exclusively with male donors and recipients, given the robustness of the male social dysfunction phenotype.

FMT recipients were housed by donor cohort in sterile caging and provided with sterile food and water for 5 weeks before behavioral analysis (Figure 4A). Consistent with previous reports (Buffington et al., 2016; Desbonnet et al., 2014), we observed impaired social behavior in age-matched, non-colonized GF control mice in the 3C assay (Figures 4B–4D and S5A–S5D). Remarkably, in contrast with our F_1 results, FMT from either MRD- or MHFD-descendant F_2 donors rescued GF social deficits in the 3C task, as indicated by a statistically significant social preference in both sociability and social novelty (Figures 4B–4D) and corroborated by automated time in chamber data (Figures S5A and S5B). Furthermore, GF hyperactivity in the 3C habituation phase and the open-field task were rescued in each recipient cohort (Figures S5E–S5H). Open-field time in center data was comparable between GF control and the two FMT-colonized cohorts (Figure S5I).

While the assessment of beta diversity revealed statistically significant clusters between fecal microbial communities from GF mice colonized with either MRD- or MHFD-descendant F_2 offspring microbiota (Figures 4E and S4B), no differences were observed in alpha diversity metrics between groups (Figures 4G and 4H), consistent with the results of alpha and beta diversity analysis in the F_2 generation offspring 16S sequencing dataset (Figures 2A and 2E–2G). These data indicate that the adolescent state of the male MHFD F_2 gut microbiome, although distinct from that of the MRD lineage, is not the primary cause of F_2 male social deficits. Importantly, however, these data do not rule out the therapeutic potential of targeting the adolescent gut microbiome to resolve social deficits in MHFD descendants.

Post-weaning *L. reuteri* treatment rescues MHFD-descendant F_2 social deficits

In our previous work, metagenomic whole-genome shotgun sequencing revealed a marked decrease in the abundance of the commensal species *L. reuteri* (Buffington et al., 2016) in the male F_1 MHFD gut. Interestingly, *L. reuteri* had been shown to increase both peripheral and central oxytocin (Poutahidis et al., 2013), a hormone that regulates social behavior through modulating the mesolimbic dopaminergic reward system (Buffington et al., 2016; Hung et al., 2017). Precision treatment with a strain of *L. reuteri* isolated from human breastmilk, *L. reuteri* (ATCC PTA-6475), rescued social behavior in MHFD F_1 males, as well as in multiple mouse models for ASD, including idiopathic and genetic models (Buffington et al., 2021; Sgritta et al., 2019). Here, we sought to determine whether *L. reuteri* could likewise rescue MHFD-descendant F_2 social deficits.

Monogamous pairs of F_1 MRD- or MHFD-descendant mice were bred to generate F_2 offspring which, upon weaning, were administered *L. reuteri* in drinking water daily through the remainder of the experiment (Figure 5A). Consistent with our results in the MHFD-descendant F_1 males, *L. reuteri* rescued MHFD-descendant F_2 male social deficits in reciprocal social interaction (Figures 5B, S6I, and S6J) and the 3C task (Figures 5C, 5D, 5G, S6A, S6B, S6E, and S6F). Remarkably, we observed an unexpected and dramatic increase in social behavior among *L. reuteri*-treated MHFD-descendant F_2 females, including a significant increase in reciprocal social interaction (Figures 5B, S6I, and S6J) and instatement of preference for social novelty in the 3C task (Figures 5F, 5H, S6C, and S6H), metrics which we found to be indistinguishable between untreated MRD- versus

MHFD-descendant F_2 females (Figures 1G, 1I, 1K, S2D, and S2H). 3C sociability was also rescued in the *L. reuteri*-treated MHFD-descendant F_2 females (Figures 5E, 5H, S6C, and S6G). *L. reuteri* treatment did not impact locomotor (Figures S6K–S6N) or anxiety-like behavior (Figure S6O) in F_2 descendants of either sex or lineage. Together, these results demonstrate that post-weaning *L. reuteri* treatment is sufficient to rescue social dysfunction in MHFD-descendant F_2 males and stimulate social function in MHFD-descendant F_2 females, thus providing further support for early adolescence as a viable therapeutic window for microbiota-targeted treatments for disorders of social dysfunction.

L. reuteri exerts differential impacts on the community ecology of the MRD- versus MHFD-descendant F_2 gut microbiota

We next assessed whether *L. reuteri* administration, which restores sociability and preference for social novelty in MHFD F_2 mice, also alters microbiota composition in the F_2 generation. *L. reuteri*-6475 is a moderate producer of the antimicrobial metabolite reuterin, which is generated through anaerobic fermentation of glycerol. Reuterin has been shown to reduce the abundance of a subset of species in the gut, including opportunistic pathogens, such as enterohemorrhagic *Escherichia coli*, that cause hemolytic-uremic syndrome (Eaton et al., 2011), and to prevent necrotizing enterocolitis in premature infants (Shelby et al., 2021). Remarkably, we observed a differential impact of *L. reuteri* treatment on both alpha and beta diversity in MRD versus MHFD F_2 descendants (Figures 5 and 6). Alpha diversity metrics observed OTU counts, Chao1 index, and Shannon index (Figures 5J–5L) each showed significant differences between MRD- versus MHFD-descendant F_2 cohorts. The distinct effect of *L. reuteri* supplementation on gut microbial ecology between groups was also shown by changes in the relative abundance of 36 taxa identified by LEfSe (Figure 6A). Notably, we observed a significant increase in the proportion of SCFA-producing taxa, such as *Limosilactobacillus, Ligilactobacillus*, and *Ruminococcus* genera among *L. reuteri*-treated F_2 MHFD descendants, compared with their *L. reuteri*-treated F_2 MRD-descendant counterparts. PCoA of unweighted and weighted UniFrac dissimilarities revealed statistically significant clusters of fecal microbial communities between maternal lineage cohorts (Figures 5I and S4C); however, visually these clusters overlapped. We thus stratified the microbiome sequencing data by sex and found a sexually dimorphic impact of *L. reuteri* on host microbiome composition, with 38 discriminant taxa identified between MRD- versus MHFD-descendant F_2 females and only 13 between male cohorts (Figure S7). Taken together, these data identify a substantial impact of *L. reuteri* treatment on the overall composition of the host gut microbiome, thus challenging the idea that the effects of *L. reuteri* on host brain and behavior are independent of its impact on the native microbial community and, moreover, revealing a relative instability of the MHFD-descendant gut microbiome, a state that could paradoxically heighten both disease susceptibility as well as therapeutic efficacy.

F_3 generation MHFD descendants display normal social behavior

Having found that MHFD-descendant F_2 gut microbiota are sufficient to rescue baseline social deficits in GF FMT recipients, despite clear social dysfunction in the F_2 males themselves, we hypothesized that HFD-associated dysbiosis of the *maternal* gut microbiome is causal in descendant social deficits, consistent with early-life pathology underlying

neurodevelopmental disorders (de la Torre-Ubieta et al., 2016). Accordingly, we reasoned that MHFD-descendant F_3 males would display neurotypical social behavior given the partial restoration, including the increase in microbial richness, of the MHFD-descendant F_2 (F_3 maternal) gut microbiome.

F_3 subjects were generated by monogamous breeding of MRD- and MHFD-descendant F_2 mice and weaned into litter-specific cages separated by sex and maternal lineage at 3 weeks of age (Figure 7A). Consistent with our hypothesis, we observed comparable reciprocal social interaction between maternal diet lineages (Figures 7B, S8I, and S8J) and statistically significant social preferences in MHFD-descendant F_3 mice in the 3C task (Figures 7C–7H and S8A–S8H). We did not observe any differences in locomotor (Figures S8K–S8N) or anxiety-like behavior (Figure S8O) between the F_3 cohorts of either sex. Collectively, these data demonstrate that the effects of HFD-induced dysbiosis in the maternal lineage can be resolved within two generations upon restoration of a healthy diet.

DISCUSSION

Traditionally, neurodevelopmental disorders and the underlying pathology were exclusively attributed to variation in the human genome; however, it is becoming increasingly evident that dysbiosis of the gut microbiome can likewise contribute to host brain and behavioral dysfunction. Microbiome-directed therapies are emerging as an innovative avenue for ameliorating maladaptive behaviors associated with neurodevelopmental disorders (Stewart Campbell et al., 2022). Here, we provide evidence in an environmental mouse model for social dysfunction that diet-induced dysbiosis of the maternal gut microbiome could causally contribute to behavioral deficits in descendants.

We report significant social deficits in the MHFD-descendant F_2 generation (Figures 1D–1I, 1K, and S2), with increased severity in the male sex, recapitulating the disproportionate prevalence of ASD in human males (Christensen et al., 2018; Ferri et al., 2018). Consistent with previous findings in male MHFD offspring (F_1) and other mouse models for social dysfunction (Buffington et al., 2016, 2021; Sgritta et al., 2019), *L. reuteri* supplementation was sufficient to normalize MHFD-descendant F_2 social behavior in both 3C and reciprocal social interaction (Figures 5B–5H and S6A–S6J). Conversely, while we observed impaired sociability in MHFD-descendant F_2 females in the 3C task (Figures 1F and 1I), neither MRD- nor MHFD-descendant F_2 female offspring demonstrated preference for social novelty (Figures 1G and 1I), and we observed no differences in reciprocal social interaction between groups (Figure 1K). Unexpectedly, *L. reuteri* administration led to a highly significant increase in both reciprocal social interaction (Figure 5B) and preference for social novelty (Figure 5F) among MHFD-descendant F_2 females, metrics that did not differ between the non-treated F_2 cohorts (Figures 1G, 1I, and 1K). We anticipate that probiotic species other than *L. reuteri* that exert similar effects on the microbial community ecology of the host and, relatedly, host physiology, would exert similar behavioral effects. The remarkable effect of *L. reuteri* on female social behavior was accompanied by a near-tripling of discriminant taxa between the MRD- versus MHFD-descendant F_2 females relative to their male counterparts (Figure S7). Given that the males and females within the respective cohorts originate from common litters, the disproportionate impact on the

female gut microbiome provides a rich opportunity for future investigation into the effects of host sex on microbiome composition and malleability. The sexually dimorphic impact of grandmaternal HFD on descendant social behavior, consistent with the milder behavioral phenotypes in human females with ASD compared with males (Baron-Cohen et al., 2005; Werling and Geschwind, 2013), may reveal redundancy in female social circuitry to ensure maternal care for young. Taken together, these data add to a growing body of evidence that host responses to probiotics are sex specific (Karunasena et al., 2014), which has important therapeutic implications.

Consistent with the finding that loss of microbial richness driven by a low MAC diet is largely recoverable within a single generation (Sonnenburg et al., 2016), we likewise observed recovery of microbial richness in MHFD-descendant F_2 males and females (Figures 2E and 2F). Nonetheless, changes in the abundance of specific taxa were observed in the MHFD-descendant F_2 generation (Figures 2A and 3), revealing enduring "scars" on microbiome configuration due to HFD exposure in the maternal lineage that could have functional consequences for the host. Finally, the absence of social deficits in the MHFD-descendant F_3 generation (Figures 7 and S8) suggests that the diet-mediated recovery of gut microbial richness in the MHFD-descendant F_2 females is sufficient to normalize fetal neurodevelopment and behavioral outcomes in their descendants.

FMT from F_2 donors to GF recipients (Figures 4 and S5) provided insight into the relative contribution of dysbiosis of the offspring versus *maternal* gut microbiome to offspring social deficits. MHFD-descendant F_2 male-derived fecal microbiota was sufficient to rescue baseline social deficits in formerly GF recipients (Figures 4 and S5), suggesting that it is not the adolescent state of the F_2 gut microbiome driving their social dysfunction, but instead dysbiosis of the *maternal* (F_1) gut microbiome. Multi-omic analyses to determine the molecular and cellular mechanisms by which HFD-induced scars on the microbiome in the maternal lineage affect brain development, function, and behavior will provide powerful insight into how diet-induced prepregnancy obesity predisposes offspring to neurodevelopmental disorders.

The differential impact of *L. reuteri* on gut community structure of MRD- versus MHFD-descendant F_2 offspring (Figures 5, 6, and S4C) reveals a relative instability of the MHFD-descendant F_2 gut microbiota when challenged by probiotic exposure. Surprisingly, we found *Limosilactobacillus* to be more abundant in the gut of treated MHFD versus MRD F_2 descendants, despite equivalent dosing (Figure 6). HFD-induced dysbiosis in the maternal lineage (Figures 2E–2G) may thus allow for *L. reuteri* to exert a greater impact on the native community. Conversely, in the absence of an environmental stressor (i.e., diet-induced dysbiosis in the maternal lineage), the effect of *L. reuteri* may be limited due to ecological stability. Interestingly, *L. reuteri* promoted the expansion of other SCFA-producing taxa, including *Ruminococcus* and *Ligilactobacillus*, which we identified as discriminant taxa preferentially increased among the MHFD versus MRD lineage F_2 animals treated with *L. reuteri* (Figures 5 and S7). In this light, we hypothesize that the *L. reuteri*-mediated and MHFD lineage-specific increase in biosynthetic potential for SCFAs might be key to the resulting behavioral rescue in treated F_2 MHFD descendants, given that SCFA receptors are broadly expressed throughout the immune and nervous systems, SCFA

supplementation relieves heightened responses to social stress (van de Wouw et al., 2018), and gut microbiota-derived SCFAs regulate the expression of the cytokine IL-17a, which is associated with social behavior (Dupraz et al., 2021; Reed et al., 2020; Shin Yim et al., 2017). Moreover, given the exaggerated effect of *L. reuteri* on the female gut microbiome and that maternal prenatal abundance of SCFA-producing bacterial taxa was recently found to be positively correlated with child behavioral outcomes in a human cohort (Dawson et al., 2021), we anticipate that targeted maternal probiotic treatment during pregnancy could have a protective role in offspring neurodevelopment. Together, these results identify an intergenerational impact of grandmaternal HFD on the stability of descendant microbial communities in response to environmental pressures, with implications for host disease susceptibility as well as therapeutic targeting of the gut microbiome.

Given that we observed (1) significant changes in female MHFD offspring F_1 gut microbiome composition, (2) MHFD-descendant F_2 male social dysfunction (Figures 1 and S2), despite partial recovery of gut microbiome diversity (Figures 2, 3, and S4), (3) resolution of baseline social deficits upon transplant of GF mice with MHFD F_2 fecal content (Figures 4 and S5), and (4) normal social behavior in the MHFD-descendant F_3 generation (Figures 7 and S8), we propose a model in which HFD-induced dysbiosis of the maternal gut microbiome and its vertical transmission to the MHFD-descendant F_1 females are respectively causal in MHFD-descendant F_1 and F_2 social dysfunction. Longitudinal metagenomic sequencing and functional profiling of the maternal gut microbiome during pregnancy and lactation will improve our understanding of the impact of diet on remodeling of the maternal gut microbiome and metabolome during the antenatal period and the precise mechanisms underlying its influence on offspring neurodevelopment.

Here, we report dysbiosis of the gut microbiome and behavioral dysfunction across multiple descendant generations in response to a single generation of HFD consumption. Translationally, our findings emphasize the need to consider family history of diet-associated metabolic dysfunction and obesity in the maternal lineage as potential risk factors for neurodevelopmental disorders, independent of the mother's own diet, lifestyle, and medical history, and highlight the potential for the implementation of policies providing universal access to nutritious food, particularly for expecting mothers, to yield multigenerational returns. Finally, they identify the maternal gut microbiome and its remodeling during pregnancy as promising therapeutic targets for improving long-term neurobehavioral outcomes in descendants.

Limitations of the study

Here, we show that HFD-induced dysbiosis in the maternal lineage contributes to social dysfunction across multiple generations of descendants and that probiotic strains can exert a sexually dimorphic impact on host gut microbial ecology. Additional experiments will be required to fully elucidate the mechanisms underlying these phenomena. Integrated multi-omic studies, including metagenomic, metabolomic, transcriptomic, and epigenomic approaches interrogating changes in both the mothers and their offspring, in each respective generation, will provide key mechanistic insight. We provide evidence that *L. reuteri* modifies the configuration of the host gut microbiome in a sex-dependent fashion. It will

be interesting to pursue the therapeutic implications of this finding. For instance, could distinct probiotic, prebiotic, or synbiotic formulations provide improved care for male versus female patients, not only in the context of neurodevelopment and behavior, but also in other disease contexts, such as metabolic disorders or irritable bowel disease? Relatedly, given the heightened remodeling of the female gut microbiome in response to *L. reuteri* and our finding that dysbiosis of the *maternal* gut microbiome modulates behavioral outcomes in descendants, the prenatal period is an obvious target for probiotic-mediated intervention aimed at reducing risk for adverse neurodevelopmental outcomes in offspring. Preclinical investigation into critical intervention periods will be of both scientific and translational significance. Cross-fostering experiments, to tease apart the impacts of gestational versus lactational exposure, will be key to identifying optimal windows for intervention. Our metataxonomic sequencing data identify multiple candidate species that may exert similar effects on host neurodevelopment and behavior as *L. reuteri*, and their therapeutic utility should be explored. Finally, careful assessment of female-specific behaviors, such as alloparenting (Carcea et al., 2021), may reveal complexity in female social behavior that is undetectable in the 3C task and reciprocal social interaction, tasks historically optimized to assess male social behaviors in genetic mouse models for ASD (Silverman et al., 2010). In this vein, how maternal environmental exposures, including HFD, affect maternal behavior, and female-specific behavior, in general, is a neglected topic of research that merits investigation, particularly in the context of the intergenerational effects of maternal environmental stressors on descendant neurobehavioral health outcomes.

STAR★METHODS

RESOURCE AVAILABILITY

Lead contact—Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Shelly Buffington (shbuffin@utmb.edu).

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

- The accession number for the 16S rRNA gene sequencing data reported in this paper is NCBI Sequence Read Archive: BioProject PRJNA878523. The original, unprocessed data reported in this manuscript can be accessed *via* Mendeley Data: https://doi.org/10.17632/z6wz8kpk3n.1.

- This paper does not report original code.

- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mouse husbandry and maternal diet—WT C57BL/6N mice were obtained from Taconic Laboratories (B6), maintained at animal biosafety level-1 under specific pathogen free (SPF) conditions, and kept on a 12-hour light/dark cycle in polysulfone cages with

access to sterile food and water *ad libitum*. Six-week-old females were placed on either a regular diet (RD) consisting of 13.4% kcal from fat, 30% kcal from protein, and 57% kcal from carbohydrates (Lab Diets, #5001) or HFD consisting of 60% kcal from fat, 20% kcal from protein, and 20% kcal from carbohydrates (Research Diets, #D12492). After six weeks on diet, females were randomly assigned to monogamous pairs with WT C57BL/6N adult males to produce subject offspring. Resulting offspring (F_1) were weaned at 3 weeks of age and all placed on RD, regardless of maternal diet (RD or HFD). Resulting F_1 maternal regular diet (MRD) pairs and MHFD offspring pairs were monogamously bred to generate F_2 offspring (Figures 1A and 1B). F_2 offspring were weaned at 3 weeks of age and fed RD. Behavioral tests were performed on male and female F_2 offspring at 7–8 weeks of age, with handling for three days prior in conjunction with stool collection. Behaviorally naïve MRD-descendant F_2 pairs and MHFD-descendant F_2 pairs were monogamously bred to generate the F_3 generation (Figures 1A and 1B). F_3 descendants were weaned at 3 weeks of age and fed RD. Behavioral tests were performed on male and female F_3 offspring at 7–8 weeks of age, with handling for three days prior in conjunction with stool collection. C57Bl/6N germ-free mice were acquired from the Baylor College of Medicine Gnotobiotic Core Facility. Animal care and experimental procedures were approved by The University of Texas Medical Branch Institutional Animal Care and Use Committee in accordance with all guidelines set forth by the U.S. National Institutes of Health.

METHOD DETAILS

Behavior—Behavioral assays were performed as previously described (Buffington et al., 2016), as detailed in brief below. Animals were acclimated to the behavioral suite for 30–60 minutes prior to initiation of all experiments. Apparatuses were spot cleaned with 70% ethanol after each animal and thoroughly cleaned at the end of the day. ANY-maze automated video tracking system software version 6.33 (Stoelting) was used for video recording and automated data acquisition.

Reciprocal social interaction: Mice were placed in a neutral, 30 cm^3 Plexiglass arena containing a shallow layer of corncob bedding with a previously unencountered age- and sex-matched conspecific matched for maternal diet. Time spent engaged in social interaction (close following, touching, nose-to-nose sniffing, nose-to-anus sniffing, and/or crawling over/under each other) over a ten-minute period was recorded by a blinded human observer and analyzed via ANY-maze.

Crawley's three-chamber (3C) test for sociability and preference for social novelty: Crawley's 3C test for sociability and preference for social novelty was performed as described (Silverman et al., 2010). Briefly, animals were habituated for 10 minutes in a $60 \times 40 \times 23$ cm arena divided into three inter-connected chambers. Sociability was measured during a second 10-minute interval in which the test subject could interact either with an empty wire cup (Empty) or a wire cup containing an age- and sex-matched stranger conspecific (Mouse 1). Stranger mice were acclimated to restraint in the wire cup during three training periods, over a period of two days prior to the test. Preference for social novelty was assayed by introducing a second stranger mouse (Mouse 2) into the previously empty wire cup. The location of the Empty Cup/Mouse 2 *versus* Mouse 1 were

counterbalanced within cohorts. Interactions were again recorded by ANY-maze software and an independent observer.

Open field test: Animal test subjects were gently lowered into an open arena ($40 \times 40 \times 20$ cm) and allowed to explore freely for 10 minutes. ANY-maze automatically measured distance traveled, speed, and position in the arena, as well as time spent in the center of the arena (defined as the interior 20×20 cm).

16S ribosomal RNA (rRNA) gene amplicon sequencing—Stool samples were aseptically collected in sterile 2mL Eppendorf tubes, immediately placed on dry ice, and stored at $-80°C$ until further processing. Bacterial DNA was extracted and sequenced by the Alkek Center for Metagenomics and Microbiome Research at Baylor College of Medicine using protocols adapted from those developed for the NIH-Human Microbiome Project (Human Microbiome Project Consortium, 2012), as described previously (Buffington et al., 2016). Briefly, bacterial genomic DNA was extracted using MagAttract PowerSoil DNA Kit (Qiagen) followed by PCR amplification of the 16S rDNA V4 region. The primers used for amplification include MiSeq adapters and single-end barcodes allowing for pooling and direct sequencing of PCR products. Sequencing was performed on the Illumina MiSeq platform using the 2×250 bp paired-end protocol yielding overlapping paired-end reads. The 16S rRNA gene read pairs were demultiplexed and merged using USEARCH v7.0.1090 (Edgar, 2010), allowing zero mismatches and a minimum overlap of 50 bases. Merged reads were trimmed at first base with Q5. A quality filter was applied to the resulting merged reads and reads containing above 0.05 expected errors were discarded. 16S rRNA gene sequences were clustered into Operational Taxonomic Units (OTUs) at a similarity cutoff value of 97% using the UPARSE algorithm (Edgar, 2013). OTUs were mapped to an optimized version of the SILVA Database (Quast et al., 2013) containing only the 16S v4 region to determine taxonomies. Abundances were recovered by mapping the demultiplexed reads to the UPARSE OTUs. A custom script constructed a rarefied OTU table from the output files generated in the previous two steps for downstream analyses of alpha-diversity, beta-diversity, and phylogenetic trends using the *Agile Toolkit for Incisive Microbial Analyses* (ATIMA2) platform (atima.research.bcm.edu).

Limosilactobacillus (L.) reuteri culture and treatment: *L. reuteri* PTA-6475™ was purchased from ATCC (MM4–1A). Cultures were grown anaerobically in De Man, Rogosa, and Sharpe (MRS) broth (MilliporeSigma) for 48 hours in a 5% CO_2, 5% H_2, remainder N_2 environment. Stocks were aliquoted and frozen at $-80°C$ then moved to $-20°C$ storage the week of use. Stock viability was confirmed and colony forming units (CFU) quantified by serial plating on MRS agar and growing anaerobically at 37°C for 48 hours. For treatment of F_2 offspring, 200 μL *L. reuteri* was added to 200 mL sterile drinking water at a concentration of 1×10^8 CFU/mL at time of weaning. Treated water was refreshed daily, Monday – Friday, for at least four weeks prior to behavior, and was continued throughout behavioral assessment until time of tissue collection.

Fecal microbiota transplants—Two-to-three fresh fecal samples were collected from each donor mouse (5 MRD- and 4 MHFD-descendant F_2 male subjects), pooled together

according to diet, and homogenized in sterile, deoxygenated PBS (1 mL PBS per stool) in an anaerobic chamber. The resulting slurry was vortexed, rested on ice for 15 minutes, and then spun at 1200 rpm for 5 minutes. Supernatants were transferred to sterile 50mL conical tubes within the anaerobic chamber and thoroughly sealed prior to exit from the chamber for transit to the recipients. 16 four-week-old C57BL/6N germ-free (GF) recipient mice were immediately colonized (8 per group) by a single 200 μL gavage. Mice were housed for 4 weeks before handling, stool collection, and behavior. Fecal samples were snap frozen on dry ice and stored at −80°C until prepared for sequencing. 8 six-week-old GF control (GF-C) mice, not subjected to FMT, were used to establish a baseline for behavioral and molecular analysis.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analyses—Power analyses to establish group size were performed in GPower 3.1. Data were analyzed and visualized using GraphPad Prism version 8.4.3 for Mac OS X, GraphPad Software, San Diego, California USA, www.graphpad.com. Data are presented as individual data points throughout the figures. Bar graphs include background columns indicating group mean. Error bars represent mean ± SEM, unless otherwise indicated, with each point representing an individual mouse (biological replicate) or pair of mice, in the reciprocal social interaction task. Sample sizes are indicated in the figure legends. Litter numbers are as follows: For MRD/MHFD F_2 male and female offspring, 6–7 and 4–6 litters per group were used, respectively (Figures 1, 2, 3, S2, S3 and S4). For GF male mice, 2 litters per group were used (Figures 4 and S5). For MRD/MHFD + *L. reuteri* F_2 male and female offspring, 3–4 and 3 litters per group were used, respectively (Figures 5, 6, S6 and S7). For MRD/MHFD F_3 male and female offspring, 5 and 3 litters per group were used, respectively (Figures 7 and S8). Statistics are reported in figure legends as *t(degrees of freedom)* = *t-statistic* or *q-statistic, p* = *p-value*. $p < 0.05$ was considered significant, where *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$. For data without repeated measures (3C locomotor data and open field test data), data were visualized as column data but analyzed using a two-tailed nested t-test to account for cage effects. No significant differences between sex-matched cages within the same cohort were identified. For Crawley's 3C assay data, to retain the precision of estimated variability while still accounting for litter effects, a mixed-effects model with repeated measures was applied, with the Šídák correction for multiple comparisons (Jimenez and Zylka, 2021; Lazic and Essioux, 2013). Random (litter) effects with a variance of zero were removed from the model and analyzed by repeated measures two-way ANOVA. For reciprocal social experiments, an unpaired, two-tailed *t*-test was performed unless otherwise indicated. In cases where data were not normally distributed, the non-parametric Mann-Whitney test was used. Locomotor data was analyzed by one-way ANOVA with Tukey's adjustment for multiple comparisons. Social Preference Indexes were calculated as in (Rein et al., 2020) and analyzed using a nested *t*-test. Principal coordinate analysis (PCoA) plots were constructed based on calculated UniFrac distances and the Monte Carlo permutation test was used to estimate *p* values. Beta diversity was assessed based on both unweighted and weighted UniFrac distances, visualized by principal coordinate analysis (PCoA), and statistically analyzed using permutational multivariate analysis of variance (PERMANOVA). Differentially abundant bacterial taxa were determined by Linear Discriminant Analysis

(LDA) Effect Size (LEfSe) (Segata et al., 2011) using the Galaxy computational tool (http://huttenhower.sph.harvard.edu/galaxy) (Afgan et al., 2018) and applying the all-against-all strategy with a minimum logarithmic LDA score (i.e., biomarker effect size) of 2.0.

Summary of outliers and excluded data—A limited number of animals were removed from analysis due to statistical identification as outliers and/or failure to meet pre-established criteria. Outliers were identified by Grubbs' test (alpha = 0.05, http://graphpad.com/quickcalcs/grubbs1/) and were removed from that specific dataset (*e.g.*, OF, RS, or 3C); therefore, sample sizes may vary slightly by test. For 3C, Grubbs' test was run on the difference between interaction times ($M_1 - E$, and $M_2 - M_1$). Additionally, mice that failed to interact for a minimum of 10 seconds with either social or non-social object in the 3C task were removed from the entire 3C dataset.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

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Highlights

- MHFD reduces microbial richness in female offspring (F_1)

- Social deficits persist in MHFD-descendant F_2, despite recovery of microbial richness

- Post-weaning L. reuteri treatment rescues MHFD-descendant F_2 social deficits

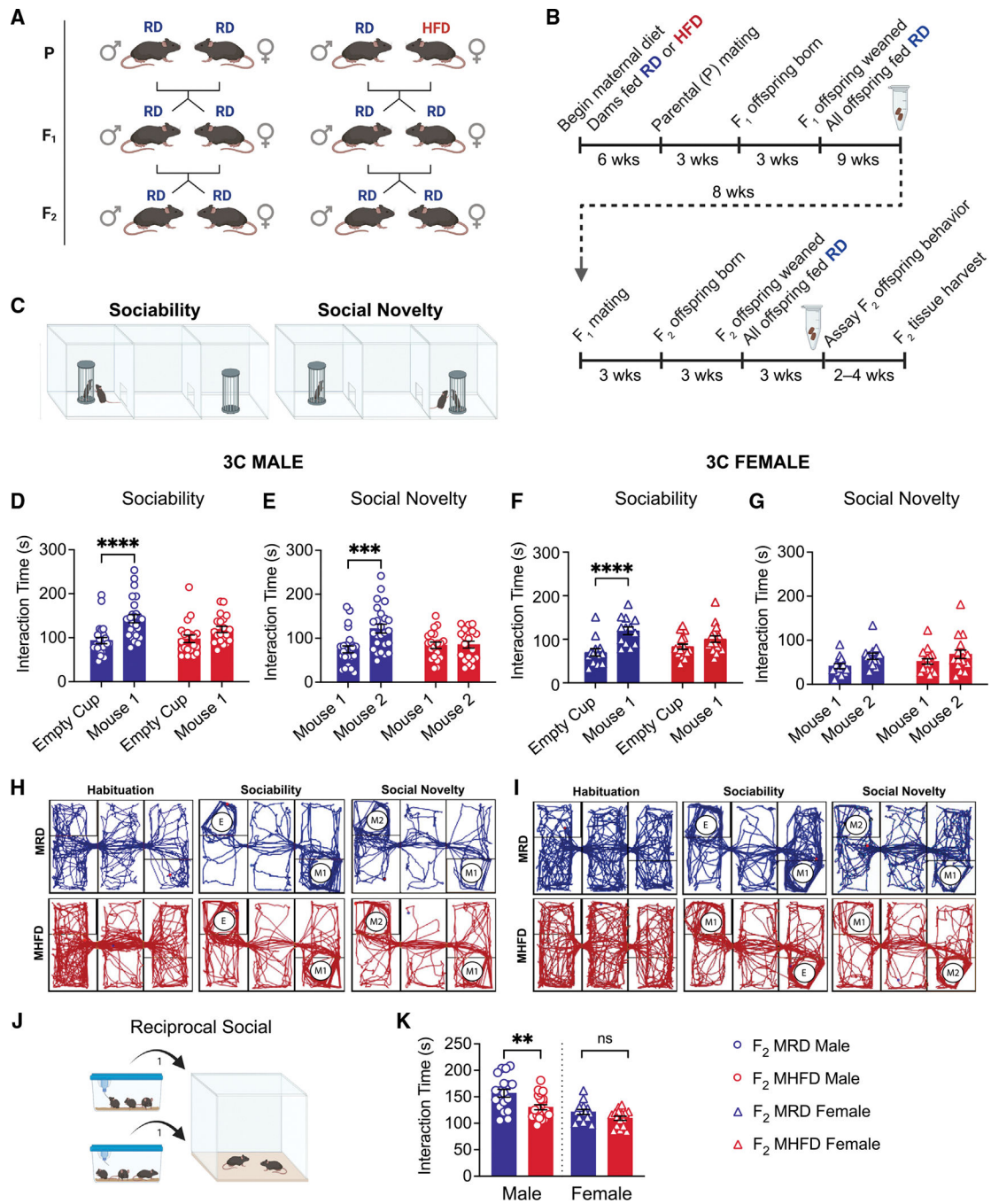- *L. reuteri* exerts a sexually dimorphic impact on host microbiota configuration

Figure 1. MHFD-descendant F$_2$ offspring display social dysfunction

(A) Schematic of breeding scheme to produce MRD- and MHFD-descendant F$_2$ generations with HFD exposure indicated.

(B) Timeline of mating and behavior. Eppendorf tubes represent time of stool sample collection.

(C) Schematic of Crawley's 3 chamber (3C) test for sociability and preference for social novelty.

(D and E) (D) MHFD F_2 male offspring show impaired sociability (MRD: $t(86) = 4.517$, p < 0.0001; MHFD: $t(86) = 1.735$, p = 0.1651) and (E) preference for social novelty (MRD: $t(43) = 4.208$, p = 0.0003; MHFD: $t(43) = 0.1159$, p = 0.9916) compared with MRD F_2 males.

(F) MRD F_2 females show a strong preference in 3C sociability (MRD: $t(28) = 5.406$, p < 0.0001), with MHFD F_2 female offspring showing mild deficits in sociability (MHFD: $t(28) = 2.181$, p = 0.0741).

(G–I) (G) Neither MRD nor MHFD F_2 female offspring showed a statistically significant preference for social novelty (MRD: $t(28) = 1.934$, p = 0.1224; MHFD: $t(28) = 1.613$, p = 0.222). Representative MRD-descendant (blue) or MHFD-descendant (red) F_2 offspring 3C track plots for (H) males and (I) females.

(J) Reciprocal social interaction schematic.

(K) Comparison of reciprocal social interaction times revealed decreased interaction among MHFD-descendant F_2 male, but not female, stranger pairs (male MHFD versus MRD: $t(39) = 3.103$, p = 0.0036; female MHFD versus MRD: $t(28) = 1.918$, p = 0.0653). Bar graphs show mean ± SEM with individual data points representing biological replicates. (D–G) N = 13–25 subjects per group; (K) N = 14–22 pairs per group.
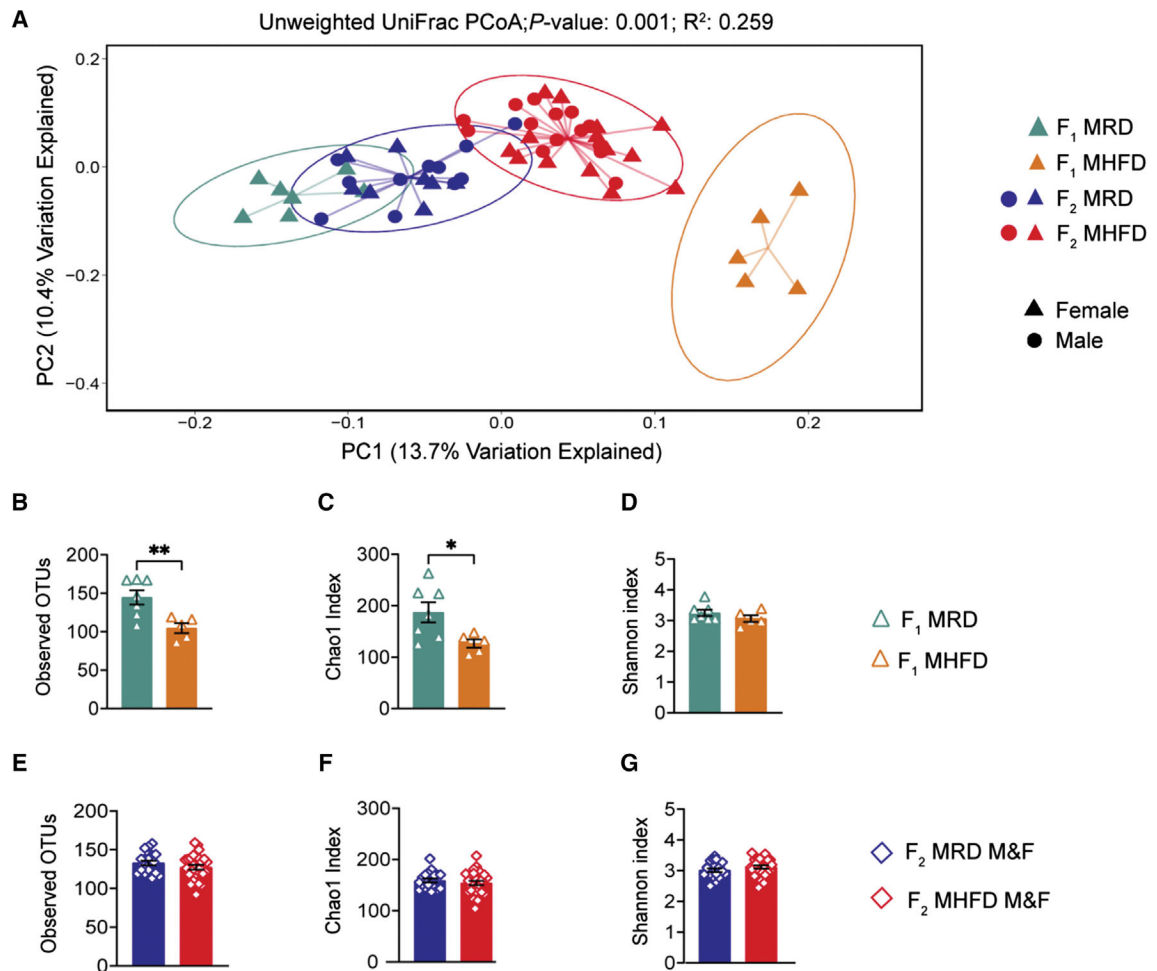
Figure 2. F$_2$ MHFD descendants harbor a distinct gut microbiome from F$_2$ MRD descendants but show a partial recovery of richness

(A) Principal-coordinate analysis (PCoA) of unweighted UniFrac distances from the averaged rarefied 16S rRNA gene amplicon sequencing dataset (8,310 reads/sample; n = 1,000 rarefactions) revealed statistically significant clusters based on diet and generation (p = 0.001, R^2 = 0.259).

(B–D) Alpha diversity metrics of F$_1$ females used to generate F$_2$ offspring revealed a statistically significant loss of microbial diversity in MHFD compared with MRD dams, as measured by observed OTUs (MHFD F$_1$ versus MRD F$_1$: $t(10)$ = 3.272, p = 0.0084) and Chao1 index (MHFD F$_1$ versus MRD F$_1$: $t(10)$ = 2.494, p = 0.0317). The Shannon diversity index did not differ between cohorts (MHFD F$_1$ versus MRD F$_1$: $t(10)$ = 1.204, p = 0.2564).

(E–G) No significant differences in alpha diversity between F$_2$ groups were observed, as measured by observed OTUs (MHFD F$_2$ versus MRD F$_2$: $t(45)$ = 1.192, p = 0.2395), Chao1 index (MHFD F$_2$ versus MRD F$_2$: $t(45)$ = 0.8097, p = 0.4224), and Shannon index (MHFD F$_2$ versus MRD F$_2$: $t(45)$ = 1.274, p = 0.2093). Bar graphs show mean ± SEM with individual data points representing biological replicates. (A–G) N = 5–15 subjects per group.
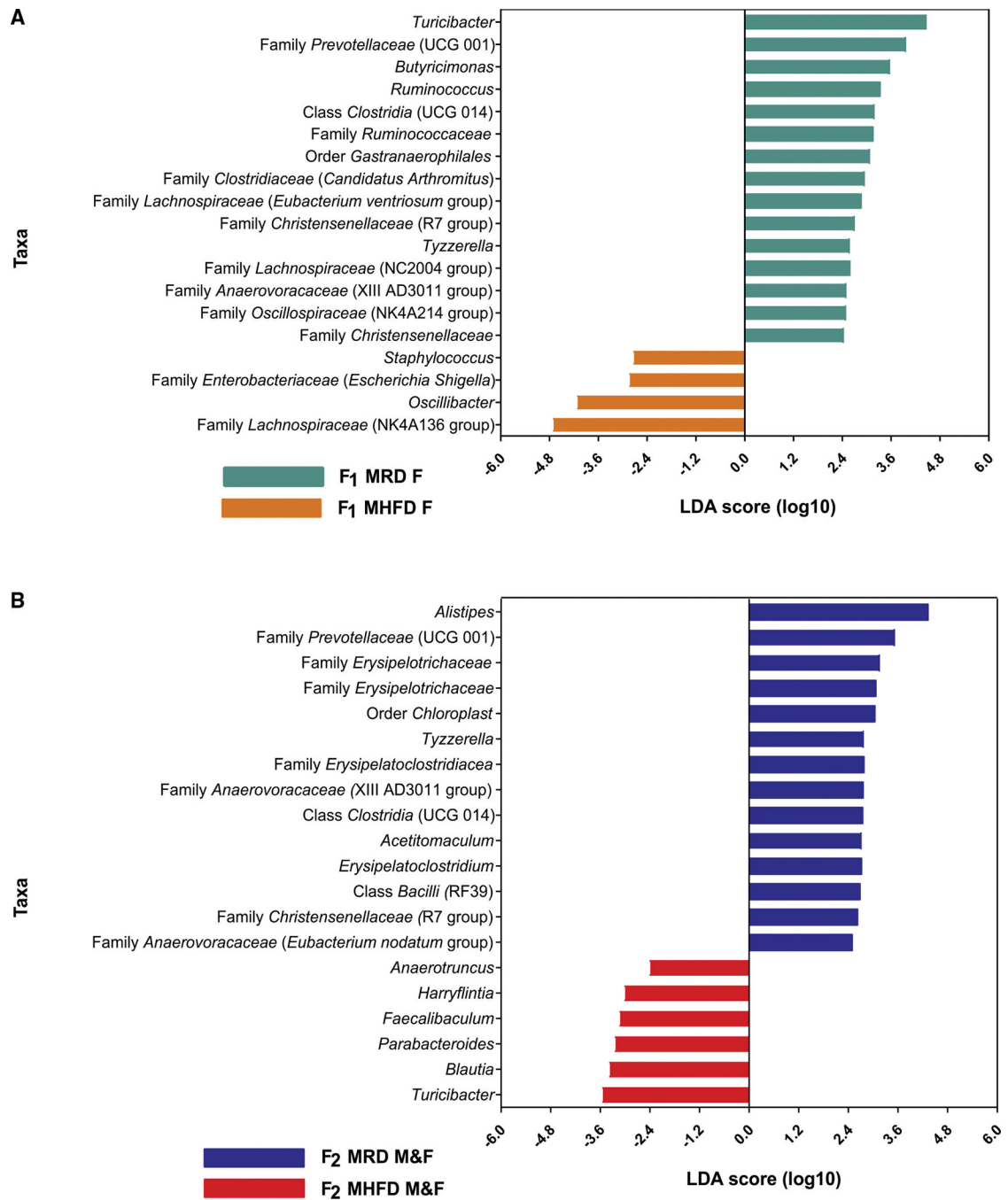
Figure 3. LEfSe analysis indicates differentially abundant genus-level taxa between diet cohorts in F₁ and F₂ generations

(A) Histogram of the LDA scores (log10) computed for genus-level taxa with differential abundance in MHFD and MRD F$_1$ dams.

(B) Histogram of the LDA scores (log10) computed for genus-level taxa with differential abundance in MHFD and MRD F$_2$ offspring. LDA, linear discriminant analysis; LEfSe, LDA effect size. (A and B) N = 5–15 subjects per group.
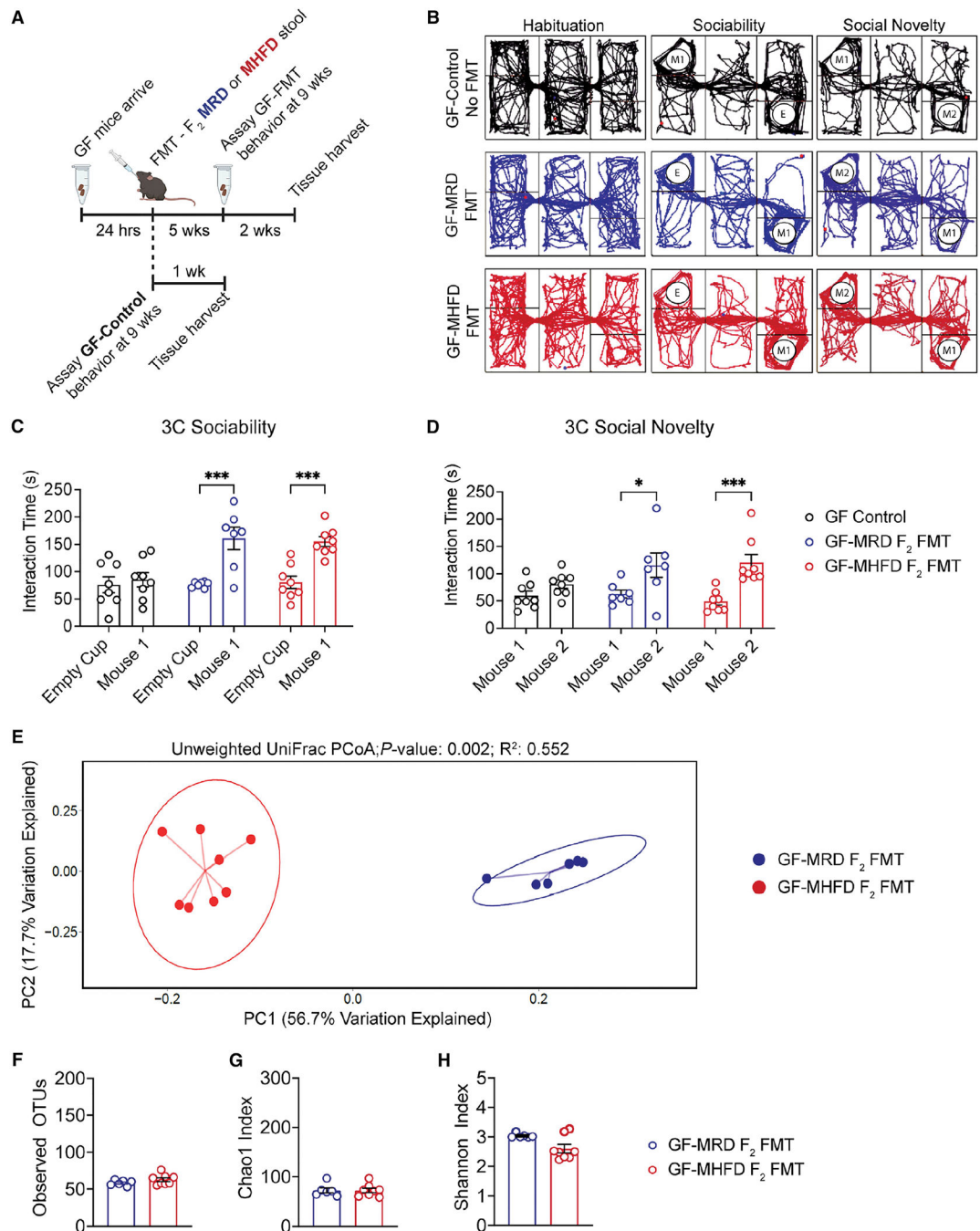
Figure 4. Fecal microbiota transplants from both F₂ MRD and MHFD descendants rescue germ-free social deficits

(A) Timeline of germ-free (GF) fecal microbiota transplant (FMT), stool collection for 16S rRNA gene sequencing, and behavioral analysis.

(B–D) (B) Representative 3C track plots for GF-Control (black), GF MRD-FMT (blue), or GF MHFD-FMT (red) males. 3C interaction times for (C) sociability (GF-Control: t(20) = 0.5364, p = 0.9349; MRD-FMT: t(20) = 4.688, p = 0.0004; MHFD-FMT: t(20) = 4.429, p = 0.0008) and (D) preference for social novelty (GF-Control: t(20) = 1.355, p = 0.4696; MRD-FMT: t(20) = 3.155, p = 0.0149; MHFD-FMT: t(20) = 4.558, p = 0.0006) showing

social dysfunction in GF males is rescued by FMT from either MRD- or MHFD-descendant F$_2$ donors. Bar graphs show mean ± SEM with individual data points.

(E) PCoA of unweighted UniFrac distances from the averaged rarefied 16S rRNA gene amplicon sequencing dataset (3,370 reads/sample; n = 1,000 rarefactions) revealed statistically significant clusters based on diet (p = 0.002, R^2 = 0.552).

(F–H) Alpha diversity metrics did not differ between GF MRD-FMT or GF MHFD-FMT males, as measured by observed OTUs (GF MRD-FMT versus GF MHFD-FMT: $t(12)$ = 1.391, p = 0.1895), Chao1 index (GF MRD-FMT versus GF MHFD-FMT: Mann-Whitney U = 22, p = 0.8518), and Shannon diversity index (GF MRD-FMT versus GF MHFD-FMT: Mann-Whitney U = 12, p = 0.1419). Bar graphs show mean ± SEM with individual data points representing biological replicates.
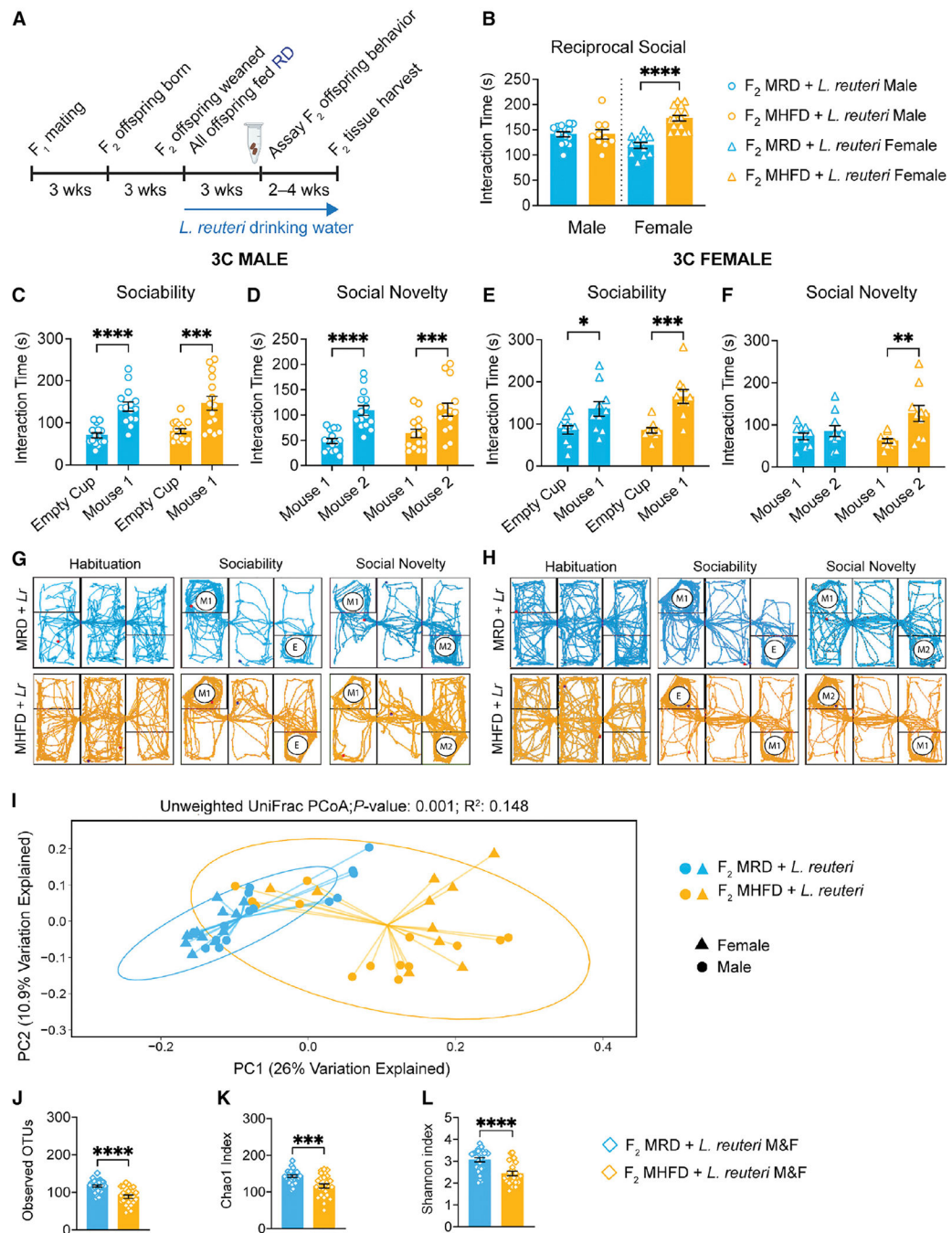
(C–H) N = 7–8 subjects per group.

Figure 5. *L. reuteri* rescues social deficits in male F₂ MHFD-descendant offspring and exerts differential effects on F₂ MRD versus MHFD offspring gut microbiota composition

(A) Experimental schematic. Three monogamous MRD lineage or MHFD lineage F_1 breeder cages were used to establish MRD- and MHFD-descendant F_2 generations, respectively. Upon weaning at 3 weeks old, all mice were given drinking water containing 10^8 CFU/mL *L. reuteri* daily through behavior assessment.

(B) Analysis of the reciprocal social interaction times between pairs revealed no differences between males (male MHFD + *L reuteri* versus MRD + *L. reuteri*: $t(22) = 0.01855$, p = 0.9854), with increased interaction times between F_2 MHFD + *L. reuteri* females compared

with F_2 MRD + *L. reuteri* females (female MHFD + *L. reuteri* versus MRD + *L. reuteri*: t(25) = 6.340, p < 0.0001).

(C and D) (C) F_2 male MHFD + *L. reuteri* offspring show typical preference during sociability (MRD: *t*(56) = 4.515, p < 0.0001; MHFD: *t*(56) = 4.373, p = 0.0001) and (D) social novelty (MRD: *t*(28) = 5.472, p < 0.0001; MHFD: *t*(28) = 4.28, p = 0.0004).

(E–H) (E) Likewise, MHFD + *L. reuteri* F_2 female offspring show a statistically significant preference during sociability (MRD: *t*(36) = 2.633, p = 0.0246; MHFD: *t*(36) = 4.253, p = 0.0003) and (F) preference for social novelty (MRD: *t*(18) = 0.7226, p = 0.7228; MHFD: *t*(18) = 3.763, p = 0.0028). Representative MRD + *L. reuteri* (light blue) or MHFD + *L. reuteri* (light orange) track plots for (G) males and (H) females, respectively. Bar graphs show mean ± SEM with individual data points.

(I) PCoA of unweighted UniFrac distances from the averaged rarefied 16S rRNA gene amplicon sequencing dataset (5,508 reads/sample; n = 1,000 rarefactions) revealed statistically significant clusters based on diet (p = 0.001, R^2 = 0.148).

(J–L) *L. reuteri* administration differentially affects MRD and MHFD F_2 gut microbiome alpha diversity, as measured by observed OTUs (MHFD F_2 + *L. reuteri* versus MRD F_2 + *L. reuteri*: *t*(50) = 5.285, p < 0.0001), Chao1 index (MHFD F_2 + *L. reuteri* versus MRD F_2 + *L. reuteri*: *t*(52) = 4.143, p = 0.0001), and Shannon index (MHFD F_2 + *L. reuteri* versus MRD F_2 + *L. reuteri*: *t*(50) = 4.436, p < 0.0001). Bar graphs show mean ± SEM with individual data points representing biological replicates. B, N = 10–16 pairs per group; (C–F) N = 10–15 subjects per group; (I–L) N = 24–28 subjects per group.

A

Figure 6. LEfSe analysis indicates differentially abundant genus-level taxa between diet lineage in *L. reuteri*-treated F$_2$ groups

Histogram of the LDA scores (log10) computed for genus-level taxa with differential abundance in MHFD and MRD F$_2$ offspring treated with *L. reuteri*. LDA, linear discriminant analysis; LEfSe, LDA effect size. N = 24–28 subjects per group.
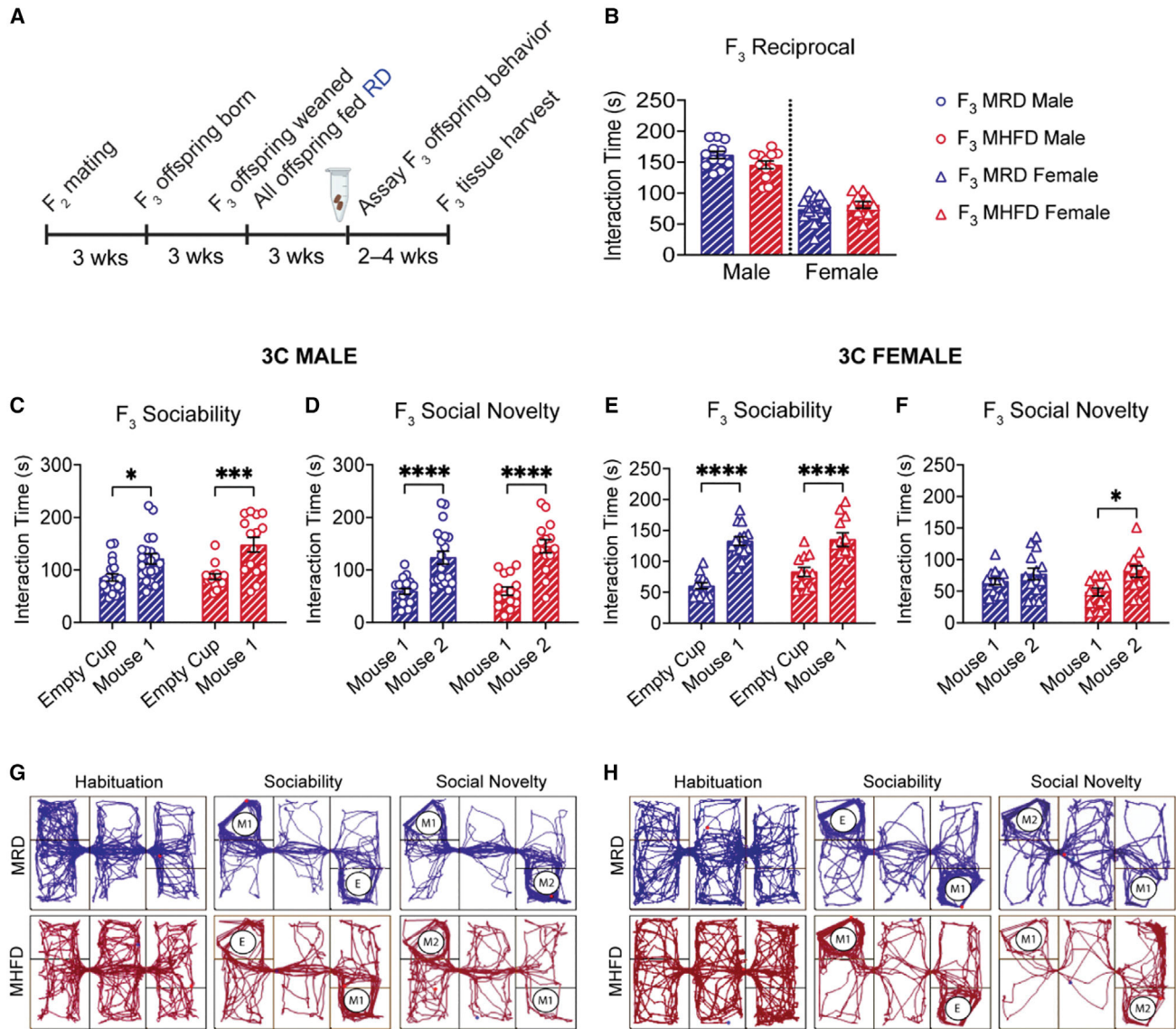
Figure 7. Neurotypical social behavior is observed in the MHFD-descendant F$_3$ generation

(A) Breeding schematic. MRD control and MHFD-descendant F$_2$ mice were bred to produce the MRD- and MHFD-descendant F$_3$ generations, respectively.

(B–H) (B) Comparison of reciprocal social interaction times revealed no differences between male (F$_3$ MRD versus MHFD: t(22) = 1.824, p = 0.0817) or female pairs (F$_3$ MRD versus MHFD: t(24) = 0.9925, p = 0.3309). Both MRD- and MHFD-descendant F$_3$ males demonstrate normal sociability (C) (MRD: t(33) = 2.934, p = 0.0120; MHFD: t(33) = 4.322, p = 0.0003) and preference for social novelty (D) (MRD: t(33) = 5.493, p < 0.0001; MHFD: t(33) = 6.271, p < 0.0001) in the 3C task. F$_3$ MRD and MHFD female offspring show a statistically significant preference for mouse 1 during the sociability phase of the 3C task (E) (MRD: t(48) = 6.979, p < 0.0001; MHFD: t(48) = 4.663, p < 0.0001), while only MHFD-descendant F$_3$ females show a preference for social novelty (F) (MRD: t(48) = 1.722, p = 0.4632; MHFD: t(48) = 2.879, p = 0.0118). Representative MRD and MHFD track plots for (G) males and (H) females, respectively. Bar graphs show mean ± SEM with

individual data points representing biological replicates. (B) N = 11–15 pairs per group; (C–F) N = 12–20 subjects per group.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
Limosilactobacillus reuteri PTA-6475	ATCC	Strain MM4-1A
Chemicals, peptides, and recombinant proteins		
De Man, Rogosa, and Sharpe (MRS) Media	MilliporeSigma	Cat# MHA00MRS2
De Man, Rogosa, and Sharpe (MRS) Agar	MilliporeSigma	Cat# 110660
5% H_2, 5% CO_2, 90% N_2 anaerobic gas mixture	Linde Gas & Equipment, Praxair Distribution	Cat# BI NICDHYC1-K
Critical commercial assays		
MagAttract PowerSoil DNA Kit	Qiagen	Cat #27100-4-EP
Deposited data		
16S ribosomal RNA (rRNA) gene amplicon sequencing data	NCBI SRA	BioProject PRJNA878523
Original unprocessed data	This paper; Mendeley	https://doi.org/10.17632/z6wz8kpk3n.1
Galaxy Computation Tool	Huttenhower Lab	http://huttenhower.sph.harvard.edu/galaxy
Agile Toolkit for Incisive Microbial Analyses (ATIMA2) Platform	Petrosino Lab	https://atima.research.bcm.edu
Experimental models: Organisms/strains		
C57Bl/6N	Taconic Biosciences	Cat# B6-M; Cat# B6-F
Germ-free mice (C57Bl/6N background)	UTMB Germ Free Mouse Facility	N/A
Software and algorithms		
ANY-maze Video Tracking Software	Stoelling	https://www.stoellingco.com/anymaze/video-tracking/software.html
Prism 9.0	Graphpad	https://www.graphpad.com/scientific-software/prism
Adobe Illustrator CS6	Adobe	https://www.adobe.com
Miseq Software	Illumina	https://support.illumina.com/sequencing/sequencing_software/miseq_reporter/downloads.html
R	The R Foundation	https://www.r-project.org/about.html
R Studio	R Studio	https://rstudio.com/

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Other		
Ugo Basile Sociability Cage, Mouse, w/Gray Enclosures	Stoelting	Cat# 60450
Small Mouse 'Stranger' Enclosures (wire cages), set/2, Gray	Stoelting	Cat# 60451
Open Field, 40cm, Gray	Stoelting	Cat# 60101
Reciprocal Social Interaction Box, Clear Acrylic	The Container Store	Cat# 10071077
Regular diet chow	Lab Diets	Cat# 5001
High-fat diet chow	Research Diets	Cat# D12492
Bed-O'cobs 1/8 mouse home cage bedding	The Andersons Plant Nutrient	Cat# 8B