High-Fiber Diet Restores Maternal Obesity-Induced Cognitive and Social Behavioral Deficits in Offspring via Regulating Gut Microbiota-Metabolites-Brain Axis

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SUMMARY

Maternal obesity has been reported to be related to the neurodevelopmental disorders in offspring. The current study is aimed to examine the roles of diet on the gut microbiota and validating the microbiota-metabolites-brain axis as a major mechanism associated with cognitive and social behavioral changes in the offspring of maternal obesity. Here, a cross-sectional study on 778 children aged 7-14 years in two cities of China indicates that maternal obesity may lead to cognitive and social impairments. The animal research indicates that maternal obesity in mice disrupts cognitive and social behaviors and alters the microbiota composition in the offspring, which are prevented by a high-dietary fiber diet in either dams or offspring. Co-housing and feces microbiota transplantation experiments reveals a causal relationship between the microbiome- and the behavioral changes. Moreover, treatment of the microbial metabolites short-chain fatty acids exhibits a similar beneficial effect on alleviating the behavioral deficits in offspring. Together, our study purports the microbiota-metabolites-brain axis as a mechanism that could enable therapeutic strategies against maternal obesity-induced cognitive and social dysfunctions.

Keywords

Maternal obesity; Cognitive and social behavioral deficits in offspring; Dietary fiber; Gut microbiota; SCFAs

INTRODUCTION

Obesity in women of reproductive age is increasing in prevalence worldwide due to intake of high-calorie, low-fiber diets, increasing consumption of manufactured foods with high in sugar, and sedentary lifestyle. Maternal obesity before and during pregnancy is widely recognized to have short-term and long-term adverse health outcomes for both the mothers and their children. Evidences from both animal and human studies indicate that maternal obesity induced by dietary intervention leads to obesity, diabetes, increased blood pressure, and even behavioral changes in the offspring (Drake and Reynolds, 2010; Godfrey et al., 2017), which indicates maternal obesity may be one of the influences contributing to the “developmental origins of health and disease” (Drake and Reynolds). The high prevalence of maternal obesity means that the determination of any such long-term effects is now an urgent priority (Heslehurst et al.). Recent UK and US nationally distributed longitudinal studies showed increased risks for poorer cognitive outcomes and autism spectrum disorders (ASD) in children of mothers with obesity before pregnancy (Basatemur et al., 2013; Pugh et al., 2015). Work in rodents also showed that maternal high-fat diet (MHFD) induces long-term cognitive deficits across several generations recently (Sarker and Peleg-Raibstein, 2018). Despite the potential public health importance, few cohort studies have been done to examine the neurobiological mechanism by which maternal obesity affects offspring behavior and brain function.

A non-genetic, yet heritable contributor to behaviors may be the microbiota, a community of microorganisms harbored in gastrointestinal (GI) tract that impacts development and function of the immune, metabolic, and nervous systems (Cho and Blaser; Moeller et al., 2018; Santiago et al.). The intestinal ecosystem is thought to be established at or soon after birth, facilitated by vertical transmission and exposure to and/or ingestion of environmental flora (Ferretti et al., 2018; Moran et al., 2018). Thus, maternal influences on the offspring's microbiome are significant, and may potentially alter the risk of mental impairment. Indeed, maternal obesity has been associated with alterations in the gut microbiome in offspring in both human and non-human primates (Batterham et al.; Chu et al.). A study on *Macaca fuscata* (Japanese macaque) report that a high-fat maternal diet, but not obesity *per se*, re-structured the offspring's intestinal microbiome. Specifically, maternal obesity negatively impacts a subset of bacteria in the offspring gut and selective re-introduction of *Lactobacillus (L.) reuteri* restores social deficits in offspring (Buffington et al.). A recent study also identifies differences between AD and normal controls in 11 genera from the feces and 11 genera from the blood (Li et al., 2019). Given emerging reports that link gut microbiota to brain function, it has been speculated that changes in the gut microbiome may also be relevant to cognitive impairments in offspring induced by maternal obesity.

Dietary fiber as the seventh nutrient is widely reported to regulate the gut microbiome, providing an important substrate to the community of microbes

(microbiota) that inhabits the distal gut (Sonnenburg and Sonnenburg, 2014). Unlike humans, who produce ~17 gastrointestinal enzymes to digest mostly starch, the gut microbiota produces thousands of complementary enzymes with diverse specificities, enabling them to depolymerize and ferment dietary polysaccharides into host-absorbable – short chain fatty acids (SCFAs) (Kaoutari et al., 2013). A recent study reported that SCFAs could regulate the activity of hypothalamic orexigenic neurons (Farhadi et al.). Notably, SCFAs are key molecules that modulate microglia maturation, morphology and function (Erny et al.). Additional evidence in human beings suggests that dietary manipulation of the maternal microbiome in pregnancy with dietary fiber has beneficial effects for both offspring immune function and metabolism (Thorburn et al., 2014). However, it still is unknown whether dietary fiber restores cognitive and social behavioral deficits in offspring from obesity mothers.

Here we report that a) maternal obesity induces cognitive and social behavioral deficits in human and mouse offspring; b) maternal dietary fiber supplement improves offspring social behavioral deficits induced by maternal high fat diet (MHFD) by restructuring the gut microbiome transmitted from mother to offspring; c) mother-to-offspring gut microbiome mediates maternal high fat diet (MHFD) induced learning and memory impairments in offspring; d) offspring directly dietary fiber administration improves gut microbiome, aberrant spliceosome alterations and restores HFD-induced cognitive and social behavioral deficits in MHFD-CD offspring; e) oral treatment with a mixture of

acetate and propionate reverses behavioral deficits in MHFD-CD Offspring.

RESULTS

Maternal Pregnancy Overweight and Obesity Are Associated with Impaired Child Neurodevelopment

To examine the association between maternal pregnancy weight and neurodevelopment in offspring, 778 children (403 boys and 375 girls) were eligible for follow-up at 7-14 years of age, whose parents completed the social competence scale of the Child Behavior Checklist, which includes 20 social competence items with three social competence subscales measure competencies: activities (e.g., sports and hobbies); social subjects(e.g., friendships and interpersonal skills); and school performance (e.g., performance, learning ability and school problems). Demographics of the study population are shown in **Table 1**. Compared with mothers with normal weight, overweight & obese mothers had the highest proportions of overweight & obese children (χ^2 test, $p < 0.05$). Additionally, overweight & obese mothers had on average lower educational attainment and lower family income. On average, all underweight, normal and overweight & obese mothers gained pregnant weight gain as recommended. Compared with children of normal weight mothers, children of overweight and obese mothers had significantly lower score of social competence (Kruskal-Wallis with Bonferroni's test, $p < 0.01$) **(Table1; Figure S1A)**. Consistent with other countries cohort studies of poorer cognitive performance and increased risk of autism spectrum disorders in children of

obese mothers, children of Chinese overweight & obese mothers had significantly reduced scores of social subjects and school performance, but not activities, indicating lowered social and learning ability (Kruskal-Wallis with Bonferroni's test, $p < 0.05$) **(Table1; Figures S1B-S1D)**. Further analysis revealed that significantly reduced social competence were more pronounced in boys than in girls, which could be due to lower school performance scores (Kruskal-Wallis with Bonferroni's test, $p < 0.01$) **(Figures S1A and S1B)**. Taken together, these data indicate that maternal obesity could be more likely to cause cognitive and social impairments in their male offspring.

Table1 Participant Characteristics and Social Competence among Children

Characteristics	Maternal Pre-pregnancy BMI			P value
	Underweight (BMI<18.5) N=132	Normal (BMI 18.5-23.9) N=533	Overweight & Obesity (BMI >23.9) N=79	
Child's age (years) (Mean ± SD) [a]	10.32±2.01	10.39±1.86	10.42±1.95	0.955
Child's gender [b]				
Male, %	52.3	47.3	49.4	0.941
Child's BMI [b]				0.012
Underweight, %	15.4	11.6	15.9	
Normal, %	73.2	69.1	53.6	
Overweight & Obesity, %	11.4	19.4	30.4[+#]	
Breast feeding [a]	81.8	82.7	79.7	0.801
Maternal Education level [b]				0.001
Secondary high school or lower, %	8.3	14.8	36.7	
High school, %	30.3	19.9	15.2	
Diploma in higher education or Bachelor's degree, %	59.1	63.8	48.1	
Master's degree or higher, %	2.3	1.5	0.0	
Maternal pregnant weight gain (Mean ± SD) [a]	15.70±5.97	14.24±5.51	11.57±6.10	0.000
Family income (RMB) [b]				0.000
< 80,000, %	5.3	9.6	27.8	
80,000–150,000, %	33.3	32.5	32.9	
150,000-300,000, %	39.4	39.0	30.4	
> 300,000, %	21.9	19.0	8.9	
Children's social competence (Mean ± SD) [a]				
Total	17.05±4.06	17.10±3.72	15.65±3.79[*]	0.005
Ability	4.89±2.36	4.90±2.18	4.36±2.21	0.147
Social	6.80±2.00	6.78±1.87	6.16±1.92*	0.045
School	5.36±0.63	5.43±0.67	5.13±0.95*	0.013

[a] Kruskal-Wallis test;

[b] χ^2 test;

[+] Significant difference from underweight maternal BMI category with significance level at < 0.05;

[#] Significant difference from normal maternal BMI category with significance level at < 0.05;

[*] Significant difference from normal maternal BMI category with significance level at < 0.05, and Bonferroni correction for multiple comparisons. See also Figure S1.

Maternal Obesity Induces Cognitive and Social Behavioral Deficits in Mouse Offspring

In human studies, confirmation of causation and identification of mechanisms linking maternal obesity with offspring neurodevelopment are difficult. To investigate how maternal obesity affects offspring neurodevelopment, female C57BL/6 mice were fed either control diet (CD) or high-fat diet (HFD) for 12 weeks. As expected, MHFD significantly increased maternal weight **(Figure S2A)**. Females were then paired with males to produce offspring that were fed with a control diet after weaning (3 weeks) (**Figure 1A**). There was no significant difference in offspring weight between maternal diet groups at 8–10 weeks old or at 7 months old (**Figure S2B**), at which the behavioral experiments were performed. There were no sexual differences between male and female offspring in the behavior test. At 8-10 weeks old, to assess working memory and long-term memory, Y-maze test and novel object recognition test were performed, respectively. Compared with MCD-CD offspring, MHFD-CD offspring had fewer spontaneous alternation and showed a discrimination index between the novel and familiar object close to -0.2, indicating impairments of working memory and long-term memory (**Figures 1B, 1C, S2C and S2D**). Consistent with a previous report, MHFD-CD offspring had impaired sociability and showed no preference for social novelty by a three-chamber sociability test (two-tailed paired Student's t tests, $p < 0.01$) (**Figures 1D and 1E**). Furthermore, MHFD-CD mice still showed significant cognitive impairment but not social,

compared with MCD-CD offspring at 7 months of age (**Figures 1B-1E**) (ANOVA with Tukey's test, $p < 0.05$). Taken together, these data indicate that maternal obesity leads to offspring's memory and social deficits.

Maternal Dietary Fiber Intake Restores Maternal Obesity-Induced Cognitive and Social Behavioral Deficits in Offspring

To investigate whether dietary fiber alleviates maternal obesity-induced offspring neurodevelopment dysfunction, female mice were fed with one of four diets: control diet (MCD), high-fat diet (MHFD), high-fat/high-fiber diet (a high-fat diet with inulin as a source of fiber, MFFD), and a control diet with inulin (MFD) for 12 weeks. Females were then paired with males to produce offspring that were fed with a control diet after weaning (**Figure 1F**). Consistent with reports of lower fertility in obese mothers (Poston et al., 2016), the litter size was reduced (**Figure S2G**) and latency to first litter increased in female mice fed HFD (**Figure S2H**). Relative to the "standard" HFD, a diet comprised of dietary fiber reduced weight gain and markedly increased fertility (ANOVA with Tukey's test, $p < 0.01$) (**Figures S2E-S2H**). There was no significant difference in offspring weight between maternal diet cohorts at 3 weeks old, or at 8–10 weeks old (**Figures S2I and S2J**). Fecal samples were collected and behaviors in offspring were assessed when mice were 8-10 weeks old. Strikingly, maternal dietary fiber intake significantly improved working memory and long-term memory (ANOVA with Tukey's test, $p < 0.01$), as well as sociability and the preference for social novelty in MHFD-FD offspring (two-tailed paired

Student's t tests, $p < 0.05$) **(Figures 1G-1J, S2K-S2M)**.

Taken together, these data indicate that maternal dietary fiber intake could protect against maternal obesity-induced cognitive and social behavioral deficits in offspring.

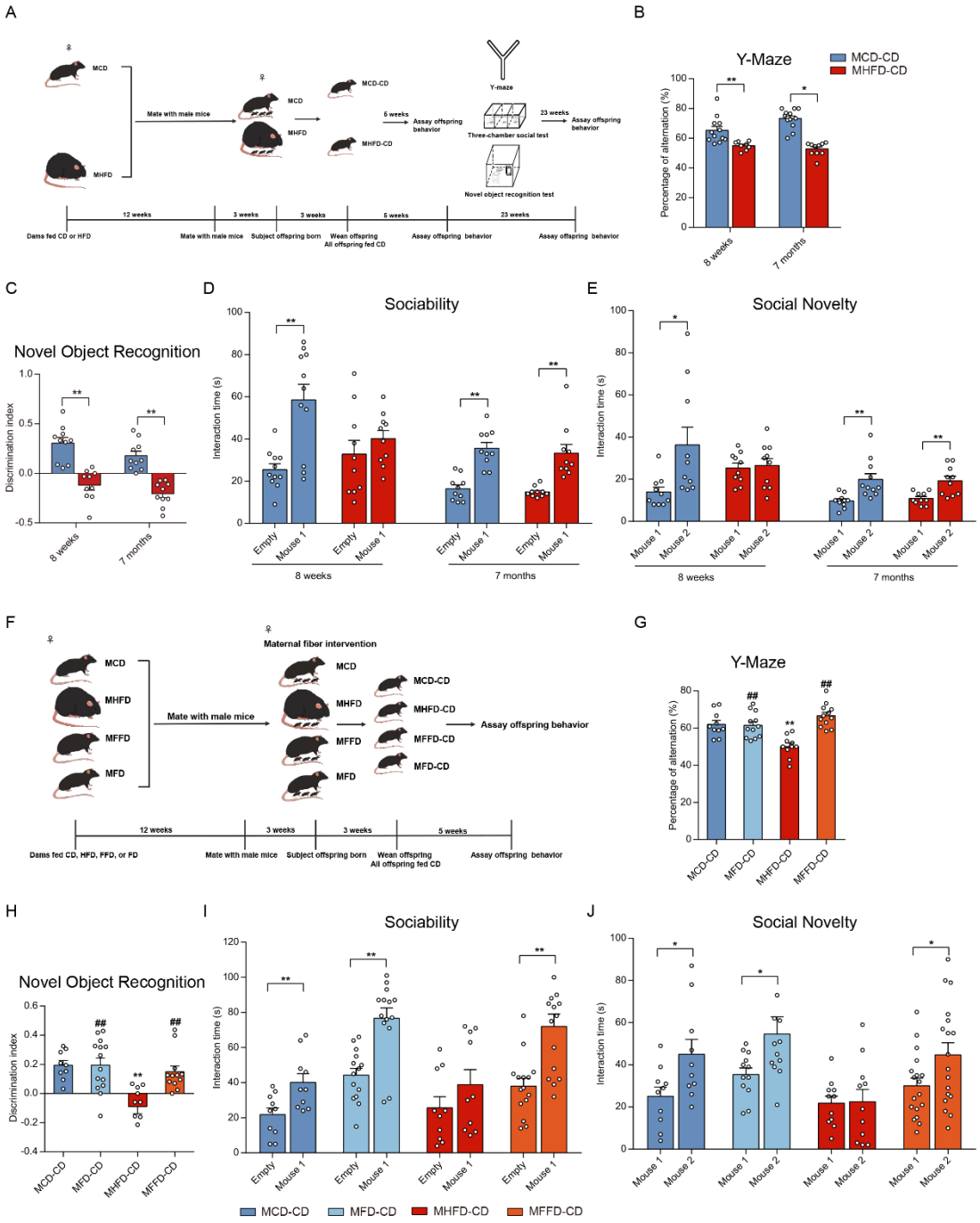

Figure1 Maternal Obesity and Dietary Fiber Intake Imprint the Cognitive and Social behaviors of Mouse Offspring

(A) Schematic of the maternal diet regimen and breeding.

(B) For Y-maze, spontaneous alternations were recorded.

(C) For the novel object recognition test, the discrimination index between the novel and

familiar object were recorded.

(D) In the sociability test, the time spent on interacting with a mouse or with an empty wire cage were recorded.

(E) In the social novelty test, the time spent on interacting with a novel versus a familiar mouse.

(F) Schematic of the maternal dietary fiber supplementation.

(G-J) Behavioral phenotypes in offspring after maternal dietary fiber supplementation, including (G) Y-maze, (H) the novel object recognition test, (I) sociability, and (J) preference for social novelty.

Data presented as mean ± SEM. Statistical analyses were performed by one-way ANOVA with Tukey's multiple comparison test (for B, C, G and H) or by paired two-tailed Student's t test (for D, E, I, and J). For Tukey's multiple comparison test, $^{*}p < 0.05$, $^{**}p < 0.01$, compared with the MCD-CD group, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, compared with the MHFD-CD group. For Student's t test, $^{*}p < 0.05$, $^{**}p < 0.01$. See also Figure S2.

Maternal Dietary Fiber Intake Restores Maternal Obesity-Induced Synaptic Impairment in Offspring

The ultrastructures of synapses in the hippocampus were examined. An analysis of postsynaptic density (PSD) ultrastructure revealed that the length and width of PSD were significantly elevated in the MFFD-CD group, compared with the MHFD-CD group (ANOVA with Tukey's test, $p < 0.01$) (**Figures 2A and 2B**). In line with PSD ultrastructural alterations, the expressions of *PSD-95*, a major component of PSD, was also restored in MFFD-CD offspring (**Figures 2C and S3A**), compared with MHFD-CD offspring as assessed by qPCR and immunofluorescence. Accordingly, the mRNA levels of synapse-related *FXR1*, *GluA2* and *BDNF* were decreased in the offspring derived from the HFD-treated dams, but not in the MFFD-CD offspring (**Figures 2C and S3B**).

Social behaviors are mediated by the prefrontal cortex (PFC). Given that microglia dysregulation was reported in a range of neuropsychiatric conditions including ASD, the expression levels of microglia surface molecule *CD31*, *CSF1R*, and *F4/80*, as well as *Mafb*, an important transcription factor of the adult microglia program (Matcovitch-Natan et al., 2016), were assessed. Maternal obesity lowered *Mafb* mRNA level and increased *CD31* mRNA level in MHFD-CD offspring, which were restored by enrichment of the diet with dietary fiber (**Figure S3C**). Furthermore, the microglia-neuron interaction dysfunction, such as reductions in the levels of *CX3CL1*, *NGF* and *DAP12*, in MHFD-CD offspring were ameliorated in MFFD-CD offspring (**Figure S3D**).

Likewise, unlike the MHFD-CD offspring, the expression of *FXR1*, *FXR2*, *PSD-95* and *Tdp2* were increased in MFFD-CD offspring (**Figures S3E andS3F**). These findings were consistent with reports of shared mechanisms underlying both social and cognitive impairments (State and W). Taken together, these data indicate that maternal high fiber intake protects offspring against synaptic impairment and disruption of microglia maturation in hippocampus and PFC, which were induced by maternal obesity.

Maternal Dietary Fiber Intake Re-shapes the Gut Microbiome of Both Mother and Offspring

Maternal obesity has been shown to alter the gut microbiome of offspring (Chu et al., 2016; Ma et al., 2014; Steegenga et al., 2017) and microbial reconstitution could reverse social deficits (Buffington et al., 2016a). Dietary fiber is a well-recognized prebiotic known to promote growth of select beneficial bacteria in colon (Gibson, 1999). Hence, we next examined whether dietary fiber's protection against HFD-induced behavioral deficits would correlate with microbiota alterations. Analysis of microbiota composition by 16S sequencing indicated that there was a remarkable change in the maternal microbiome composition but not community richness between MHFD group and MFFD group **(Figures S4A and S4B)**. Maternal dietary fiber intake also corrected some of the HFD-induced changes in microbiota that were observed at the phylum level and order level **(Figures S4C and S4D)**. Specifically, S24-7 (OUT_73_S24-7, OTU_127_S24-7, OUT_187_S24-7 et al.), *Bifidobacterium*

animalis (424_Bifidobacterium_animalis), *Prevotella* (OUT_21_Prevotella) and Clostridiales (OTU_276_Clostridiales and OTU_435_Clostridiales and OTU_277_Clostridiales) were prevalent in most MFFD samples and absent from most or all MHFD samples (**Figure S4E**).

While bacterial α-diversity did not differ between the offspring from any diet group (**Figures S4F**), the unweighted Unifrac distance of the MFFD-CD mice was different from MHFD-CD mice, similar to that in observed in their dams, which revealed that dietary fiber regimen altered the microbiota composition (**Figures 2Dand S4A**). Differentiating bacterial taxa in offspring belong predominantly to the Clostridiales and Bacteroidales orders, with single representatives from the Actinobacteria, Firmicutes and Tenericutes phyla (**Figures 2E, S4G and S4H**). Specifically, 21 differentiating bacterial taxa were identified between MHFD and MFFD by Wilcoxon rank-sum test analyzing OTUs, nine of which belonged to S24_7 family (**Figure 2F**). The abundance of five bacterial OTUs positively correlated with increased cognitive and social behaviors in mice using Spearman's rank correlation (**Figure 2G**) ($R > 0.4$, $p < 0.05$). The association of specific bacterial species with MFFD-CD samples, which are also highly correlated with increased cognitive- or social-relevant behaviors, supports the hypothesis that specific bacteria may contribute to the protection of dietary fiber against cognitive and social deficits in offspring, induced by maternal high fat diet.

Microbial metabolites in the gut could impact neurological outcomes (Wang et al., 2018). SCFAs in offspring feces were next examined, which are derived from microbial fermentation of dietary fibers and are likely to have broad impacts on various aspects of host physiology. Dietary fiber treatment marked improved MHFD-induced decreases in the feces levels of acetate, propionate, but not butyrate in offspring (**Figure 2H**) (ANOVA with Tukey's test, $p < 0.05$).

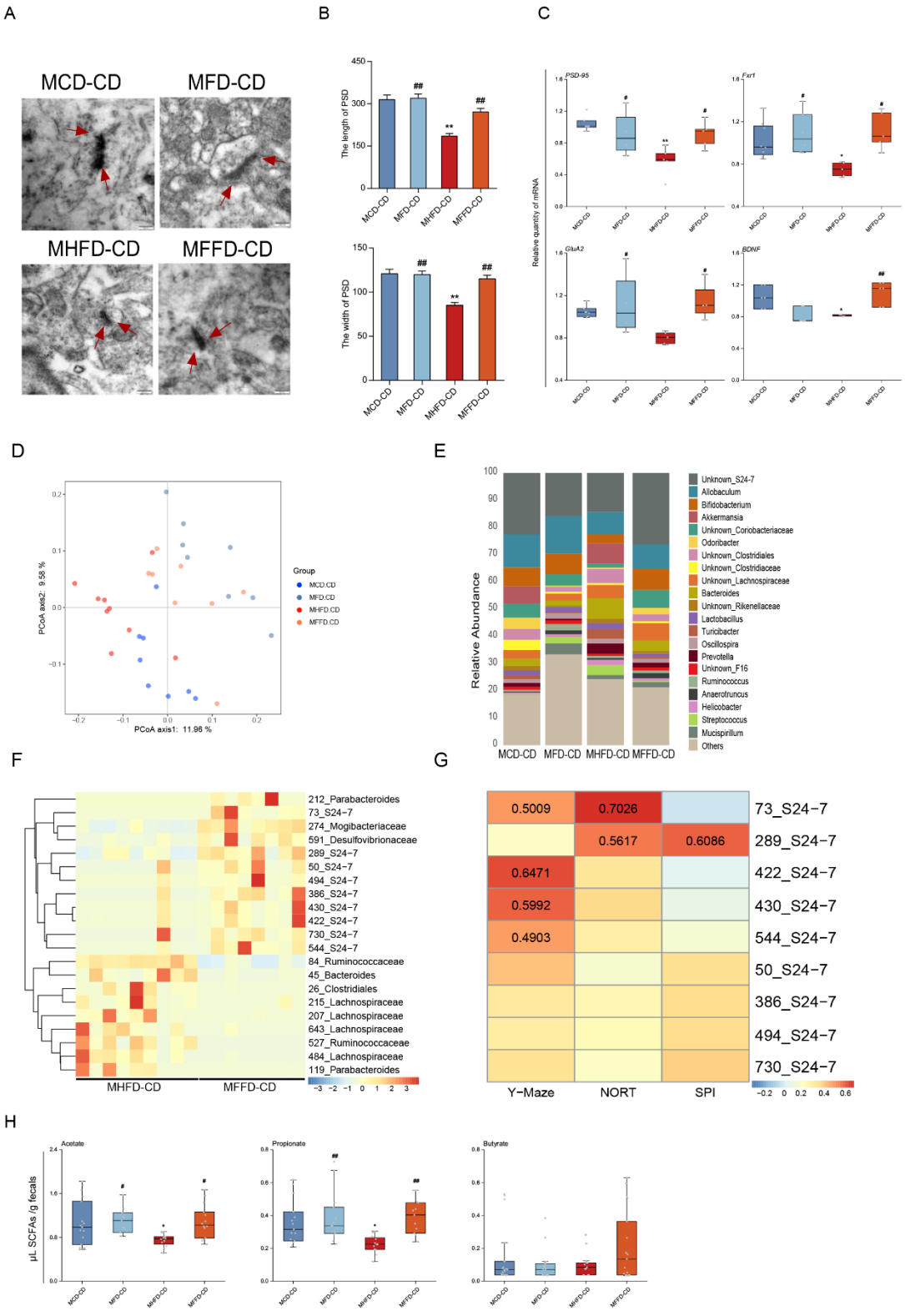

Figure 2 Maternal Dietary Fiber Intake Restores Synaptic Impairment and Re-shapes the Gut Microbiome of Offspring

(A) Representative images of ultrastructure of synapse.

(B) The length (up) and width of PSD (down).

(C) The mRNA expressions of *PSD-95*, *Fxr1*, *GluA2*, and *BDNF* in the hippocampus (n=8 per group).(D) Principal coordinates analysis (PCoA) of unweighted UniFrac distances from the averaged rarefied 16S rRNA gene dataset (p = 0.0001, R^2 = 0.1993).

(E) The relative abundance of bacteria at the genus level. All genera with an average relative abundance below 1% were grouped to “others”.

(F) A Z-score scaled heatmap of different OTUs identified by Wilcoxon rank-sum test between MHFD-CD and MFFD-CD with $p \leq 0.01$.

(G) Abundance of selected taxas in the offspring microbiome is correlated with behavior of offspring. Spearman’s rank correlation between the microbiome and mouse behavior. If p value < 0.05 for significant correlations, R is noted.

(H) The concentrations of short-chain fatty acid (SCFA) including acetate, propionate and butyrate in offspring feces.

Data of (B) and (C) presented as mean ± SEM. Data of (H) presented as median ± interquartile range. Statistical analyses were performed by one-way ANOVA with Tukey’s multiple comparison test (for B, C and H). $^{*}p < 0.05$, $^{**}p < 0.01$, compared with the MCD-CD group, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, compared with the MHFD-CD group. See also Figure S3 and Figure S4.

Gut microbiota mediates maternal obesity-induced social and cognitive deficits in offspring

The maternal gut microbiome can be vertically transmitted to their offspring by breastfeeding and lead to longer-lasting colonization of the offspring gut (Ferretti et al., 2018; Moran et al.; Yassour et al., 2018). To investigate whether gut microbiota plays an important role in MHFD-induced behavioral abnormalities, the fecal microbiota from female MHFD and MFFD mice were transplanted into antibiotics-treated adult female mice, respectively (**Figures 3A, S5A and S5B**). Offspring from the dams that inheriting MHFD donor microbiota did show impaired memory and social behaviors (**Figures 3B-3E, S5C and S5D**). By contrast, offspring from the dams that inheriting MFFD donor microbiota had no behavioral abnormalities (**Figures 3B-3E, S5C and S5D**). Subsequently, to investigate that maternal colonization with MHFD donor microbiota or MFFD donor microbiota influence behaviors in offspring after birth, cross-fostering experiments were performed by switching newborns between recipients of MHFD and MFFD's microbiota. Strikingly, offspring derived from MHFD fecal microbiota-transplanted mothers, but reared by MFFD fecal microbiota-transplanted mothers, exhibited behavioral improvements; those from MFFD fecal microbiota-transplanted mothers that were reared by MHFD fecal microbiota-transplanted mothers exhibited behavioral impairments (**Figures 3A-3E, S5C and S5D**). Accordingly, MHFD offspring were co-housed with MFFD offspring, thus allowing the transfer of microbiota via coprophagy

(**Figure 3F**). Similarly, MHFD offspring co-housed with MFFD offspring exhibited increased cognition, as well as improved sociability and preference for social novelty, as determined by behavioral tests (**Figures 3G-3J, S5E - S5G**). Altogether, these results indicate that there is a causality relationship between the gut microbiota disturbances and behavioral deficits in the offspring of maternal obesity, and maternal dietary fiber intake could protect against behavioral deficits of offspring via regulating gut microbiota.

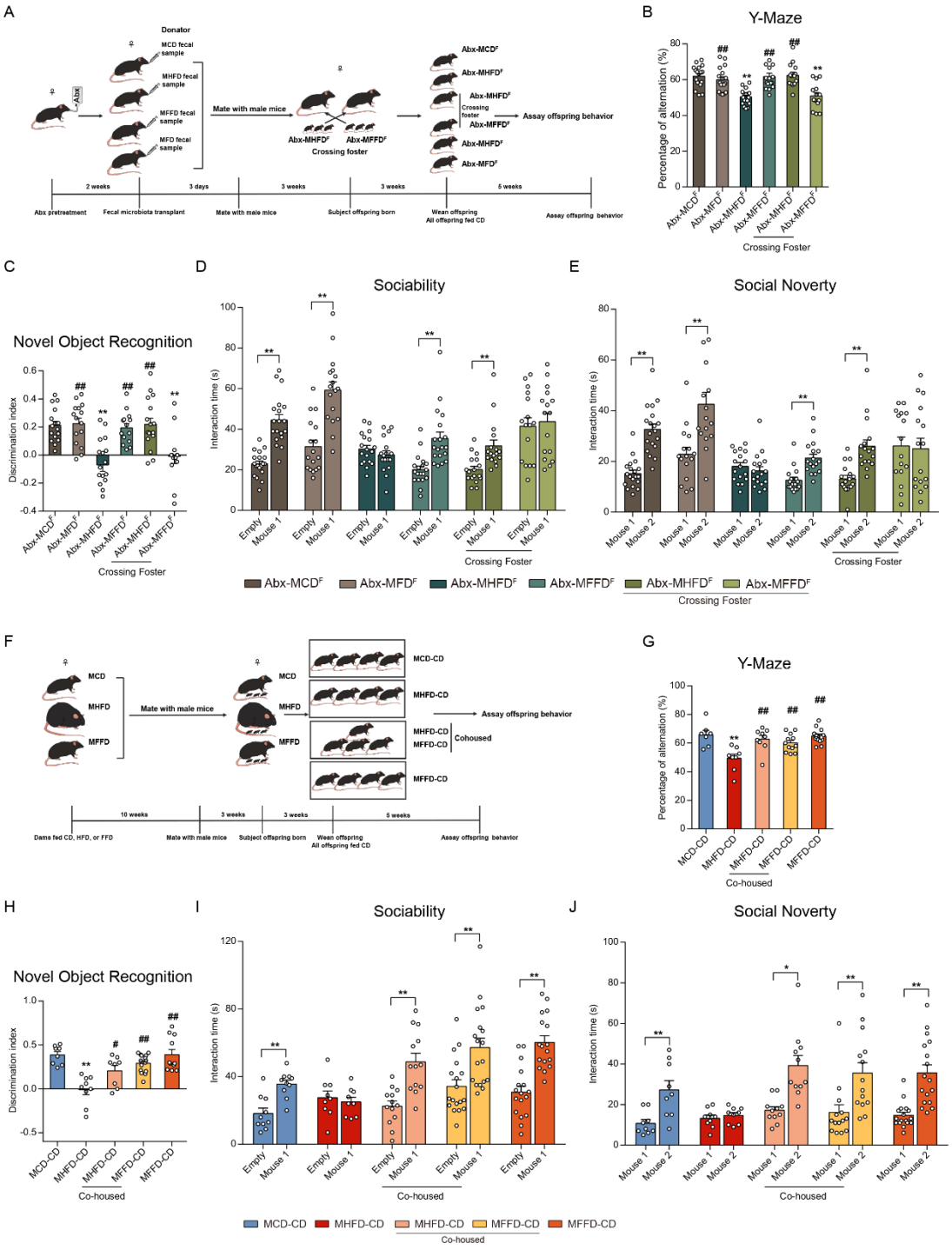

Figure 3 Gut microbiota mediates maternal obesity-induced social and cognitive deficits in offspring

(A) Schematic of the maternal fecal microbiota transplant and crossing foster.

(B-E) The offspring from fecal microbiota-transplanted mothers were tested by (B)Y-maze,

(C)the novel object recognition test, (D)sociability, and (E)preference for social novelty.

(F) Schematic of the offspring co-housing experiment.

(G-J) The co-housed offspring were tested by (G)Y-maze, (H)the novel object recognition test, (I)sociability, and (J)preference for social novelty.

Data presented as mean ± SEM. Statistical analyses were performed by one-way ANOVA with Tukey's multiple comparison test (for B, C, G and H) or by paired two-tailed Student's t test (for D, E, I, and J). For Tukey's multiple comparison test, $^{*}p < 0.05$, $^{**}p < 0.01$, compared with the MCD-CD group, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, compared with the MHFD-CD group. For Student's t test, $^{*}p < 0.05$, $^{**}p < 0.01$. See also Figure S5.

Offspring Dietary Fiber Intake Restores Maternal Obesity-Induced Behavioral Deficits and Aberrant Spliceosome Alterations

We next sought to examine whether directly dietary fiber application to offspring could also reverse the behavioral and neurobiological deficits characteristic of MHFD offspring. MHFD offspring were fed FD from weaning (3 weeks) until adult, after which behaviors were tested (**Figures 4A, S6A and S6B**).Treatment with dietary fiber significantly improved cognition(ANOVA with Tukey's test, $p < 0.01$), as well as sociability and the preference for social novelty(two-tailed paired Student's t tests, $p < 0.05$) (**Figures 4B-4E, S6C-S6D**). Gene expression profiling of RNA-seq identified 564 downregulated and 247 upregulated genes in the hippocampus of MHFD-FD and MCD-FD mice, using differentially expressed gene (DEG) analysis **(Figure S6E)** (FDR q-value < 5%). There were relatively few sex-specific DEGs **(Figure S6F)**. Gene set enrichment analysis (GSEA) indicated that KEGG pathways involving transcription, translation, and protein quality control and export were upregulated in the hippocampus of MHFD-CD group mice, relative to MHFD-FD mice **(Figure 4F)**. A KEGG pathway involving RNA processing by the spliceosome was significantly upregulated in MHFD-CD mice (**Figures 4G and 4H**) (FDR q-value < 25%). These data reveal that directly dietary fiber administration to 3 weeks old offspring could also effectively improve behavior and RNA splicing processes.

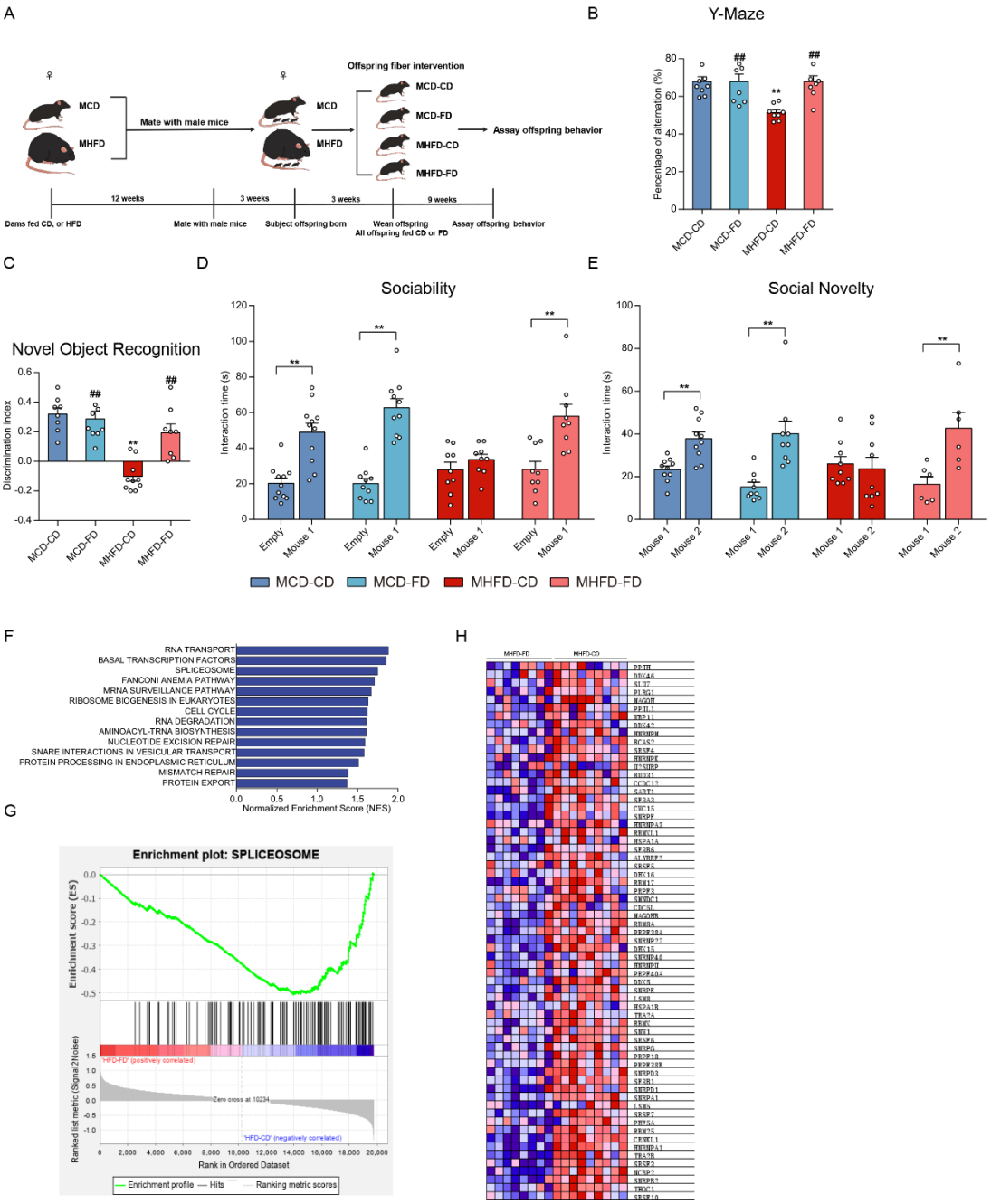

Figure 4 Offspring Dietary Fiber Intake Restores Maternal Obesity-Induced Behavioral Deficits and Aberrant Spliceosome Alterations

(A) Schematic of the offspring directly dietary fiber administration.

(B-E) Behavioral phenotypes in offspring after directly dietary fiber administration, including (B)Y-maze, (C) the novel object recognition test, (D) sociability, and (E) preference for social novelty.

(F) KEGG pathways upregulated in the hippocampus of MHFD-CD mice by Gene set enrichment analysis (GSEA) (FDR < 25%).

(G-H) The enrichment plot (G) and the heat map of the leading-edge subset (H) for the spliceosome set.

Data of (B) - (E) presented as mean ± SEM. Statistical analyses were performed by one-way ANOVA with Tukey's multiple comparison test (for B and C) or by paired two-tailed Student's t test (for D and E). For Tukey's multiple comparison test, $^{*}p < 0.05$, $^{**}p < 0.01$, compared with the MCD-CD group, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, compared with the MHFD-CD group. For Student's t test, $^{*}p < 0.05$, $^{**}p < 0.01$. See also Figure S6.

Offspring Dietary Fiber Intake Re-shapes the Gut Microbiome

We also examined whether dietary fiber could correcte directly the changes in the microbiota of MHFD offspring. The gut microbiome from MHFD-FD offspring shifted away from that of MHFD-CD offspring **(Figures 5A and S6G**). Differentiating bacterial taxa belong predominantly to the Clostridiales and Bacteroidales orders, with single representatives from the Firmicutes phyla (**Figures 5B, S6H and S6I**). Consistent with maternal dietary fiber supplement, directly dietary fiber treatment improved the level of S24-7 (OTU_436_S24-7, OTU_516_S24-7 and OTU_573_S24-7 et al.) (**Figure 5C**). Specifically, *Bacteroides* (OTU_1_Bacteroides_acidifaciens, OTU_403_Bacteroidales, OTU_448_Bacteroides_acidifaciens, and OTU_672_Bacteroides_acidifaciens, et al.) were prevalent in all MHFD-FD samples and absent from most or all MHFD-CD samples (**Figure 5C**). Conversely, *Ruminococcus* (OTU_120_Ruminococcus, OTU_157_Ruminococcus and OTU_148_Ruminococcus_gnavus) was prevalent among most MHFD-CD mice and absent from MHFD-FD groups (**Figure 5C**). The abundance of the eight OTUs belonging to S24_7 positively correlated with increased long-term memory ($R > 0.4$, $p < 0.05$). Additionally, the level of *Bacteroides* significantly correlated with increased long-term memory and social behaviors ($R > 0.5$, $p < 0.05$). *Ruminococcus* showed the opposite effects, as it correlated with reduced memory behavior and social interaction deficits ($R > 0.4$, $p < 0.05$) (**Figure 5D**). These findings suggest that offspring dietary fiber intake could also correct the

microbiota depletion in the offspring born to obese mothers.

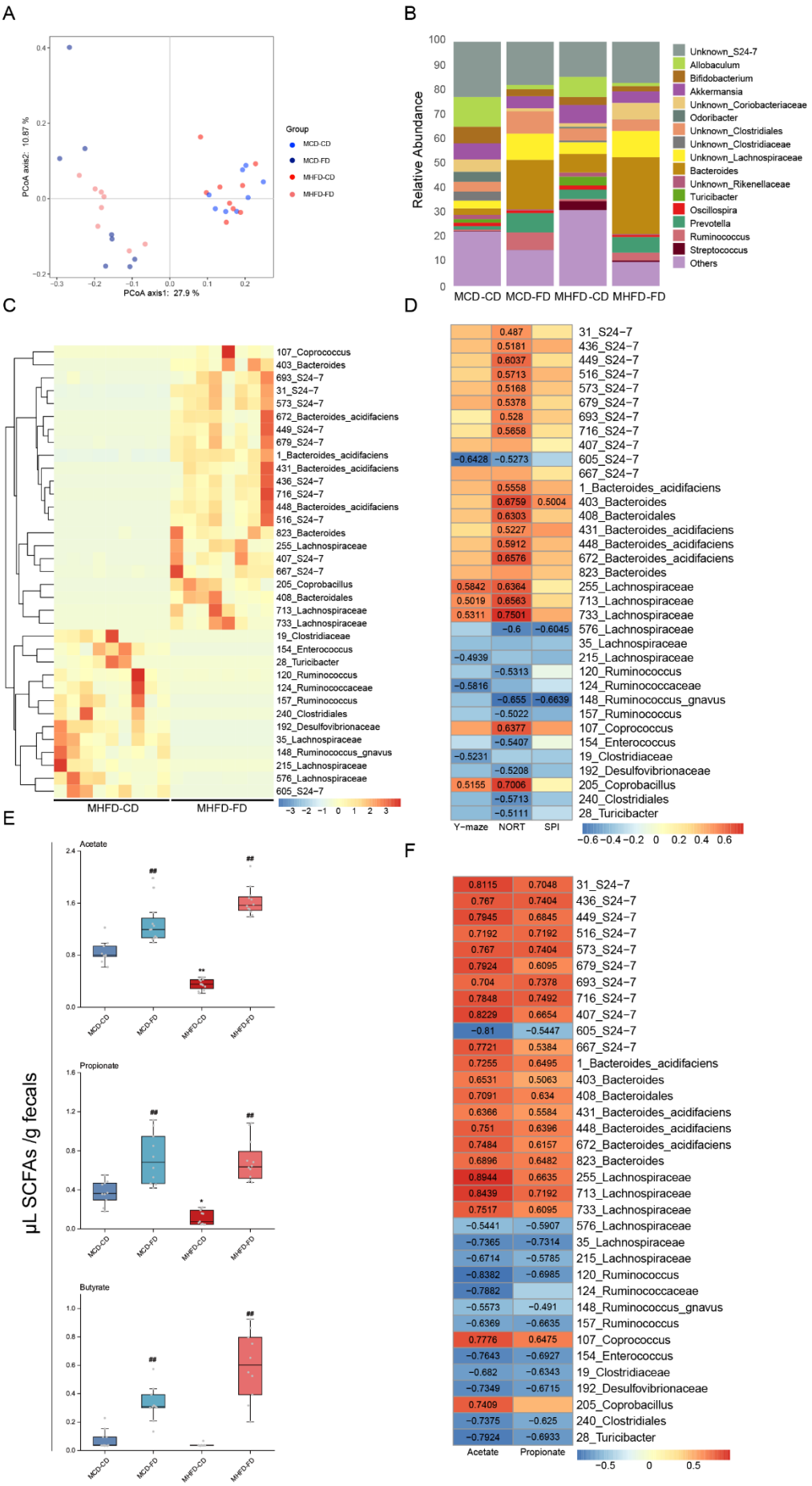

Figure 5 Offspring Dietary Fiber Intake Re-shapes the Gut Microbiome

(A) UniFrac-based phylogenetic clustering($p < 0.0001$, R2=0.3395) (n=8, 8, 9, 8 respectively).

(B) The relative abundance of bacteria at the genus level. All genera with an average relative abundance below 1% were grouped to “others”.

(C) A Z-score scaled heatmap of different OTUs identified by Wilcoxon rank-sum test between MHFD-CD and MHFD-FD with $p \leq 0.001$.

(D) Abundance of selected taxas in the offspring microbiome is correlated with behavior of offspring. Spearman’s rank correlation between the microbiome and mouse behaviors. If p value < 0.05 for significant correlations, R is noted.

(E) Short-chain fatty acid (SCFA) concentrations in feces. Data presented as median ± interquartile range. $^{*}p < 0.05$, $^{**}p < 0.01$, compared with the MCD-CD group, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, compared with the MHFD-CD group. Significant differences between mean values were determined by one-way ANOVA with Tukey’s multiple comparison test.

(F) Spearman’s rank correlation between the selected taxas of microbiome and acetate/propionate. If p value < 0.05 for significant correlations, R is noted. See also Figure S6.

SCFAs Supplementation Restores Maternal Obesity-Induced Cognitive and Social Behavioral Deficits in Offspring

In accord with maternal dietary fiber supplement, directly dietary fiber treatment elevated levels of acetate, propionate, but not butyrate in MHFD-FD offspring (**Figure 5E**). Furthermore, the level of acetate and propionate were significantly correlated with most differentiating bacterial taxa using Spearman's rank correlation (**Figure 5F**) ($R>0.4$, $p<0.05$). We hypothesized that the selective decrease of acetate and propionate in the gut of MHFD offspring was causally related to their behavioral deficits. To test this hypothesis, acetate and propionate were introduced into the drinking water of MHFD offspring at weaning for 5 weeks, after which behaviors were tested (**Figures 6A and S7A**). Remarkably, treatment with a mix of acetate and propionate significantly improved cognition (ANOVA with Tukey's test, $p < 0.001$), sociability and preference for social novelty in MHFD offspring (two-tailed paired Student's t tests, $p < 0.001$) (**Figures 6B-6E, S7B and S7C**). Accordingly, SCFAs treatment significantly improved the length and width of PSD (**Figures 6F and 6G**) (ANOVA with Tukey's test, $p < 0.01$). Collectively, Together, these observations define the importance of SCFAs, which renders offspring resistant to social and cognitive impairments.

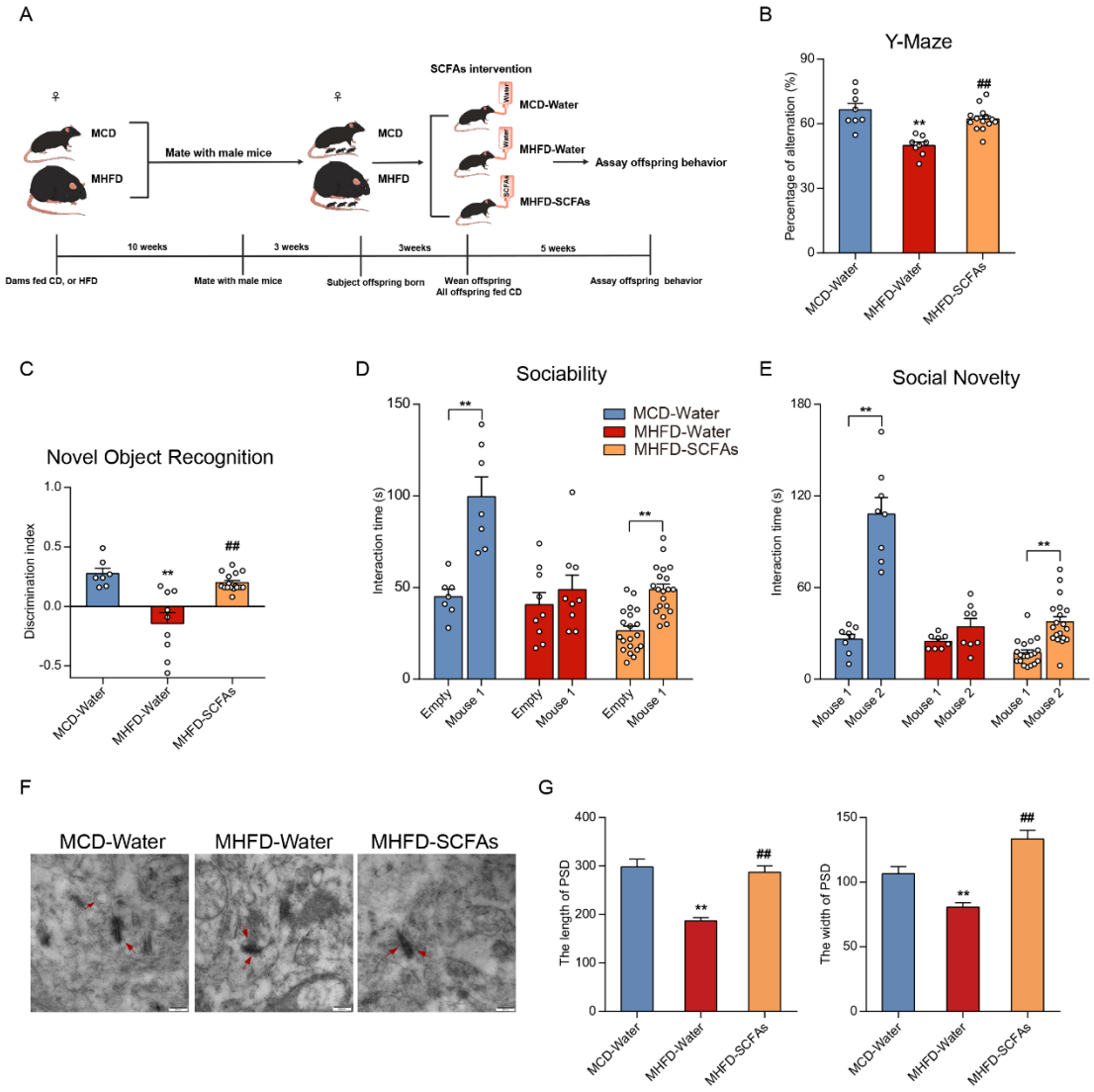

Figure 6 SCFAs Supplementation Restores Maternal Obesity-Induced Cognitive and Social Behavioral Deficits in Offspring

(A) Schematic of treatment with a mixture of acetate and propionate.

(B-E) Behavioral phenotypes in the offspring after treatment with a mixture of acetate and propionate, including (B) Y-maze, (C) the novel object recognition test, (D) sociability, and (E) preference for social novelty.

(F) Representative images of ultrastructure of synapse.

(G)The length (left) and width of PSD (right).

Data of (B) - (E) and (G) presented as mean ± SEM. Statistical analyses were performed

by one-way ANOVA with Tukey's multiple comparison test (for B, C and G) or by paired two-tailed Student's t test (for D and E). For Tukey's multiple comparison test, $^{*}p < 0.05$, $^{**}p < 0.01$, compared with the MCD-CD group, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, compared with the MHFD-CD group. For Student's t test, $^{*}p < 0.05$, $^{**}p < 0.01$. See also Figure S7.

DISCUSSION

In this study, we demonstrated that the cognitive and social dysfunction associated with maternal obesity is induced by alterations in the offspring intestinal microbiota, and maternal or offspring directly dietary fiber supplement could reverse behavior dysfunction by regulating the bacterial composition and their metabolites SCFAs.

Increasing evidence implicates maternal obesity as a major determinant of offspring health during childhood and later adult life, particularly in terms of neurodevelopmental disorders. While most of the focus in the field has been on inflammation (Bolton and Bilbo, 2014), or epigenetic processes in the brain (Mathers and McKay, 2009), the biological mechanism which links maternal obesity with neurocognitive outcomes in the offspring remains to be determined. Additionally, there is a paucity of intervention studies focused on moderation of the effects of maternal obesity on offspring. This study found that maternal obesity induced work memory and long-term memory impairments in offspring at adult (8–10 weeks old) and middle age (7 months old). The effect was consistent with a previous report that spatial memory performance in the Barnes maze was decreased in 10 weeks old male offspring born to dams exposed to a high fat diet (32% fat) from pre-conception to lactation period. Previous studies assessing the effect of maternal obesity on offspring learning and memory have largely focused on the acquisition and retention of spatial memory. Furthermore, we founded that treatment of high fat diet-fed mothers

with dietary fiber could repair cognition impairment in adult and older male offspring, which to the best of our knowledge, has not been reported before. Besides, both adult and older female offspring from obese dams also showed cognition impairment. For female offspring, maternal dietary fiber supplement improved work and long-term memory impairments in adulthood, but long-term memory impairment only in middle age (data not shown); future work will address the mechanical differences between long-term and work memory impairment in mice that were induced by maternal obesity.

Consistent with a previous report (Buffington et al., 2016b), adult male offspring born to obese mothers showed sociability and preference for social novelty deficits, and the same goes for female mice. However, both male and female mice from any MHFD-CD group showed normal social behavior in middle age, which may be due to more than one mouse living in one cage, unlike humans, could choose their own independent living space. Future studies comparing the effects of single cage or not will help resolve this question. That is different from ASD patients who could choose to stay with others or not. Of note, maternal dietary fiber intake could also restore the social behavior as well as preference for social novelty in both adult male and female offspring.

Consistent with maternal dietary fiber intake, direct dietary fiber treatment to offspring from obese mothers could also normalize the cognitive and social behavior. The transcriptional program in AD brains is distinct from that of control brains (Dube et al., 2019). RNA-seq analysis of the offspring's brains showed

several disease-related pathways were regulated. Notably, for the offspring born to obese mothers, a KEGG pathway related to the spliceosome was significantly upregulated in offspring fed control diet, compared with the offspring fed dietary fiber. Recent studies have highlighted the importance of aberrant alternative splicing of mRNA in the brains of subjects with an AD (Apicco et al., 2019) or ASD diagnosis (Gandal et al., 2018). These results suggested that the dietary fiber contribute to splicing regulation in offspring brain.

The structures of the bacterial communities differed markedly between the offspring from any diet group. Notably, a great many OUTs belonging to S24_7 significantly correlated with cognitive and social behaviors in mice. While S24_7 family had not been cultured until recently and the family classification is still ambiguous, a recent study showed that shotgun metagenomics allowed reconstruction of 59 molecular species about S24_7 family, and the S24-7 spp. were versatile with respect to complex carbohydrate degradation (Lagkouvardos et al., 2019). A recent study found significant increases in abundance of unclassified member of S24-7 in APP/PS1 mice, a mouse model of AD. Besides S24-7, directly dietary fiber treatment to offspring from obese mothers lowed the relative abundance of *Ruminococcus* and improved the *Bacteroides*. The effect on the microbiota composition of offspring directly dietary fiber administration was not fully equivalent to the maternal dietary fiber supplement, it suggests that the introduction of dietary fiber to offspring was

insufficient to regain 'lost' taxa from birth, which was consistent with previous reports (Sonnenburg et al., 2016). However, offspring directly dietary fiber administration could substitutability increase some other beneficial bacteria and decrease some harmful positively regulating behavioral outcomes. These results indicated that a combination of dietary fiber and probiotics could show better effects on restoring microbiota. These data also raised the possibility that directly dietary fiber application could contribute to the improvement of cognitive and social behaviors through the regulation of the abundance of specific bacterial species.

Indeed, offspring from dams that received fecal microbiota from MHFD, but not MCD or MFFD female mice, showed impaired memory and social behavior. Interestingly, offspring from MHFD fecal microbiota-transplanted mothers, but reared by MFFD fecal microbiota-transplanted mothers, significantly improved cognition, as well as sociability. By contrast, offspring from MFFD fecal microbiota-transplanted mothers, but reared by MHFD fecal microbiota-transplanted mothers, exhibited deficient cognitive and social behavior. Consistent with this notion, we found that MHFD offspring co-housed with MFFD offspring exhibited normal cognitive and social behavior. These findings demonstrate that a dysbiotic gut microbiota could promote defective cognitive and social behavior in mice, but do not conclude that gut bacteria are entirely causal for symptoms. Factors such as altered host genetics and perinatal events, coupled with an altered microbiota, may together influence the etiology

of cognition and/ or sociability by compounding risks that enhance symptom severity.

Gut bacteria can affect gene expression and host behavior through various pathways, including the production of neuroactive small molecules (Hsiao et al.). We found that the concentrations of SCFAs acetate and propionate in feces were reduced in offspring from obese mothers but can be restored by dietary fiber treatment in maternal or offspring's diet-specific manners. Acetate and propionate are key molecules that modulate microglia maturation, morphology and function, so they could affect CNS function. Previous studies indicated that neurodevelopmental abnormality in ASD patients accompanied with impaired acetate and propionic acid metabolism (Liu et al., 2019; Wang et al., 2020). Interestingly, the above SCFAs in children with autism significantly elevated after probiotics + Fructo-oligosaccharide intervention. Indeed, administration of the mixture of acetate and propionate to MHFD mice attenuated their cognitive and social impairments. On the basis of these observations, we reason that microbiota–derived SCFAs, particularly acetate and propionate, play a vital role in preventing the development of neurodevelopmental disorders in offspring. While the precise mechanism by which acetate and propionate rescues social and cognitive behavior and related neural adaptations in the brain remain to be determined, we favor the idea that epigenetic modifications in brain could be the key causal mechanism linking the SCFAs and behavioral outcomes in offspring. Histone deacetylases (HDACs) are involved in brain development

and a range of neuropsychiatric diseases, including depression, schizophrenia, Alzheimer disease and addiction (Volmar and Wahlestedt, 2015). Furthermore, preclinical studies in rodents suggest that HDAC inhibitors act as cognitive enhancers in fear, anxiety and trauma-related processes (Whittle and Singewald, 2014). It is known that intracellular acetate and propionate could inhibit the activity of HDACs, promoting hyperacetylation of brain histones (Soliman and Rosenberger, 2011; Waldecker et al., 2008). Moreover, previous reports show that histones modifications have also been shown to tether spliceosome components and impact splicing (Lipscombe and Lopez Soto, 2019). Specifically, Histone modification alters alternative splicing of the neurexin 1 gene (Nrxn1) specifically in memory-activated neurons in the dentate gyrus of the hippocampus (Ding et al., 2017).

In human studies, emerging evidence suggests that maternal obesity could be associated with an increased risk of ASD (Krakowiak et al., 2012) and poorer cognition in offspring, including poorer motor, spatial, and verbal skills (Godfrey et al., 2017). This risk is further exacerbated in the offspring of obese women who gain excess weight during pregnancy (Li et al., 2016). Moreover, insights from experimental studies support a role for maternal obesity on offspring neurodevelopmental outcomes across the life-course. As it's known, confirmation of causation and identification of mechanisms linking maternal obesity with offspring neurodevelopment is difficult. Accordingly, our results provide new insight into the mechanism by which a marked shift in microbial

ecology, caused by maternal high fat diet, can negatively impact both cognitive and social behaviors in offspring. These results may assist our deeper understanding the causation and underlying mechanisms linking maternal obesity with offspring neurodevelopment in human beings. Furthermore, we found that dietary fiber supplementation to obesity dams before and during pregnancy, or to 3-week old offspring from obesity mothers could both restore abnormal neurocognitive and social outcomes in offspring, which suggest that we have the opportunity to rectify some behavioral abnormalities of children during prenatal brain development, as well as postnatally. Overall, this finding opens new research avenues into preemptive therapies for neurodevelopmental disorders by targeting the maternal and/ or offspring gut microbiota. Our study also provides evidence that dietary fiber may be useful as a potential non-invasive, timely and tractable treatments for patients suffering from neurodevelopmental disorders.

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AUTHOR CONTRIBUTIONS

X.L. (Xiaoning Liu), X.L(Xiang Li), B.X., X.J., Z.Z., S.Y. (Shikai Yan), L.L., S.Y. (Shufen Yuan), S.Z., X.D., M.H., Z.L., and X.L. (Xuebo Liu) performed the experiments and analyzed the data; X.L. (Xiaoning Liu), F.Y., E.C., R.L., B.Z., M.H., Z.L., and X.L. (Xuebo Liu) wrote the manuscript; X.L. (Xiaoning Liu), X.L(Xiang Li), X.J., Z.Z., S.Y. (Shikai Yan), M.H., Z.L., and X.L. (Xuebo Liu) prepared the figures. X.L. (Xiaoning Liu), H.M., Z.L., and X.L. (Xuebo Liu) supervised the project. All authors read and approved the final manuscript.

COMPETING INTERESTS

The authors declare no competing interests.