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Maternal sleep deprivation induces gut microbial dysbiosis and neuroinflammation in offspring rats

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ABSTRACT

Maternal sleep deprivation (MSD) is a global public health problem that affects the physical and mental development of pregnant women and their newborns. The latest research suggests that sleep deprivation (SD) disrupts the gut microbiota, leading to neuroinflammation and psychological disturbances. However, it is unclear whether MSD affects the establishment of gut microbiota and neuroinflammation in the newborns. In the present study, MSD was performed on pregnant Sprague-Dawley rats in the third trimester of pregnancy (gestational days 15–21), after which intestinal contents and brain tissues were collected from offspring at different postnatal days (P1, P7, P14, and P56). Based on microbial profiling, microbial diversity and richness increased in pregnant rats subjected to MSD, as reflected by the significant increase in the phylum *Firmicutes*. In addition, microbial dysbiosis marked by abundant *Firmicutes* bacteria was observed in the MSD offspring. Furthermore, quantitative real-time polymerase chain reaction (qRT-PCR) and enzyme-linked immunosorbent assay (ELISA) showed that the expression levels of proinflammatory cytokines interleukin 1β (IL-1β) and tumor necrosis factor α (TNF-α) were significantly higher in the MSD offspring at adulthood (P56) than in the control group. Through Spearman correlation analysis, IL-1β and TNF-α were also shown to be positively correlated with *Ruminococcus_1* and *Ruminococcaceae_UCG-005* at P56, which may determine the microbiota-host interactions in MSD-related neuroinflammation. Collectively, these results indicate that MSD changes maternal gut microbiota and affects the establishment of neonatal gut microbiota, leading to neuroinflammation in MSD offspring. Therefore, understanding the role of gut microbiota during physiological development may provide potential interventions for cognitive dysfunction in MSD-impacted offspring.

Keywords: Maternal sleep deprivation; Gut microbiota; Neuroinflammation; Gut-brain axis; Cognitive function; *Firmicutes*

INTRODUCTION

Sufficient time and quality of sleep are basic requirements for maintaining a healthy body. Maternal sleep deprivation (MSD) in pregnant women caused by hormonal changes and mental stress has become a critical public health issue (Mindell et al., 2015). According to epidemiological surveys in recent decades, many women live with severe sleep disruption, sleep loss, and sleep disorder symptoms during pregnancy, particularly in late gestation (Du et al., 2021; Mindell et al., 2015; Pien & Schwab, 2004). Studies have shown that MSD can have detrimental consequences in pregnant women and newborns, such as increased maternal risk of gestational hypertension, gestational diabetes, and postpartum depression, as well as increased neonatal risk of premature or overdue delivery and dystocia (Pires et al., 2021).

Daily acclimation handling is often used to induce brief sleep deprivation (SD) in rodents to mimic the status of sleep loss in humans (Vecsey et al., 2013), with such studies revealing the detrimental effects of MSD on offspring development. For instance, MSD can induce long-lasting metabolic perturbations (Harskamp-van Ginkel et al., 2020; Khalyfa et al., 2014), reduce reproductive capability (Alvarenga et al., 2013), increase cardiovascular disease risk (Argeri et al., 2016; Lima et al., 2014; Thomal et al., 2010), and increase aberrant immune responses (Baratta et al., 2020) in offspring. In addition, MSD can also cause neurophysiological disturbances in offspring, such as altered sleep-wake patterns (Aswathy et al., 2018), impaired hippocampal neurogenesis (Zhao et al., 2014), increased neuroinflammation (Zhao et al., 2015), and hostile neuropsychological behaviors (Radhakrishnan et al., 2015). Consistently, our recent studies revealed that MSD induces impairment of synaptic plasticity and cognition in offspring rats by facilitating endocytosis of postsynaptic GluA2-containing α-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid receptors (AMPAR) (Peng et al., 2016; Yu et al., 2018).

A growing body of evidence has shown that the gut microbiota plays an indispensable physiological role in the pathogenesis of various human metabolic, immunological, and neurological diseases, suggesting that gut microbiota homeostasis largely determines host health (Li et al., 2016). Importantly, the gut microbiota plays a regulating role in the central nervous system (CNS) via the gut-brain axis, which forms a bidirectional information network between the gut microbiota and brain (Wang & Wang, 2016). Therefore, the gut microbiota is critical for psychological activities such as cognition, sociability, anxiety-like behaviors, and normal stress response, and maintains CNS homeostasis by regulating immunological function and blood-brain barrier integrity (Luczynski et al., 2016). Furthermore, circadian rhythm is intimately correlated with the gut microbiota, with SD impacting the gut microbiota in various ways (Matenchuk et al., 2020). For example, chronic SD (Poroyko et al., 2016) or brief acute SD (El Aidy et al., 2020) can lead to microbial dysbiosis, thereby altering metabolic patterns associated with cognitive capability. A recent study reported that an absence of gut microbiota can suppress the inflammatory response and cognitive impairment induced by SD in germ-free mice (Wang et al., 2021), suggesting a critical role of gut microbiota in sleep regulation and brain function.

Disturbances during either prenatal or postnatal periods may impair the vertical transmission of microbiota and cause microbial dysbiosis in newborns, leading to aberrant behavioral changes and impaired cognitive development (Tochitani, 2021). However, the colonization and development of gut microbiota as well as the mechanism of gut microbiota colonization in neurodevelopment in early life remain unclear. Accordingly, we hypothesized that alterations in gut microbiota from mothers may be augmented in newborns via vertical transmission, leading to neuropsychological impairment. Studying the colonization and development of gut microbiota from sleep-deprived mothers to offspring will improve our understanding of early gut microbiota development and the reasons for impaired neonatal neurodevelopment.

MATERIALS AND METHODS

Animals

Female and male Sprague-Dawley rats were obtained from the Chongqing Medical University Animal Care Centre (Chongqing, China) and mated in the laboratory colony of the Children's Hospital of Chongqing Medical University. Pregnant rats were housed individually in independent ventilated cages under a controlled temperature (23–25 °C) and 12/12 h light/dark cycle (0730h–1930h), with unlimited access to food and water. On day 9 of gestation, all animals were acclimated by gentle individual handling (5 min/cage/day) for 6 days (Vecsey et al., 2013). On day 15 of gestation, the animals were paired by weight and randomly divided into two groups, including 10 pregnant rats in the MSD group and eight pregnant rats in the normal rearing control group (C). All animal experiments were conducted in accordance with the Chongqing Science and Technology Commission guidelines and approved by the Chongqing Medical University Animal Care Committee (Approval No. CHCMU-IACUC202101 14017). Every effort was made to minimize both animal suffering and number of animals used.

MSD and sample collection

MSD was performed in the third trimester of pregnancy (gestational days 15–21) by gentle handling for 6 h per day (1200h–1800h) as described previously (Peng et al., 2016; Radhakrishnan et al., 2015; Vecsey et al., 2009; Yu et al., 2018). Briefly, pregnant rats were kept awake by gently tapping on the outer surface of the cage, lightly rattling the wire cage lid, removing the cage cover to gently pat the rats as they moved freely in the cage, or rummaging through the bed of wood shavings. Maternal fecal samples (C-M: n=10; MSD-M: n=8) were collected before delivery on day 21 of gestation. The pups were separately sacrificed on postnatal day 1 (C-P1: n=11; MSD-P1: n=8), day 7 (C-P7: n=11; MSD-P7: n=8), day 14 (C-P14: n=11; MSD-P14: n=8), and day 56 (C-P56: n=11; MSD-P56: n=8). Cecum and colon contents as well as brain tissues were collected on a sterile clean bench, immediately frozen with liquid nitrogen, and stored at −80 °C for subsequent analysis (Figure 1).
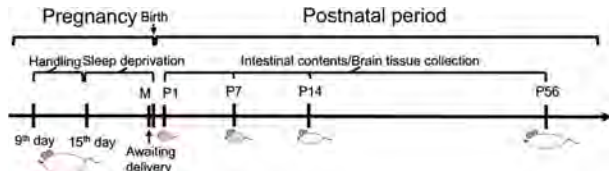
Figure 1 Experimental design

Pregnant rats were subjected to MSD for 6 h per day by acclimation to gentle handling in third trimester of pregnancy (gestational days 15–21). Fecal samples of mother rats (M) and intestinal contents and brain tissues of offspring were collected at different postnatal days (P1, P7, P14, and P56).

Microbiota profiling

DNA from collected samples was extracted according to the manufacturer's instructions using the EZNA® Stool DNA Kit (Omega, USA). Total DNA was eluted in 50 µL of elution buffer and stored at −80 °C until polymerase chain reaction (PCR) using primers Bakt_341F (CCTACGGGNGGCW GCAG) and Bakt_805R (GACTACHVGGGTATCTAATCC) (Herlemann et al., 2011) obtained from LC-Bio Technology Co., Ltd (China). The PCR products were purified by AMPure XT beads (Beckman Coulter Genomics, USA) and quantified by Qubit (Invitrogen, USA). The amplicon pools were prepared for sequencing, and the size and quantity of the amplicon library were measured using an Agilent 2 100 Bioanalyzer (Agilent, USA) and a Library Quantification Kit for Illumina (Kapa Biosciences, USA), respectively. The NovaSeq PE250 platform was used for high-throughput sequencing of the V3–V4 hypervariable region of the prokaryotic 16S rRNA gene.

Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from the offspring brain tissue samples collected at P1, P7, and P56 (n=6 per group) to determine the expression of the microglia-originated proinflammatory factors interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α) using a Bioteke RNA Extraction Kit (Bioteke, Beijing, China) following the manufacturer's protocols. RNA was subjected to reverse transcription using a Takara PrimeScript™ RT Reagent Kit (Takara Bio, China). qRT-PCR was performed for 40 cycles (95 °C for 2 min, 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 20 s) using TB Green Premix Ex Taq™ Ⅱ (Takara Bio, China) and Bio-Rad CFX Connect™ Real-Time System (Bio-Rad, USA). Primer sequences from the reference research included: IL-1β (forward: TGAGGCTGACAGACCCCA AAAGAT, reverse: GCTCCACGGGCAAGACATAGGTAG); IL-6 (forward: AGCCACTGCCTTCCCTACTTCA, reverse: GCCATTGCACAACTCTTTTCTCA); TNF-α (forward: TGGCG TGTTCATCCGTTCTCTACC, reverse: CCCGCAATCCAGG CCACTACTT); and β-actin (forward: GTCCACCCGCGA GTACAACCTTCT, reverse: TCCTTCTGACCCATACCC ACCATC) (Roque et al., 2016). The data were normalized and analyzed using the $2^{-\Delta\Delta Ct}$ method. Each sample was run in triplicate.

Enzyme-linked immunosorbent assay (ELISA)

Offspring brain tissues were collected at P1, P7, and P56 (n=5 per group). Residual blood was washed away, and the tissue was then homogenized in 1×PBS (100 mg tissue/1 mL PBS). After performing two freeze-thaw cycles to break the cell membranes, the homogenates were centrifuged at 5 000 g for 5 min at 4 °C. The supernatant was collected and the concentrations of IL-6, IL-1β, and TNF-α were measured using an ELISA Kit (Cusabio, Wuhan, China) according to the manufacturer's instructions.

Bioinformatics and statistical analysis

The Illumina NovaSeq platform (provided by LC-Bio Technology, China) was used for sequencing following the manufacturer's guidelines. Briefly, the resulting paired-end reads were assigned to samples based on their unique barcodes, truncated by cutting the barcodes and primer sequences, and then merged using FLASH software. High-quality clean tags were then obtained by filtering raw reads under specific conditions using fqtrim (v0.9.4), and chimeric sequences were identified and deleted using Vsearch (v2.3.4). After dereplication using DADA2, we obtained the feature table and feature sequence. Sequence alignment of species annotation was performed by BLAST with the SILVA and NT-16S databases. Alpha and beta diversities were calculated and analyzed using QIIME2, and alpha diversity indices were reduced by relevant integral multiples to present them in the same graph (Observed OTU and Chao1 indices were divided by 1 000, Shannon index was divided by 10, Simpson index was left unprocessed). Relative abundances other than the Wilcoxon test (only phylum and genus with relative abundances greater than 0.1% were included, P<0.05 for significance) were used to describe bacterial taxonomy, with the top 20 bacterial genera shown in the graphs in descending order. ELISA results were analyzed by one-way analysis of variance (ANOVA) with treatment (group) as the between-subjects factor and presented as mean±standard deviation (SD). Spearman correlation analysis was performed using IBM SPSS Statistics (v23.0), with the top 30 bacterial genera in descending order. Graphs were drawn by R package (v3.5.2), Prism (v9.2.0), and OmicStudio tools (https://www.omicstudio. cn/tool).

RESULTS

MSD increases diversity and richness of gut microbiota in mother rats

To verify whether MSD in the third trimester altered maternal gut microbiota, we collected maternal fecal samples before delivery on day 21 of gestation. The alpha diversity indices (Observed OTUs, Shannon, Simpson, and Chao1) were determined to compare microbial richness and diversity. Weighted UniFrac principal coordinate analysis (PCoA) of beta diversity was calculated to evaluate microbial community similarity. Compared with the controls (C-M), richness and diversity of gut microbiota increased significantly in the MSD mother rats (MSD-M) (Figure 2A), although microbial composition remained unchanged (Figure 2B). Further analysis revealed that *Firmicutes* abundance increased in the MSD-M group, while *Proteobacteria* abundance decreased at the phylum level (Figure 2C). Abundance of genera *Ruminococcaceae_UCG-005*, *Ruminococcus_1*, *Lachnospira*
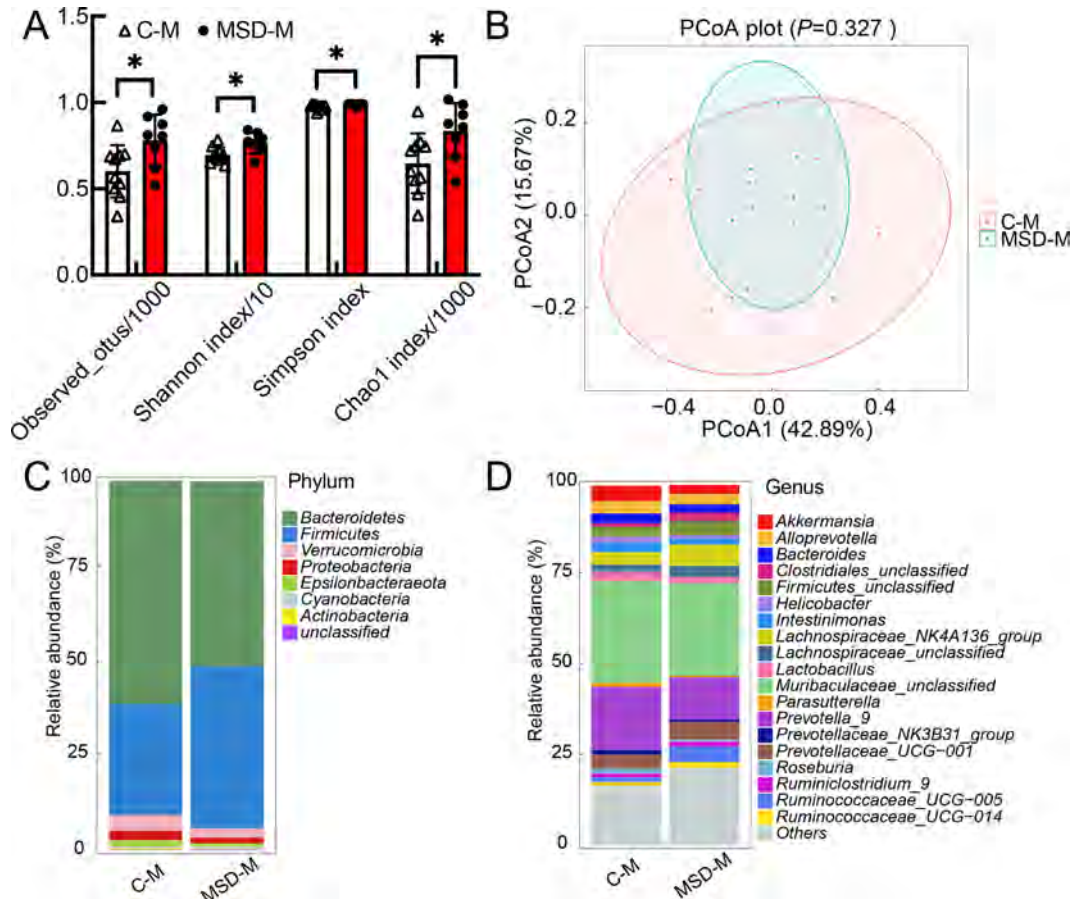
Figure 2 Microbial richness and diversity increased in sleep-deprived pregnant rats

A: Alpha diversity indices increased in both richness and diversity of gut microbiota in MSD group (MSD-M). B: Weighted UniFrac PCoA showed no significant difference in microbial composition between MSD-M and control (C-M) groups. C, D: Relative abundance of top 20 microbial phyla (C) and genera (D) were analyzed to show subtle differences between MSD-M and C-M groups. All OTUs under 1% were classified as "others". Data are expressed as means±*SD*, *: *P*<0.05.

ceae_UCG-001,*Robinsoniella*,*Ruminococcaceae_unclassified*, *Christensenellaceae_R-7_group* *Lachnospiraceae_UCG-008*, *Ruminiclostridium_6*, *Acetivibrio*, and *Clostridiales_Family_XIV_Incertae_Sedis_unclassified* increased, while abundance of *Escherichia-Shigella* decreased in the MSD-M group (Figure 2D). These results suggest that MSD in the third trimester increases the diversity and richness of gut microbiota in mother rats, as emphasized by the abundance of bacterial phylum *Firmicutes*.

First two weeks of early life are critical for development of gut microbiota

As maternal resident bacteria are a primary source for gut microbiota colonization in offspring during perinatal periods (Francis & Dominguez-Bello, 2019), we next explored the diversity and composition of gut microbiota in MSD offspring. Results showed that microbial diversity and richness steadily increased with bacterial community change in both control (Figure 3A, B) and MSD offspring (Figure 3C, D) during microbial colonization and development. Further analysis of relative abundance of gut microbiota revealed bacterial succession from dam to pup to adult offspring (Figure 3E, F). At the phylum level, *Proteobacteria* gradually decreased to the

maternal level from P1 to P56 in both control and MSD offspring (Figure 3E, G). *Firmicutes* gradually increased in control offspring (Figure 3E), but not in MSD offspring (Figure 3G). At the genus level, *Burkholderia-Caballeronia-Paraburkholderia* gradually decreased in the control offspring (Figure 3F), whereas MSD offspring showed a sudden decline at P7 (MSD-P7) and continued low abundance (MSD-P14 and MSD-P56) (Figure 3H). *Lactobacillus* first increased and then declined in both groups, but with high abundance at P7 (MSD-P7) in MSD offspring (Figure 3F) and P14 (C-P14) in control offspring (Figure 3H).

We next explored microbial changes in offspring subjected to MSD at each time point. Results showed that the gut microbiota was already complex at P1 in both the control (C-P1) and MSD (MSD-P1) pups (Figure 4A). Even though no significant difference was found between the microbial communities (Figure4B), there were still hundreds of different bacterial species and high abundance of the phylum *Proteobacteria* (Figure 4C) and genus *Burkholderia-Caballeronia-Paraburkholderia* (Figure 4D). Further analysis revealed that no significant differences were observed at the phylum level (Figure 4C), and only the abundance of the
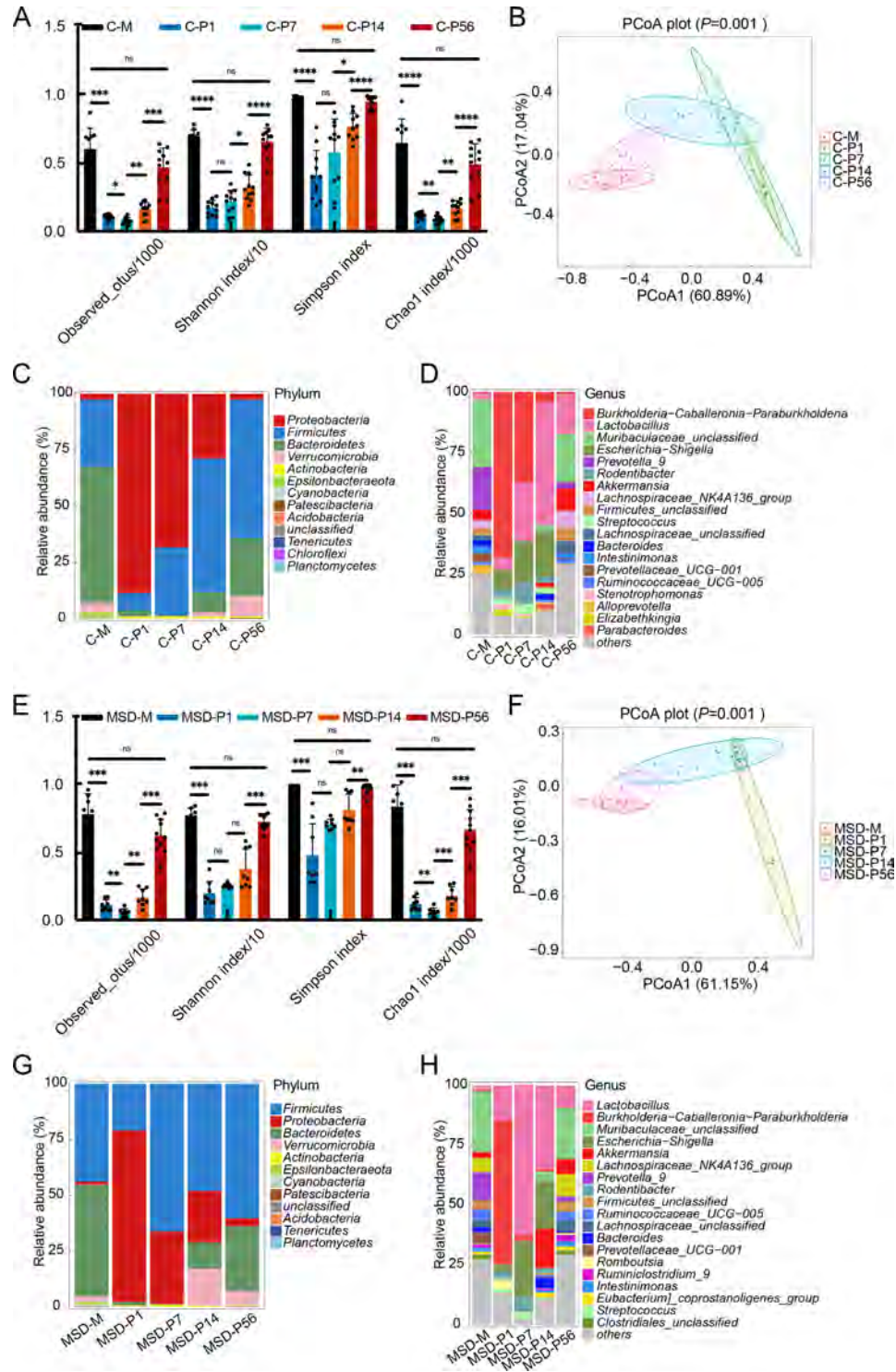
Figure 3 Microbial diversity and richness increased in both control and MSD offspring

A: Alpha diversity indices steadily increased in diversity and richness of gut microbiota in control offspring. B: Weighted UniFrac PCoA analysis showed significant differences in microbial composition at different postnatal time points in control offspring. C, D: Relative abundances of top 20 microbial phyla (C) and genera (D) were clustered to show changes from mothers to offspring in control group. E: Alpha diversity indices showed a steady increase in diversity and richness of gut microbiota in MSD offspring. F: Weighted UniFrac PCoA showed significant differences in microbial composition at different postnatal time points in MSD offspring. G, H: Relative abundances of top 20 microbial phyla (G) and genera (H) were clustered to show changes from mother rats to offspring in MSD group. Data are expressed as means±*SD*, *: *P*<0.05; **: *P*<0.01; ***: *P*<0.001; ****: *P*<0.0001.
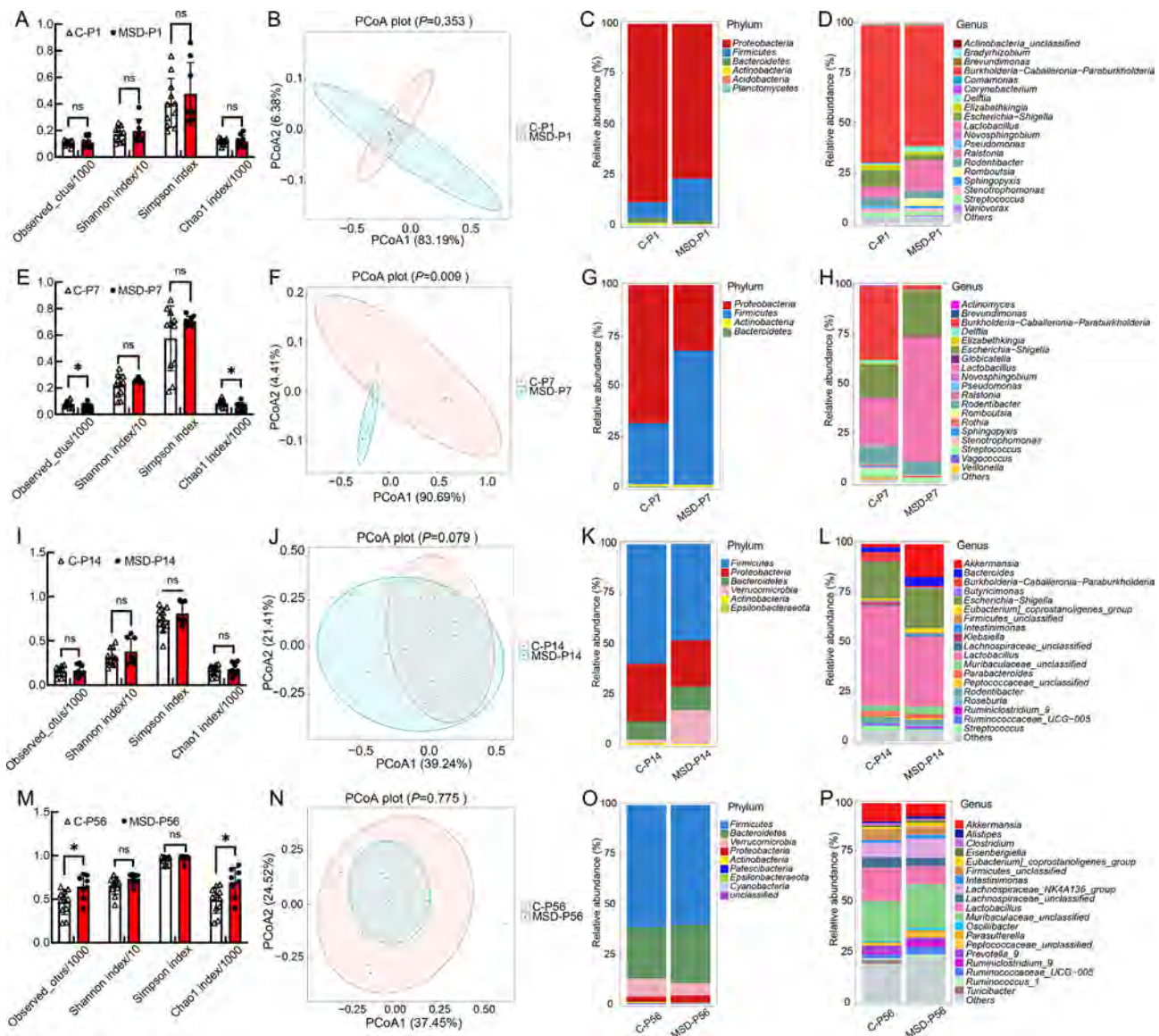
Figure 4 Changes in gut microbiota of MSD offspring

Alpha diversity indices showed changes in richness and diversity of microbiota at P1 (A), P7 (E), P14 (I), and P56 (M) in both control and MSD offspring. Weighted UniFrac PCoA showed microbial composition at P1 (B), P7 (F), P14 (J), and P56 (N) in both control and MSD offspring. Relative abundances of top 20 microbial phyla (C, G, K, O) and genera (D, H, L, P) were clustered to show differences between control and MSD offspring. Data are expressed as means±*SD*, *: *P*<0.05.

genus *Romboutsia* increased in the MSD-P1 group compared with the C-P1 group (Figure 4D). Notably, marked differences in the microbial community at P7 were observed between groups, with lower richness (Figure 4E) and largely different microbial composition (Figure 4F) in the MSD-P7 group compared to the C-P7 group. At the phylum level, *Proteobacteria* abundance decreased substantially, whereas *Firmicutes* abundance increased substantially in the MSD-P7 group (Figure 4G). At the genus level, *Lactobacillus* abundance increased, whereas *Burkholderia-Caballeronia-Paraburkholderia*, *Sphingopyxis*, *Stenotrophomonas*, *Ralstonia*, *Novosphingobium*, *Pseudomonas*, *Brevundimonas*, and *Delftia* abundance decreased in the MSD-P7 group (Figure 4H). However, there were no significant differences in

microbial richness, diversity, or composition at P14 between the control (C-P14) and MSD (MSD-P14) groups (Figure 4I, J), although the abundances of the phylum *Verrucomicrobia* (Figure 4K) and genera *Akkermansia* and *Marvinbryantia* increased, while those of *Stenotrophomonas* and *Veillonella* decreased in the MSD-P14 group (Figure 4L). At P56, there was no difference in microbial community between the control (C-P56) and MSD (MSD-P56) groups, but the MSD-P56 group showed higher richness (Figure 4M, N). Further analysis revealed no significant differences at the phylum level between groups, with only slight increases in the *Ruminococcaceae_unclassified*, *Fournierella*, *Acetatifactor*, *Oscillibacter*, *Ruminococcaceae_UCG-009*, *Odoribacter*, and *UBA1819* genera in the MSD-P56 group (Figure 4O, P).

Taken together, these results suggest that gut microbiota colonization occurs during the first 2 weeks of early life, and the microbial community in young adult offspring is similar to that in mother rats. Although the transmission and development of gut microbiota from mothers to offspring were similar in the control and MSD offspring, subtle differences were also found, especially in the highly abundant *Firmicutes*, which may be related to vertical transmission of microbial dysbiosis from mother to offspring.

Bacterial phylum *Firmicutes* is dominant in MSD

Linear discriminant analysis (LDA) effect size (LEfSe) was used to identify the differentially enriched bacterial taxa associated with MSD, which could provide information about the transmission and development of gut microbiota in offspring. Different bacteria were defined by LDA scores with a cut-off of 3.5. Results showed that MSD-M rats exhibited high levels of the phylum *Firmicutes* (class *Clostridia*, order

Clostridiales, family *Ruminococcaceae*, family *Lachnospiraceae* and genus *Ruminococcus*), while C-M rats exhibited high levels of the phylum *Proteobacteria* (Figure 5A, B). At P1 and P7 (Figure 5C–E), the MSD-P1 and MSD-P7 offspring were still dominated by *Firmicutes* (class *Firmicutes_unclassified*, class *Bacilli*, order *Lactobacillales*, order *Firmicutes_unclassified*, family *Lactobacillaceae*, genus *lactobacillus*, genus *Roseburia*, and genus *Romboutsia*), while the control offspring were still dominated by *Proteobacteria* (family *Sphingomonadaceae*, family *Xanthomonadaceae*, family *Burkholderiaceae*, genus *Delftia*, genus *Stenotrophomonas*, and genus *Burkholderia_Caballeronia_ Paraburkholderia*). At P14 (Figure 5F, G), the MSD offspring contained microbial biomarkers of the phylum *Verrucomicrobia* (class *Verrucomicrobiae*, order *Verrucomicrobiales*, family *Akkermansiaceae*, and genus *Akkermansia*), while both groups were dominated by microbial biomarkers of *Actinobacteria* and *Firmicutes* at P14 and P56 (Figure 5F–H).
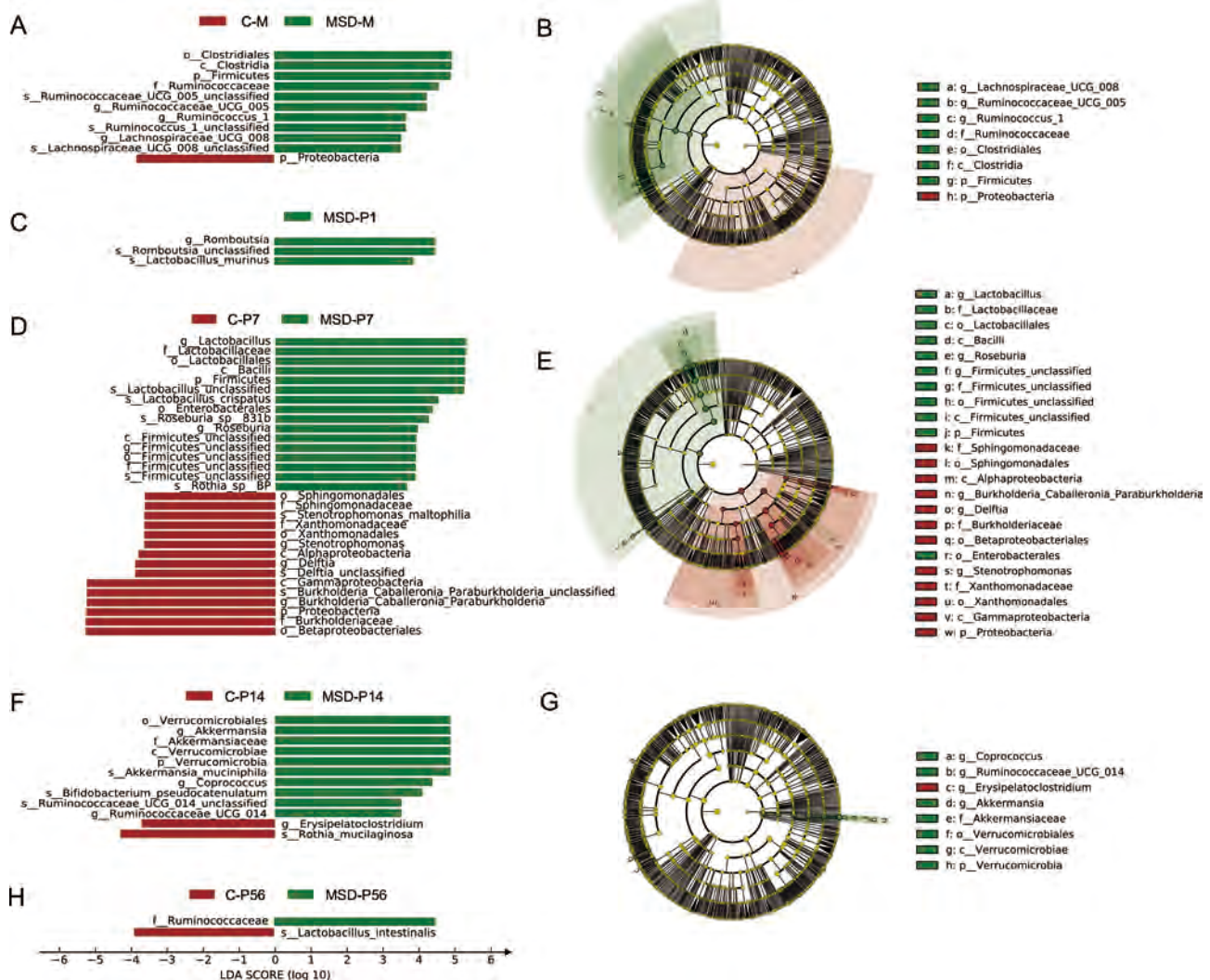
Figure 5 Dominant bacteria in MSD offspring at different postnatal time points

Linear discriminant analysis (LDA) effect size (LEfSe) showed different abundant bacterial taxa in mother rats (A) and offspring at P1 (C), P7 (D), P14 (F), and P56 (H). Different bacteria were defined by LDA scores with a cut-off of 3.5. Pie diagram summarized phylogenetic changes in mother rats (B) and their offspring at P7 (E) and P14 (G).

Thus, MSD-M rats (Figure 5A, B) and their pups, especially MSD-P7 (Figure 5D, E), were dominated by microbial biomarkers mainly found in the phylum *Firmicutes*. These results suggest that *Firmicutes* bacteria are strongly associated with MSD, and bacterial biomarkers are likely associated with physical dysfunction in the host.

MSD increases neuroinflammation in offspring

The gut microbiota exerts protective effects for the development of the host immune system (Hajela et al., 2015), and microbial dysbiosis leads to fluctuations in inflammatory cytokines in the brain (Chassaing & Gewirtz, 2014; Li et al., 2020). Thus, we performed qRT-PCR to assess the expression of proinflammatory cytokines IL-6, IL-1β, and TNF-α at P1, P7, and P56. Compared with the control offspring, the expression of IL-1β increased at P56 (*P*=0.020), but not at P1 (*P*=0.100) or P7 (*P*=0.087) (Figure 6A). No differences were observed in the expression of IL-6 between the two groups at the different time points (P1, *P*=0.064; P7, *P*=0.845; P56, *P*=0.699; Figure 6B). Furthermore, compared with the control offspring, the expression of TNF-α increased at P1 (*P*=0.038) and P56 (*P*=0.049), but decreased at P7 (*P*=0.023) (Figure 6C). To further confirm the qRT-PCR and ELISA results, we measured the proinflammatory cytokines in the brains of MSD offspring. Results showed that, compared with control offspring, the concentration of IL-1β increased in the MSD offspring at P1 (MSD-P1; *P*=0.006) and at P56 (MSD-P56; *P*=0.012), but not at P7 (MSD-P7; *P*=0.093) (Figure 6D). Furthermore, the concentration of IL-6 decreased in the MSD offspring at P7 (MSD-P7; *P*=0.003), but not at P1 (MSD-P1; *P*=0.070) or P56 (MSD-P56; *P*=0.077) compared with the control offspring (Figure 6E). The concentration of TNF-α increased in the MSD offspring at P1 (MSD-P1; *P*=0.026) and at P56 (MSD-P56; *P*=0.016), but not at P7 (MSD-P7; *P*=0.117) compared with control offspring (Figure 6F). Overall, the concentration of proinflammatory cytokines increased in the brains of young adult MSD offspring, although no significance was found at several time points, suggesting that neuroinflammation is activated in MSD offspring.

To further explore the relationship between gut microbiota and neuroinflammation, Spearman correlation analysis was performed to determine the correlations between the top 30 bacterial genera and concentrations of IL-1β, IL-6, and TNF-α at each time point (Figure 6G, I). Results showed that IL-1β was positively correlated with bacterial genera in the phylum *Firmicutes* at P1 (genus *Romboutsia*) and P56 (genera *Ruminococcaceae_UCG_005* and *Ruminococcus_1*), but negatively correlated at P7 (genus *Globicatella*). TNF-α was positively correlated with the genera *Ruminococcaceae_UCG-005* and *Ruminococcus_1* at P56. IL-6 was associated with the phylum *Firmicutes*, showing positive correlation with *Vagococcus* (P7) and *Turicibacter* (P56) and negative correlation with *Lactobacillus* (P7). These results suggest that activation of neuroinflammation is positively correlated with offspring abundance of bacterial genera in *Firmicutes*, which is also a biomarker associated with MSD, as shown in LEfSe analysis.

DISCUSSION

Sleep loss has become a global concern in modern society, especially during pregnancy (Hutchison et al., 2012) due to a series of complicated factors such as physiological status (Chien & Chen, 2015), psychological concerns (Jiang et al., 2021) and living conditions (Nam et al., 2018). Both pregnant women and their babies can suffer severe consequences caused by MSD (Pires et al., 2021). Evidence accumulated from recent studies suggests that MSD can cause metabolic disorders, decrease reproductive capacity, and increase cardiovascular disease risk, as well as abnormal immune response and cognitive impairment in offspring (Alvarenga et al., 2013; Argeri et al., 2016; Baratta et al., 2020; Harskamp-van Ginkel et al., 2020; Khalyfa et al., 2014; Lima et al., 2014; Peng et al., 2016; Thomal et al., 2010; Yu et al., 2018). In addition, the gut microbiota plays an important role in neuropsychological development in newborns (Tochitani, 2021), suggesting that MSD may affect the cognitive development of offspring via the gut-brain axis. In the present study, we found that both gut microbiota richness and diversity increased after MSD in mother rats (Figure 2A). These results are contradictory to findings in nonpregnant SD animals (Gao et al., 2019; Maki et al., 2020). These discrepancies between previous studies and our work need to be resolved but may be at least partially explained by pregnancy, which impacts hormonal changes and metabolic patterns (Qi et al., 2021). Indeed, pregnancy is associated with profound alterations in the gut microbiota (Crusell et al., 2018; Grech et al., 2021), particularly in the third trimester, which may lead to metabolic, immunological, and hormonal changes necessary to support a healthy pregnancy and fetal growth (Koren et al., 2012). These changes in hormone levels, such as estrogen and progesterone, may also affect the microbial community, accompanied by unique inflammatory and immunological changes (García-Gómez et al., 2013; Nuriel-Ohayon et al., 2019).

Accumulating evidence indicates that the gut microbiota provides protective effects for the development of the host immune system (Hajela et al., 2015), and microbial dysbiosis can lead to fluctuations in inflammatory cytokines in the brain (Chassaing & Gewirtz, 2014; Li et al., 2020). Studies have revealed that fecal transplantation with healthy gut microbiota can alleviate symptoms caused by stress and reduce aberrant inflammatory responses (Rao et al., 2021). Although disrupted sleep is also associated with an increase in proinflammatory biomarkers (Okun & Coussons-Read, 2007) and abundance of the phylum *Firmicutes* (Benedict et al., 2016; Poroyko et al., 2016), the relationship between MSD and gut microbiota development in offspring is unknown. We found that bacteria in the phylum *Firmicutes* (such as class *Clostridia*, order *Clostridiales*, family *Ruminococcaceae*, family *Lachnospiraceae*, and family *Lactobacillaceae*) were highly abundant in the MSD mothers and their offspring. With the increase in *Firmicutes*, the *Firmicutes* to *Bacteroidetes* (FB) ratio increased in the MSD group, especially in MSD-P7. An increase in the FB ratio is associated with abnormal lipid metabolism and higher body mass index (John & Mullin, 2016; Koliada et al., 2017). Combined with previous study on the increase in the FB ratio in young adults with insufficient sleep (Benedict et al., 2016), we speculate that MSD-induced microbial dysbiosis in offspring may lead to changes in metabolism and immune response through microbiota-host
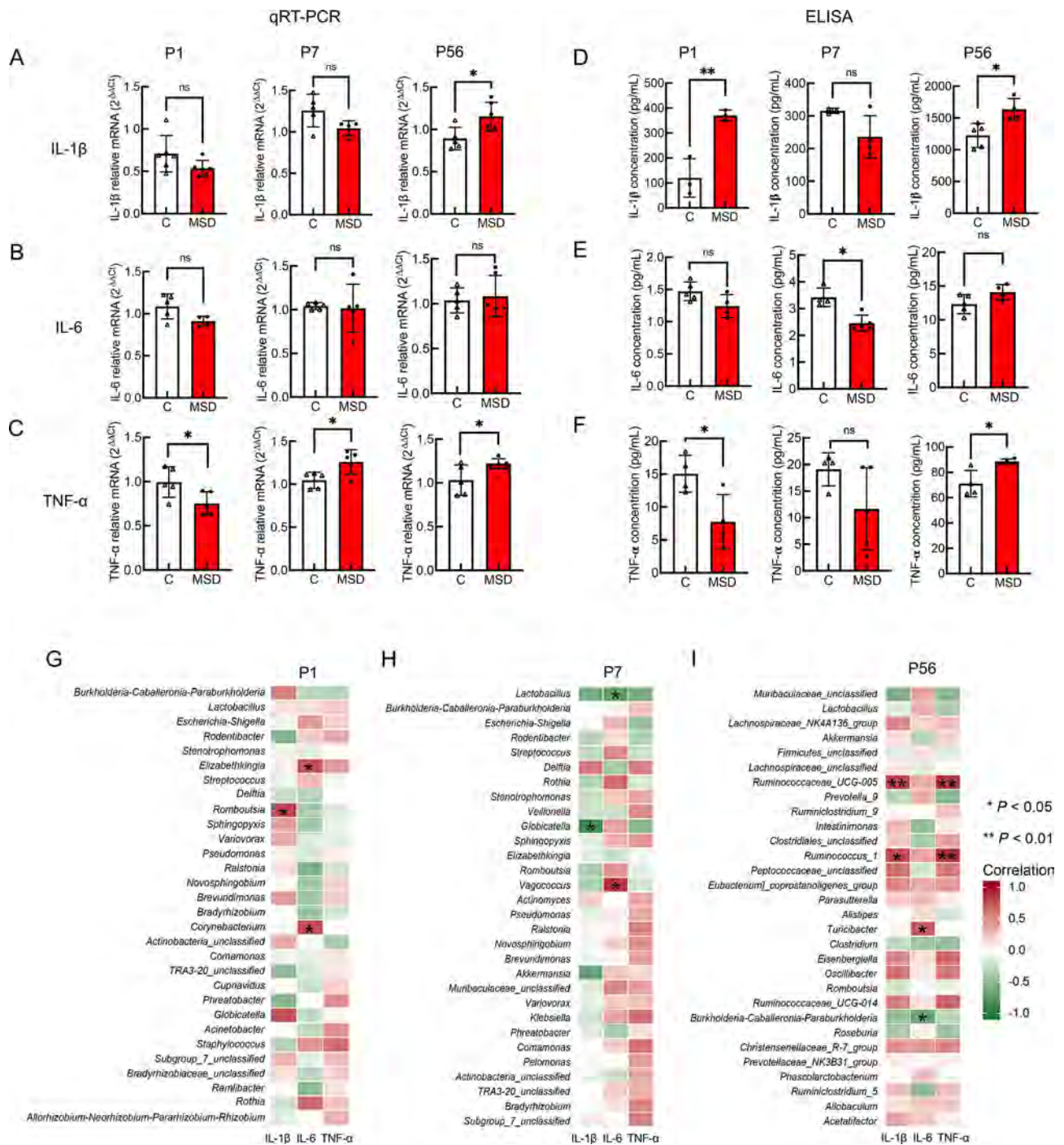
Figure 6 MSD increased proinflammatory cytokines in brains of MSD offspring

A–C: mRNA expression of proinflammatory cytokines IL-1β (A), IL-6 (B), and TNF-α (C) in brains of MSD offspring measured by qRT-PCR. D–F: Proinflammatory cytokines IL-1β (D), IL-6 (E), and TNF-α (F) in brains measured by ELISA. G–I: Spearman heat-map showed correlations among top 30 microbial genera and proinflammatory cytokines IL-1β, IL-6, and TNF-α at P1 (G), P7 (I), and P56 (H). Data are expressed as means±*SD*, *: *P*<0.05; **: *P*<0.01.

interactions, thereby affecting physiological functions. Indeed, we revealed a significantly altered microbial community in MSD-P7 offspring, which may be related to the transfer of microbial dysbiosis from MSD mothers to their offspring.

Dysregulation of microglial activation may also contribute to MSD-induced neuroinflammation and cognitive impairment in young offspring (Zhao et al., 2014, 2015). However, how MSD causes such dysregulation in offspring is not clear. In the present study, we found that microbial dysbiosis in MSD mothers may be passed to their offspring, which may disrupt interactions between the microbiota and microglia through the gut-brain axis, subsequently leading to aberrant

neuroinflammation. IL-6, IL-1β, and TNF-α are proinflammatory biomarkers of microglia, which play critical roles in regulating brain homeostasis and maturation of neural circuits. Thus, we performed qRT-PCR and ELISA to evaluate the state of neuroinflammation in MSD offspring. Consistent with previous research (Zhao et al., 2015), we found that MSD increased the expression and concentration of IL-1β and TNF-α in young adult offspring (MSD-P56). Interestingly, low concentrations of IL-6, IL-1β, and TNF-α were observed in early life (P1 and P7) of the MSD group (Figure 6D, E). One possible explanation is that the immature immune system was incapable of generating enough proinflammatory cytokines due to microbial dysbiosis in early life (MSD-P1 and MSD-P7) as gut microbiota homeostasis is very important for the development and maturation of the host immune system and immune regulation (Goyal et al., 2021; Yoo et al., 2020; Zhao & Elson, 2018). In addition, some proinflammatory cytokines may be obtained from the mother through the blood-placental barrier during the prenatal period (Fricke et al., 2018). For example, a higher concentration of IL-1β was observed although IL-1β mRNA expression remained unchanged in the MSD group at P1. In addition, Spearman correlation analysis revealed that the phylum *Firmicutes* played an important role in neuroinflammation in the MSD offspring (Figure 6G–I), indicating a close relationship between proinflammatory cytokines and gut microbiota.

In summary, MSD increased microbial richness and diversity in mother rats, with high abundance of bacteria in the phylum *Firmicutes*, and induced microbial community disturbance and neuroinflammation in offspring. Combined with recent research (Peng et al., 2016; Yu et al., 2018; Zhao et al., 2014, 2015), these findings suggest that MSD may cause cognitive disability and aberrant inflammatory response in offspring by disrupting gut microbiota development and the gut-brain axis. Although our findings provide potential interventions for the clinical treatment of cognitive dysfunction in MSD offspring, we only detected a correlation between gut microbiota and neuroinflammation. Thus, further studies, such as fecal transplantation and metabolic profiling, should be conducted to determine the underlying mechanisms of these neurophysiological and behavioral phenotypes in offspring subjected to MSD.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

Z.Y.Y. and Z.F.D. conceived the study and wrote the manuscript. Z.Y.Y. performed MSD. Z.Y.Y., X.H.L., and Q.X. collected the intestinal contents and brain tissues. Z.Y.Y. and L.Z. performed biochemical assay. Z.Y.Y., W.T.H., and D.X.L. analyzed the data. All authors read and approved the final version of the manuscript.

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