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Manganese-induced Sex-specific Gut Microbiome Perturbations in C57BL/6 Mice

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Abstract

Overexposure to manganese (Mn) leads to toxic effects, such as promoting the development of Parkinson's-like neurological disorders. The gut microbiome is deeply involved in immune development, host metabolism, and xenobiotics biotransformation, and significantly influences central nervous system (CNS) via the gut-brain axis, *i.e.* the biochemical signaling between the gastrointestinal tract and the CNS. However, it remains unclear whether Mn can affect the gut microbiome and its metabolic functions, particularly those linked to neurotoxicity. In addition, sex-specific effects of Mn have been reported, with no mechanism being identified yet. Recently, we have shown that the gut microbiome is largely different between males and females, raising the possibility that differential gut microbiome responses may contribute to sex-selective toxicity of Mn. Here, we applied high-throughput sequencing and gas chromatography–mass spectrometry (GC-MS) metabolomics to explore how Mn^{2+} exposure affects the gut microbiome and its metabolism in C57BL/6 mice. Mn^{2+} exposure perturbed the gut bacterial compositions, functional genes and fecal metabolomes in a highly sex-specific manner. In particular, bacterial genes and/or key metabolites of neurotransmitter synthesis and pro-inflammatory mediators are significantly altered by Mn^{2+} exposure, which can potentially affect chemical signaling of gut-brain interactions. Likewise, functional genes involved in iron homeostasis, flagellar motility, quorum sensing, and Mn transportation/oxidation are also widely changed by Mn^{2+} exposure. Taken together, this study has demonstrated that Mn^{2+} exposure perturbs the gut microbiome and its metabolic functions, which highlights the potential role of the gut microbiome in Mn^{2+} toxicity, particularly its sex-specific toxic effects.

Keywords

Manganese exposure; gut microbiome; sex-specific toxicity; gut-brain axis; metabolome; metagenomics

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Introduction

Trillions of bacteria reside in human gastrointestinal tract and they are deeply involved in human metabolism and health (Ley *et al.*, 2006). Besides food digestion and energy harvest, the gut microbiome plays a crucial role in neurodevelopment, immune response, inflammation and xenobiotic biotransformation (Guarner and Malagelada, 2003; Bäckhed *et al.*, 2005). The gut microbiome is a highly dynamic system and can be influenced by environmental factors, such as heavy metals and antibiotics (Jakobsson *et al.*, 2010; Lu *et al.*, 2014). Multiple xenobiotics can alter bacteria community compositions and disturb the production of key metabolites, which can largely influence the interactions between the gut microbiome and host (Maurice *et al.*, 2013; Lu *et al.*, 2014). On the other hand, gut bacteria can modulate the effects of xenobiotics on the host. For example, gut bacteria transform Hg^{2+} and Cr (VI) to Hg^{0} and Cr(III) to reduce their toxicity, while other xenobiotics, such as nitrazepam, are converted to more toxic species by gut bacteria (Takeno and Sakai, 1991; Upreti *et al.*, 2004; Monachese *et al.*, 2012; Younan *et al.*, 2016). Therefore, bi-directional interactions between the gut microbiome and exposure actually exist.

Manganese (Mn) is an essential trace element for mammals and many microorganisms (Jakubovics and Jenkinson, 2001; Aschner and Aschner, 2005). Mn is necessary for normal brain function and amino acid, lipid, and carbohydrate metabolism (Greger, 1998; Aschner and Aschner, 2005). Mn also functions as the cofactor of numerous key enzymes, such as arginase, glutamine synthetase, manganese catalase, and manganese superoxide dismutase (Greger, 1998; Jakubovics and Jenkinson, 2001). However, Mn overload is toxic and associated with a series of diseases, including chronic liver failure, cardiovascular diseases, bone loss and neurodegeneration (Roth and Garrick, 2003; Crossgrove and Zheng, 2004; Milatovic *et al.*, 2009). Mn can cross the blood-brain barrier, accumulate in the brain and cause neurodegenerative disorders, such as Parkinson's disease (PD) (Crossgrove and Zheng, 2004; Reaney *et al.*, 2006). Mn-induced tissues and neuron damages involve multiple mechanisms, including mitochondrial dysfunction, oxidative stress (Milatovic *et al.*, 2009), activation of pro-inflammatory mediators and neuroinflammation (Chen *et al.*, 2006; Milatovic *et al.*, 2009), and alterations of ion homeostasis (Klaassen and Amdur, 1996; Zheng *et al.*, 1999; Roth and Garrick, 2003; Zhang *et al.*, 2003; Crossgrove and Zheng, 2004). Manganese toxicity is also species-dependent. Mn^{3+} is more reactive and toxic than Mn^{2+} (Crossgrove and Zheng, 2004; Reaney *et al.*, 2006). A previous study revealed that Mn^{3+} exposure caused significantly higher blood manganese levels than Mn^{2+}, and Mn^{3+} accumulated in brain more efficiently than Mn^{2+} (Reaney *et al.*, 2006). Since a considerable amount of Mn comes from food and water, gut bacteria are being exposed to Mn before it is absorbed to the body. However, it is largely unknown whether Mn exposure can perturb the gut microbiome and its functions. It is also unclear whether gut bacteria can influence the toxicity and physiological effects of Mn.

In particular, a compelling body of evidence demonstrates that the gut microbiome significantly affects central nervous system (CNS) via the gut-brain axis, *i.e.* the bidirectional biochemical signaling between the gastrointestinal tract and the CNS. The gut microbiome can largely influence behaviors and diseases in the host, such as depression and schizophrenia (Collins *et al.*, 2012; Cryan and Dinan, 2012; Foster and Neufeld, 2013;

Dinan *et al.*, 2014). Animals with depression and anxiety were generally associated with alterations of gut bacteria (O'Mahony *et al.*, 2009; Park *et al.*, 2013). Oral administration of *Lactobacilli rhamnosus* to mice could alter the GABA receptor expression in key CNS stress-related brain regions and influence anxiety-like behaviors (Foster and Neufeld, 2013). Gut microbiome perturbation has been proposed to play a role in neurodegenerative disorders such as PD (Ghaisas *et al.*, 2016). Previous studies clearly showed that the gut produced a large amount of neurotransmitters and related compounds (O'Mahony *et al.*, 2015; Yano *et al.*, 2015). For example, intestinal cells, but not brain cells, generate more than 90% of serotonin in the body (Gershon and Tack, 2007; Yano *et al.*, 2015). Gut compounds play key roles in the cross-talk of microbiome-gut-brain. Inflammatory signaling is another important type of interaction in the gut-brain axis (Bercik *et al.*, 2010; Hanamsagar and Bilbo, 2016; Rea *et al.*, 2016). It has been shown that chronic gastrointestinal inflammation induces anxiety-like behaviors and alters central nervous system biochemistry (Bercik *et al.*, 2010). However, it remains unknown whether Mn exposure perturbs the gut microbiome, which leads to altered chemical signaling involved in the gut-brain axis.

It has been reported that Mn has sex-selective toxicity (Zhang *et al.*, 2003; Madison *et al.*, 2011; Mergler, 2012). For example, $MnCl_2$ exposure had reverse effects on the body weight of male and female SD rats (Zhang *et al.*, 2003). Another study found that Mn^{2+}-exposed female mice had long-lasting effects in neuronal morphology, which was absent in male mice (Madison *et al.*, 2011). However, the mechanism underlying sex-specific effects of Mn is poorly understood. Recently, we and others have shown that the gut microbiome is largely different between male and female animals (Chi *et al.*, 2016; Cong *et al.*, 2016), raising the possibility that differential gut microbiome responses may contribute to sex-selective toxicity. In fact, toxicants, such as arsenic and organophosphate pesticides, cause sex-specific perturbations of the gut bacteria (Chi *et al.*, 2016; Gao *et al.*, 2017), which may further affect toxicity and disease susceptibility in males and females when exposed to these toxicants.

This study was designed to address three questions: Will Mn exposure alter the gut microbiome and its metabolic functions? Are there any changes of chemical signaling involved in the gut-brain interactions? Are these changes sex-specific? Therefore, both male and female C57BL/6 mice were exposed to $MnCl_2$ in drinking water for 13 weeks, followed by the assessment with multi-omics, including 16S rRNA gene sequencings, metagenomics and GC-MS metabolomics. 16S rRNA sequencing and metagenomics sequencing were used to define the alterations of bacterial compositions and functional pathways of gut bacteria. GC-MS metabolomics was employed to analyze the metabolic changes related to the gut microbiome. To the best of our knowledge, this is the first study to examine the sex-specific effects of Mn exposure on the gut microbiome and associated metabolic functions.

Materials and Methods

Animals and manganese exposure

C57BL/6 mice (7 weeks old, Jackson Laboratory, Bar Harbor, ME) were housed in the University of Georgia animal facility for a week before exposure, as well as throughout the

duration of the experiment in static microisolator cages with Bed-O-Cob combination bedding under environmental conditions of 22°C, 40–70% humidity, and a 12:12 hr light:dark cycle. Before experimentation, all mice were allowed to consume tap water ad libitum, and were provided with standard pelleted rodent diet before and during experimentation. At the experimental period, mice were randomly assigned into either the control group, or 100 ppm $MnCl_2$ treatment group (consumption of Mn is ~20 mg/kg body weight/day) (n=20, with 5 male mice and 5 female mice per group). The Mn dose used in this study was modeled according to several previous studies that demonstrated neurotoxicity of Mn at similar concentrations (Moreno *et al.*, 2009; Avila *et al.*, 2010; Krishna *et al.*, 2014). The animals were treated humanely and every effort was made to alleviate suffering. The animal protocol was approved by the University of Georgia Institutional Animal Care and Use Committee. At the start of experiment, $MnCl_2$ (Pfaltz & Bauer, Inc., Waterbury, CT) was dissolved in tap water and was administered to individual animal (~ 8 weeks of age) in drinking water for 13 weeks. The mice were allowed to consume ad libitum. Drinking water with $MnCl_2$ was made fresh every week. Control mice (~8 weeks of age) continued to receive tap water in their drinking water bottles which they were allowed to consume ad libitum.

16S rRNA sequencing

Mice fecal pellets from individual mouse were collected for 16S rRNA analysis at 0 and 13 weeks, and stored under liquid nitrogen before being transferred to -80°C until further analysis. DNA was isolated from fecal pellets using a PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA) according to the manufacturer's instructions. The resultant DNA was quantified by Nanodrop® and stored at −80°C until further analysis. Purified DNA (1 ng) was used to amplify the V4 region of 16S rRNA of bacteria using universal primers of 515 (5′-GTGCCAGCMGCCGCGGTAA) and 806 (5′-GGACTACHVGGGTWTCTAAT). The resultant DNA products were barcoded and quantified by Qubit 2.0 Fluorometer using Qubit dsDNA HS Assay kit (Life Technologies, Grand Island, NY) according to manufacturer's instructions and pooled to be sequenced. Sequencing was performed on an Illumina Miseq at the Georgia Genomics Facility to generate pair-end 250 × 250 (PE250, v2 kit) reads. The raw mate-paired fastq files were merged and quality-filtered using Geneious 8.0.5 (Biomatters, Auckland, New Zealand) with error probability limit set as 0.01. The data then were analyzed using quantitative insights into microbial ecology (QIIME, version 1.9.1). UCLUST was used to get the operational taxonomic units (OTUs) with 97% sequence similarity. The data was assigned at five different levels: phylum, class, order, family and genus.

Metagenomics sequencing

For metagenomics sequencing, DNA (10 ng/μL) was fragmented using the Bioruptor UCD-300 sonication device, followed by sequencing library construction using the Kapa Hyper Prep Kit according to the manufacturer's instructions. The resulting DNA was pooled, quantified, and sequenced at the Georgia Genomics Facility using an Illumina NextSeq High Output Flow Cell. The raw fastq files were imported into the MG-RAST metagenomics analysis server (version 4.0) with MG-RAST ID: Control (male): 4689984.3, 4689979.3, 4689977.3, 4689967.3, and 4689960.3; $MnCl_2$ treatment (male): 4707697.3,

4707696.3, 4707700.3, 4707701.3, and 4707704.3; Control (female): 4689988.3, 4689986.3, 4689982.3, 4689978.3, and 4689973.3; $MnCl_2$ treatment (female): 4707703.3, 4707702.3, 4707699.3, 4707698.3, and 4707695.3 (Meyer *et al.*, 2008). Sequences were assigned to M5NR Subsystems database for functional analysis with maximum e-Value cutoff 10–5, 75% minimum identity cutoff, and minimum alignment length cutoff of 35. Metagenomics analyzed and compared the normalized sequencing counts of bacterial genes between the controls and treatment samples.

Gas chromatography mass spectrometry metabolomics profiling

Metabolites were extracted from fecal samples collected from individual mouse using methanol and chloroform as described previously (Lu *et al.*, 2014). Briefly, 20mg feces was vortexed with 1ml of methanol/chloroform/water solution (2:2:1) for 1 hour, followed by centrifugation at 3,200 × g for 15 minutes. The resultant upper phase and lower phase were transferred to a HPLC vial and dried for about 4 hours in SpeedVac, and derivatized using N,O Bis(trimethylsilyl)trifluoroacetamide (BSTFA). The derivatized samples were analyzed using an Agilent Technologies 6890N Network GC System/5973 Mass-Selective Detector (Agilent Technologies, Santa Clara, CA) with an Agilent DB5-MS column (5% Phenyl and 95% dimethylarylene siloxane as the stationary phase, 30 m length; 0.250 mm diameter (narrowbore); film thickness 0.25 μm) (Agilent Technologies, Santa Clara, CA) under the following conditions: initial oven temperature was set at 60° C for 2 minutes, ramped to 320°C by 8°C/minute, and then held at 320°C for 10.5 minutes. 2 μL of sample solution was injected with helium as the carrier gas at a flow rate of 0.8 mL/minute. The temperature of the injector, ion source, and MS Quadrupole were set at 275°C, 230°C, and 150°C respectively. The mass spectrometer was operated in full scan mode from 50 to 600 m/z. The XCMS Online tool was used to pick up and align peaks and calculate the accumulated peak intensity. To identify the metabolite represented by a particular feature, retention time and m/z data from the XCMS Online output was used to filter the total ion chromatogram. The compounds were tentatively identified after matching with the NIST MS database and the identification of a few selected metabolites, such as amino acids, was further validated using authentic standards.

Statistical analysis

Principal coordinate analysis (PCoA) was used to compare the gut microbiome profiles between the control and treatments, while a nonparametric t-test was conducted by Metastats, integrated with Mothur software (Schloss *et al.*, 2009), to determine statistically significant changes ($p<0.05$) to the gut-microbial community structure between treatments and control as previously described (White *et al.*, 2009). DESeq2 (version 3.4) has been applied to calculate the statistically significant changes of functional genes (Love *et al.*, 2014). To generate difference of metabolic profile between the control and treatment group, a two-tail Welch's t-test ($p<0.05$) was used.

Results

1. Mn^{2+} exposure perturbed the gut microbiome in a sex-specific manner

We first used 16s rRNA gene sequencing to examine the changes of gut bacterial compositions over time using beta diversity and alpha diversity metrics. Beta diversity evaluates the diversity in microbial community between samples, while alpha diversity reflects species richness in given samples. As shown in Fig. 1A and 1B, PCoA analysis shows that gut bacterial communities of mice initially clustered together at the baseline before exposure. After a 13-week Mn^{2+} treatment, the controls and exposed animals clearly separated into their own groups, which indicated Mn^{2+} exposure perturbed the trajectories of gut microbiome development. The alpha diversities (PD whole tree) in Mn^{2+}-treated groups were lower than control groups (Fig. 1C and 1D), indicating that Mn^{2+} treatment reduced phylogenetic diversity of gut bacteria. Notably, a strong sex-specific alteration of gut microbiome was found. For example, the relative abundance of the phylum *Firmicutes* significantly increased in Mn^{2+}-exposed male mice, while it decreased in Mn^{2+}-treated female mice (Fig. 2A). Moreover, Mn^{2+} exposure significantly reduced the phylum *Bacterodetes* in male mice only. Likewise, sex-dependent perturbations of gut bacteria were also evident at the genus level, as illustrated in Fig. 2B and 2C. These results clearly show that the gut bacterial community structures have been differentially altered in male and female mice by Mn^{2+} exposure.

2. Mn^{2+} exposure altered the abundance of bacterial genes of tryptophan and GABA metabolism pathways

Perturbed gut microbiome profiles are frequently associated with the alterations of functional bacterial genes. Neurotransmitters serve as key signaling molecules for the gut microbiota to influence brain activities (Sampson and Mazmanian, 2015), so we examined whether Mn^{2+} exposure altered relevant genes in the gut microbiome. As shown in Fig. 3A, genes in tryptophan biosynthesis pathways, including anthranilate phosphoribosyltransferase, indole-3-glycerol phosphate synthase, and tryptophan synthase (beta and alpha chain), were significantly altered by Mn^{2+} in a sex-specific manner. For example, the gene encoding tryptophan synthase (beta chain) was decreased and increased in Mn^{2+}-treated female and male mice, respectively (Fig. 3A). Mn^{2+} also induced a different effect on the genes of phenylalanine synthesis, with biosynthetic aromatic amino acid aminotransferase and prephenate dehydratase being increased and decreased in females and males (Fig. 3B). Likewise, a sex-specific effect of Mn^{2+} on GABA/putrescine metabolism is evident, as shown in Fig. 3C. For instance, the gene encoding glutamate decarboxylase, which synthesizes GABA from glutamate, was significantly decreased in Mn^{2+}-exposed male mice only (Fig. 3C). As an important source of GABA, the metabolism of putrescine plays a role in GABA homeostasis (Sequerra *et al.*, 2007). The putrescine transport gene, *potA*, was increased in Mn^{2+}-exposed male mice, while *potA* and *potG* were decreased in female mice (Fig. 3C). Putrescine biosynthesis genes were also significantly changed by Mn^{2+} exposure in a sex-selective fashion, as revealed by altered agmatine deiminase in females and arginine decarboxylase and N-carbamoylputrescine amidase in males (Fig. 3C).

3. LPS synthesis and DNA repair genes were specifically enriched in female mice by Mn^{2+} exposure

LPS plays an important role in the host inflammation and contributes to the gut-brain interactions (Cryan and Dinan, 2012; Sampson and Mazmanian, 2015). We found that the abundances of LPS biosynthesis genes were widely increased in Mn^{2+}-treated female mice, but reduced in male animals, as shown in Fig. 4A and 4B. For example, bacterial genes involved in Kdo2 and lipid A synthesis, the important components of LPS, were increased in females only by Mn^{2+} treatment (Fig. 4A). Moreover, LPS assembly related genes were largely increased in female mice by Mn^{2+} exposure, but were either reduced or not significantly changed in male mice (Fig. 4B). The oxidative stress response gene, cytochrome c551 peroxidase, as well as multiple DNA repair genes, was significantly increased in female mice but decreased in male mice under Mn^{2+} exposure (Fig. 4C).

4. Genes related to iron homeostasis were altered by Mn^{2+} exposure

Mn can interact with iron and perturb normal physiological processes (Zheng *et al.*, 1999; Crossgrove and Zheng, 2004). So, we next explored whether the iron homeostasis in the gut microbiota was affected by Mn^{2+}. In fact, many iron transport related genes were significantly altered, such as ferrichrome-iron receptor, outer membrane receptor protein (Fe transport), ferric iron ABC transporter, iron compound ABC uptake transporter, and ferric uptake regulation protein (Fig. 5). Again, a sex-selective impact of Mn^{2+} exposure on iron acquisition and metabolism was found. These results suggest that Mn exposure could differentially affect the iron acquisition and metabolism in the gut microbiome of female and male mice.

5. Mn^{2+} exposure altered genes in quorum sensing, motility and chemotaxis and metal/drug resistance

Bacteria control behaviors including motility and virulence according to the population density fluctuation by the cell-cell signaling process called quorum sensing. The detection of an autoinducer at the threshold concentration leads to the alteration of gene expression. We found that quorum sensing genes were specifically increased in Mn^{2+}-exposed male mice only, including autoinducer 2 (AI-2) kinase LsrK, *S*-ribosylhomocysteine lyase and autoinducer-2 production protein LuxS (Fig. 6A). Consequently, bacterial genes involved in flagellar motility and chemotaxis were largely increased in male mice (Fig. 6B and 6C). Interestingly, multiple metal or drug resistance related genes were significantly increased in Mn^{2+}-treated female mice but consistently decreased in male mice (Fig. 6D). Our results again highlight a sex-selective effect of Mn^{2+} on these critical bacterial functional genes.

6. Sex-selective regulation of genes of Mn transportation and oxidation

As an essential trace metal, Mn can be absorbed and utilized by many bacteria species (Jakubovics and Jenkinson, 2001). We next investigated whether Mn metabolism-related bacterial genes were regulated by exogenous Mn^{2+} exposure. Interestingly, two manganese transporter genes, manganese ABC transporter SitB and SitD, were specifically enriched in the gut microbiome of Mn^{2+}-treated female mice, while they were largely reduced in male mice (Fig. 7A and 7B). The gene encoding multicopper oxidase that oxidizes Mn^{2+} to Mn^{3+}

significantly increased in females but decreased in males (Fig. 7C) (Webb *et al.*, 2005). These results suggest the gut bacteria of male and female animals may have different capacities or responses to mediate Mn utilization and oxidation.

7. Mn^{2+} exposure disturbed the fecal metabolome of mice

The communication between gut bacteria and host is largely dependent on metabolites with various functions. We further examined whether the fecal metabolomes were altered by Mn^{2+} exposure. As shown in Fig. 8, the fecal metabolomic profiles of male and female mice were perturbed by a 13-week Mn^{2+} treatment in a sex-selective manner. In particular, the majority of fecal metabolites of male mice were down-regulated, while numerous up- and down-regulated metabolites were captured for female mice (Fig. 8A and 8B). For example, we observed the aphla-tocopherol and gama-tocopherol were decreased in Mn^{2+}-treated male and female mice (Fig. 8C and 8D), with a stronger response being observed for female animals. In addition, several neurotransmitters or the precursors of neurotransmitter synthesis, such as glycine, glutamic acid and phenylalanine, were altered in a sex-selective manner, with up-regulation and down-regulation in the fecal metabolomes of female and male mice, respectively (Fig. 8E–8G). More identified significantly changed metabolites ($p<0.05$) are listed in Table S1 and S2.

Discussion

In this study, we explored the sex-specific effects of Mn^{2+} exposure on the gut microbiome compositions, functional genes and fecal metabolomes by high-throughput sequencing and GC-MS metabolomics. Our results reveal that the gut microbiome has been significantly perturbed in C57BL/6 mice by Mn^{2+} treatment. Our data also show that Mn^{2+} may affect the gut-brain axis by influencing the synthesis of several neurotransmitters or their precursors, including GABA and tryptophan. Mn^{2+} also modulates the bacteria-related pro-inflammatory mediators, such as LPS, which may also impair gut-brain interactions. Moreover, our data highlight several potential mechanisms of how Mn^{2+} perturbs the gut microbiome, including altering quorum sensing, inducing oxidative stress, and disturbing iron homeostasis. Notably, we found strong sex-specific effects of Mn^{2+} exposure on the gut bacteria. Our data also suggest that the bi-directional interactions between Mn^{2+} exposure and the gut microbiome was impaired: Mn^{2+} exposure influences the gut bacteria and their functions, and Mn^{2+}-induced perturbations of gut bacteria may mediate the toxicity of Mn in the host.

Recently, much attention has been paid to the interactions between the gut microbiome and CNS (Foster and Neufeld, 2013; Ghaisas *et al.*, 2016). Compelling evidence indicates that the gut microflora has profound effects on CNS activities and host behaviors (Collins *et al.*, 2012; Cryan and Dinan, 2012; Foster and Neufeld, 2013). For example, previous studies suggested that bacterial colonization in the gut played a critical role in neural system development (Barbara *et al.*, 2005; Stilling *et al.*, 2014; Sampson and Mazmanian, 2015). Genes of some key neurotransmitter receptors like serotonin receptor 1A in germ-free mice are different compared with conventionally raised mice (Neufeld *et al.*, 2011). Likewise, oral administration of some species such as *Lactobacillus rhamnosus* and *Bifidobacterium*

longum can alter the gene expression in CNS and influence anxiety-like behaviors (Bercik *et al.*, 2011b; Bravo *et al.*, 2011). Other experiments also demonstrated that the perturbation of gut microbiota by non-absorbable antimicrobials was associated with the changes of CNS activities and behaviors (Bercik *et al.*, 2011a). One of the main potential mechanisms for gut bacteria to interact with host nervous systems is through neurotransmitters or their metabolic precursors (Sharon *et al.*, 2014; Sampson and Mazmanian, 2015), such as GABA and tryptophan. Tryptophan is a central precursor of serotonin or 5-hydroxytryptamine (5-HT) (Leathwood, 1990). 5-HT is an important neurotransmitter involved in multiple physiological processes, such as modulating colonic motility (Fukumoto *et al.*, 2003). Previous studies show that gut microbiota participated in the 5-HT turnover modulation (Reigstad *et al.*, 2015; Yano *et al.*, 2015). Tryptophan is generated by the gut microbiota and then can cross the blood brain barrier to be synthesized to 5-HT (O'Mahony *et al.*, 2015). Here, we found multiple genes involved in synthesizing tryptophan from chorismate were increased in Mn^{2+}-treated female mice (Fig. 3A). GABA, as an inhibitory neurotransmitter, also functions in multiple physiological processes and is directly associated with anxiety and depression (Kalueff and Nutt, 2007; Hall *et al.*, 2014). Similar to 5-HT, the concentration of GABA in the host is also affected by gut microbiota (Barrett *et al.*, 2012; Sampson and Mazmanian, 2015). In this study, the gene encoding glutamate decarboxylase was altered in Mn^{2+}-treated male mice (Fig. 3C). Bacteria can synthesize GABA from glutamate by glutamate decarboxylase (Barrett *et al.*, 2012). Previous research has also found that putrescine is an important source of GABA in the brain of rats (Sequerra *et al.*, 2007). Herein, genes involved in putrescine synthesis and transportation were changed by Mn^{2+} treatment (Fig. 3C). In addition, two phenylalanine synthesis genes also have significantly higher abundances in Mn^{2+}-treated mice than controls (Fig. 3B). Consistently, at the metabolite level, several neurotransmitters or the precursors of neurotransmitter synthesis, such as glycine, glutamic acid and phenylalanine, were perturbed by Mn^{2+} exposure (Fig. 8). Glycine and glutamic acid are well-known neurotransmitters, while phenylalanine is the precursor of the neurotransmitter dopamine (Daubner *et al.*, 2011). Collectively, these results suggest that Mn-induced perturbation of gut microbiome might disturb the normal metabolism of neurotransmitters or related precursors in gut, which could further interfere with normal gut-brain interactions.

Modulation of immune response is another mechanism that the gut flora can influence the neuron systems (Collins *et al.*, 2012; Cryan and Dinan, 2012; Sampson and Mazmanian, 2015). The gut microbiome plays a critical role in host inflammation, and multiple bacteria-derived molecules can activate immune systems, such as LPS and bacterial lipoprotein (Hirschfeld *et al.*, 1999; Cani *et al.*, 2008; Hooper *et al.*, 2012). LPS can stimulate immune cells to produce various pro-inflammatory cytokines, such as IL-1b, TNFa, and IL-6 (Bruunsgaard *et al.*, 1999). Cytokines can transport to neural systems and function as signaling molecules to influence neuron activities and behaviors (Cryan and Dinan, 2012). Here, we observed that genes involved in LPS synthesis and assembly are widely increased in Mn^{2+}-treated female mice (Fig. 4A and 4B). Interestingly, a previous study found that Mn^{2+} enhanced LPS-induced NOS2 expression and cytokines release (Barhoumi *et al.*, 2004). Therefore, Mn^{2+} exposure might increase inflammatory response not only by

increasing LPS secretion in gut bacteria, but also by potentiating the physiological response of LPS, which in turn leads to a more pronounced effect on the gut-brain axis.

In this study, Mn exposure significantly altered the gut microbiome. Likewise, we also demonstrated that other environmental toxicants, like arsenic, could also largely alter the bacterial compositions, abundance and community structures of gut bacteria (Lu *et al.*, 2014; Chi *et al.*, 2016). What mechanism is responsible for regulating the bacterial community? This is an important question to be addressed in microbiome-exposure research. In this context, our data may provide new insights into how Mn causes gut microbiome perturbations. Mn exposure may result in shifted gut microbiome structures by altering the quorum sensing, Mn availability, oxidative stress and iron homeostasis, as briefly discussed as below.

Mn^{2+} exposure alters the quorum sensing system. Bacteria control behaviors such as sporulation, motility and virulence according to the population density fluctuation by quorum sensing (Nasser and Reverchon, 2007). Autoinducers play a key role in quorum sensing. Key quorum sensing genes, including AI-2 kinase LsrK, *S*-ribosylhomocysteine lyase and AI-2 production protein LuxS, were increased in Mn^{2+}-exposed male mice (Fig. 6A), indicating that Mn^{2+} exposure could perturb the bacterial community compositions via altering quorum sensing. Besides regulating the density of the bacterial population, alterations of quorum sensing could also lead to the regulation of many critical downstream genes and pathways. As the consequence, we observed genes involved in bacteria motility and chemotaxis were enriched in gut bacteria of Mn^{2+}-treated male mice (Fig. 6B and 6C). Flagellar motility and bacterial chemotaxis play an important role in bacteria survival, which allows bacteria to respond to favorable or unfavorable environmental stimuli (Haefele and Lindow, 1987). Moreover, quorum sensing and flagella genes are also necessary for the biofilm formation in many bacteria, which could promote bacteria aggregation and adhesion (Pratt and Kolter, 1998; Singh *et al.*, 2006). Chemotaxis ability is critical in the spreading of biomass in mature biofilm, which help bacteria find optimum conditions for growth and survival (Pandey and Jain, 2002; Singh *et al.*, 2006).

Mn^{2+} exposure may also perturb the gut bacteria by mediating Mn^{2+} availability. As an essential metal, Mn is critical in multiple physiological processes in mammals and bacteria (Jakubovics and Jenkinson, 2001; Aschner and Aschner, 2005). Mn in gastrointestinal tract is normally maintained at low levels and bacteria that require Mn for growth are inhibited due to limited availability of Mn. In fact, one of effective strategies for the host to fight against bacterial infections is Mn sequestration (Diaz-Ochoa *et al.*, 2014). The host can express proteins, such as calprotectin in mucosa, which can directly bind Mn^{2+} to reduce Mn available in gastrointestinal tract and then inhibit microbial growth. This process is called nutritional immunity (Diaz-Ochoa *et al.*, 2014). However, Mn^{2+} exposure significantly increases Mn in gastrointestinal tract, which might weaken or even destroy nutritional immunity. Under such a scenario, bacteria limited by Mn availability can greatly benefit from Mn^{2+} exposure. For example, previous studies found that the growth of *Lactobacillus* species required extremely high concentrations of Mn^{2+} (Archibald and Fridovich, 1981; Archibald and Duong, 1984). In this study, *Lactobacillus* was enriched in Mn^{2+}-treated female animals (Fig. 2B). Consistently, two genes encoding Mn ABC transporter were

significantly increased in Mn^{2+}-treated female mice (Fig. 7A and 7B). Mn ABC transporter genes are actually encoded in the genome of *Lactobacillus* species (Groot *et al.*, 2005). Taken together, our data suggest that Mn^{2+} may perturb gut bacterial profiles by altering the availability of Mn^{2+}.

However, overexposure to Mn^{2+} potentially affects gut bacteria by inducing oxidative stress (Milatovic *et al.*, 2009). Previous studies demonstrated that Mn^{2+} accumulated in mitochondria and could disrupt oxidative phosphorylation, leading to ROS generation (Gunter *et al.*, 2006; Milatovic *et al.*, 2009). Mitochondria dysfunction and DNA fragmentation is one of main toxic effects of Mn^{2+} on rat neurons (Malecki, 2001; Milatovic *et al.*, 2009). In this study, the oxidative stress response gene, cytochrome c551 peroxidase, and multiple DNA repair genes were significantly activated (Fig. 4C). Likewise, we found that antioxidants, such as alpha-tocopherol and gama-tocopherol, were decreased in the fecal samples of Mn^{2+}-exposed mice. These evidence suggests that Mn^{2+} may induce oxidative stress in gut bacteria, which could influence the growth and survival of selected components of gut bacteria.

In addition to oxidative stress, high concentrations of Mn^{2+} can disturb the balance of ions, especially iron homeostasis (Zheng *et al.*, 1999). Numerous evidence shows that the interaction with iron is one of the mechanisms of Mn^{2+} toxicity (Zheng *et al.*, 1999; Roth and Garrick, 2003; Crossgrove and Zheng, 2004). For example, previous studies demonstrate that Mn transportation through blood-brain barrier to CNS relies on the binding with transferrin, an iron transporting protein (Aschner and Aschner, 1990). Mn can change the catalytic function of aconitase potentially through competing with iron in the active center of this enzyme (Zheng *et al.*, 1998). Likewise, Mn exposure causes excessive accumulation of iron in neurons, leading to oxidative stress and neuron damage (Zheng *et al.*, 1999; Crossgrove and Zheng, 2004). Iron and Mn homeostasis are important for the survival of microorganisms (Jakubovics and Jenkinson, 2001). Thus, disturbance of iron acquisition and metabolism may contribute to shaping the gut microbiome compositions. Indeed, we found multiple bacterial genes involved in the uptake and transport of iron were significantly perturbed by Mn exposure (Fig. 5). Besides the effects of Mn on the gut bacteria, Mn may affect iron metabolism in gut bacteria by perturbing host genes or responses. Mn absorption is mainly occurred in gastrointestinal tract by transferrin-dependent and transferrin-independent pathways, which are also used for iron absorption (Roth and Garrick, 2003). Transferrin and transferrin receptors can also be expressed in mucosa for iron sequestration (Diaz-Ochoa *et al.*, 2014). Mn can activate the expression of host ferritin and transferrin receptor to increase the iron absorption in tissues (Zheng *et al.*, 1999). Therefore, enhanced nutritional immunity by increasing iron absorption in GI tract may potentially affect bacterial growth and microbiome community structures.

As already mentioned above, strong sex-specific effects of Mn exposure on the gut microbiome were observed. For example, *Firmicutes* significantly increased in Mn^{2+}-exposed males, but decreased in female animals (Fig. 2A). Bacterial genes in multiple pathways, such as LPS synthesis and DNA repair, were increased in females but decreased in males (Fig. 4). Fecal metabolomes were also differentially altered in male and female mice (Fig. 8). Likewise, multiple genes encoding heavy metal resistance protein and efflux

system protein, as well as several antibiotic resistance genes, were significantly increased in treated female mice (Fig. 6D). These genes can improve bacterial survival by exporting heavy metals out of cells. As discussed previously, the genus *Lactobacillus* and Mn ABC transporter genes were specifically enriched in Mn^{2+}-exposed female mice (Fig. 2B and Fig. 7). Collectively, differential enrichment of specific genes may reflect sex-specific gut microbiome responses to Mn^{2+} exposure. The mechanism underlying sex-specific gut microbiome response remain elusive. It could arise from the initial gut microbiome difference, driven by sex hormones, between male and female mice. Additionally, sex-selective host response may also participate in mediating gut-microbiome response to exposure. Nevertheless, sex-specific gut microbiome response may play a role in differential toxicity of Mn in males and females.

The bi-directional interactions between the gut microbiome and exposure exist. Besides the widespread effects of Mn^{2+} on the gut microbiome, our data suggest that the gut microbiome may also influence the toxic effects of Mn. For example, the increase of Mn transportation genes in female mice may enrich Mn^{2+} in gut bacteria, thus reducing its toxic effects in the host by limiting Mn adsorption in host cells. On the other hand, the gut microbiota may increase the toxicity by oxidizing Mn^{2+} to Mn^{3+}. Mn^{3+} is much more reactive and toxic than Mn^{2+} (Chen *et al.*, 2001). Previous studies indicated that Mn^{3+} had higher affinity with transferrin and could accumulate at higher concentrations in brain than Mn^{2+} (Reaney *et al.*, 2002; Kearns, 2010). Here, we found the gene encoding multicopper oxidase was highly enriched in Mn^{2+}-exposed female mice only (Fig. 7C). Bacteria can oxidize Mn^{2+} to Mn^{3+} by multicopper oxidase (Webb *et al.*, 2005). These data suggest that the gut microbiota in females may have a higher capability of oxidizing Mn^{2+} to more toxic Mn^{3+}, which can enhance the toxic effects of Mn in the host. Interestingly, previous studies show that the toxic effects of manganese are different in males and females (Madison *et al.*, 2011; Mergler, 2012). Thus, differential biotransformation of Mn^{2+} by gut bacteria in males and females may play a role in the sex-specific effects of Mn exposure. Future studies are warranted to elucidate the role of the gut microbiome in mediating Mn toxicity in the host, particularly via a sex-specific manner.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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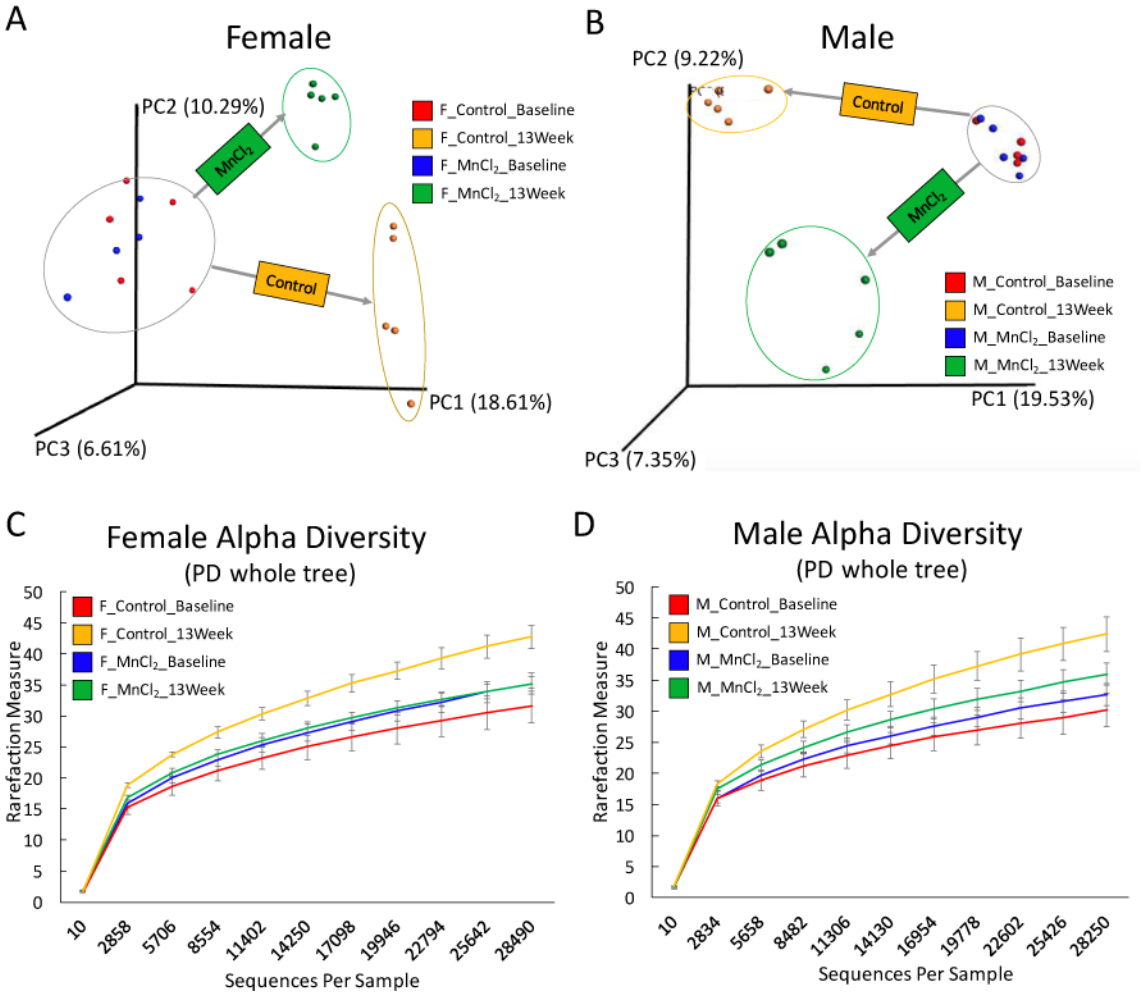

Fig. 1.

The effects of Mn^{2+} exposure on the gut microbiome in male and female C57BL/6 mice, as revealed by 16S rRNA gene sequencing. Based on the PCoA analysis of beta diversity, the gut microbiome community structures of female (A) and male (B) mice were significantly altered by Mn^{2+} exposure. The phylogenetic diversity, as evaluated by PD whole tree (alpha diversity), of the gut microbiome was significantly decreased in Mn^{2+}-treated female (C) mice and male (D) mice.

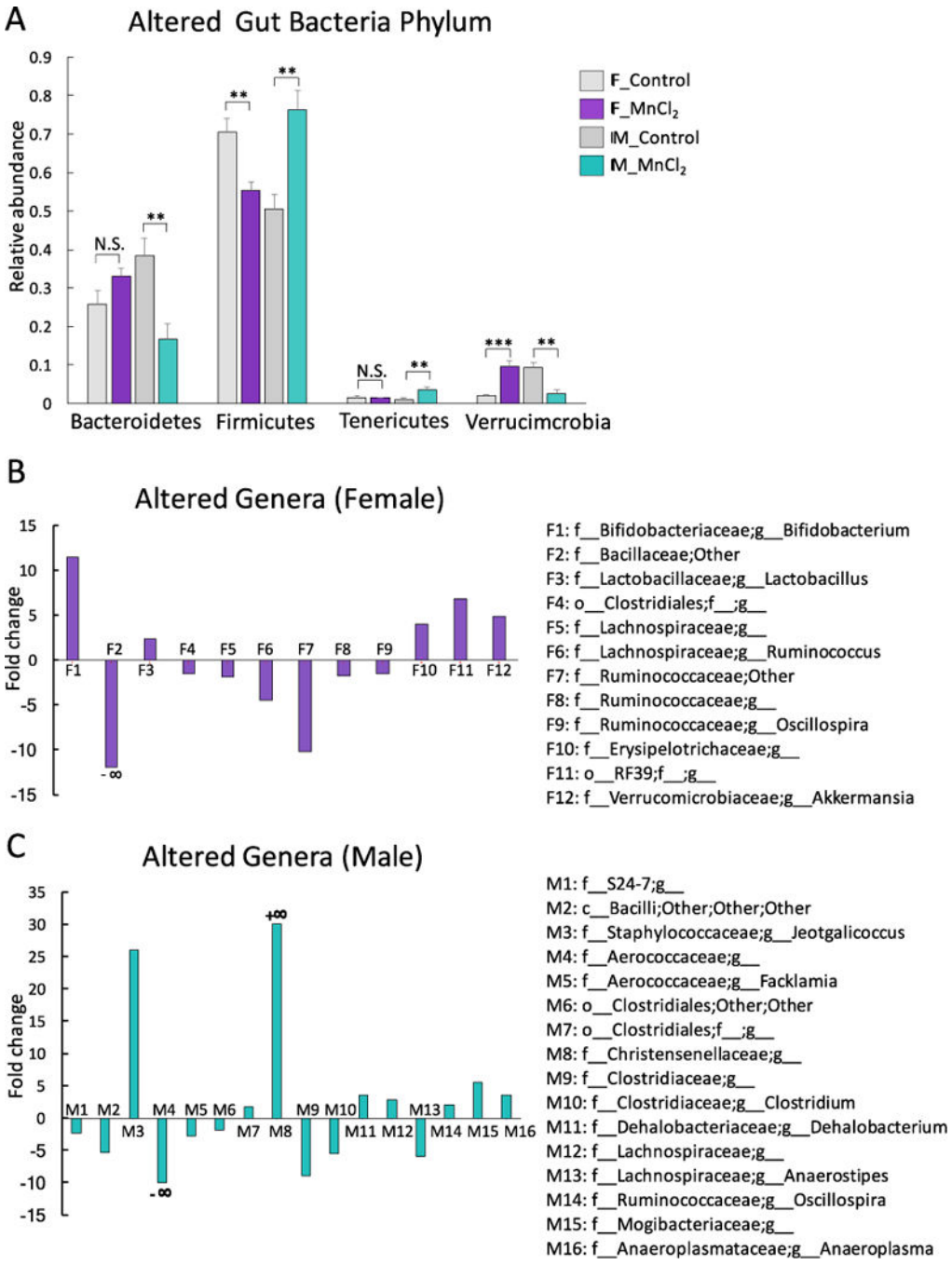

Fig. 2.

Mn^{2+} altered the relative abundance of gut bacteria at the phylum level in a sex-specific manner (A) (***p<0.001, **p<0.01, *p<0.05, N.S., no statistically significant change); Sex-dependent perturbations of gut bacteria at the genus level, as illustrated by the fold changes of significantly altered gut bacterial genera (B: Females; C: Male) (Only genera with p<0.05 are listed here; Fold changes were calculated using the relative abundance of each genus in Mn^{2+}-treated mice divided by the relative abundance of the same genus in control mice. c:

class; f: family; O: order; G: genus. +∞: only appeared after Mn exposure; -∞: abolished by Mn exposure)

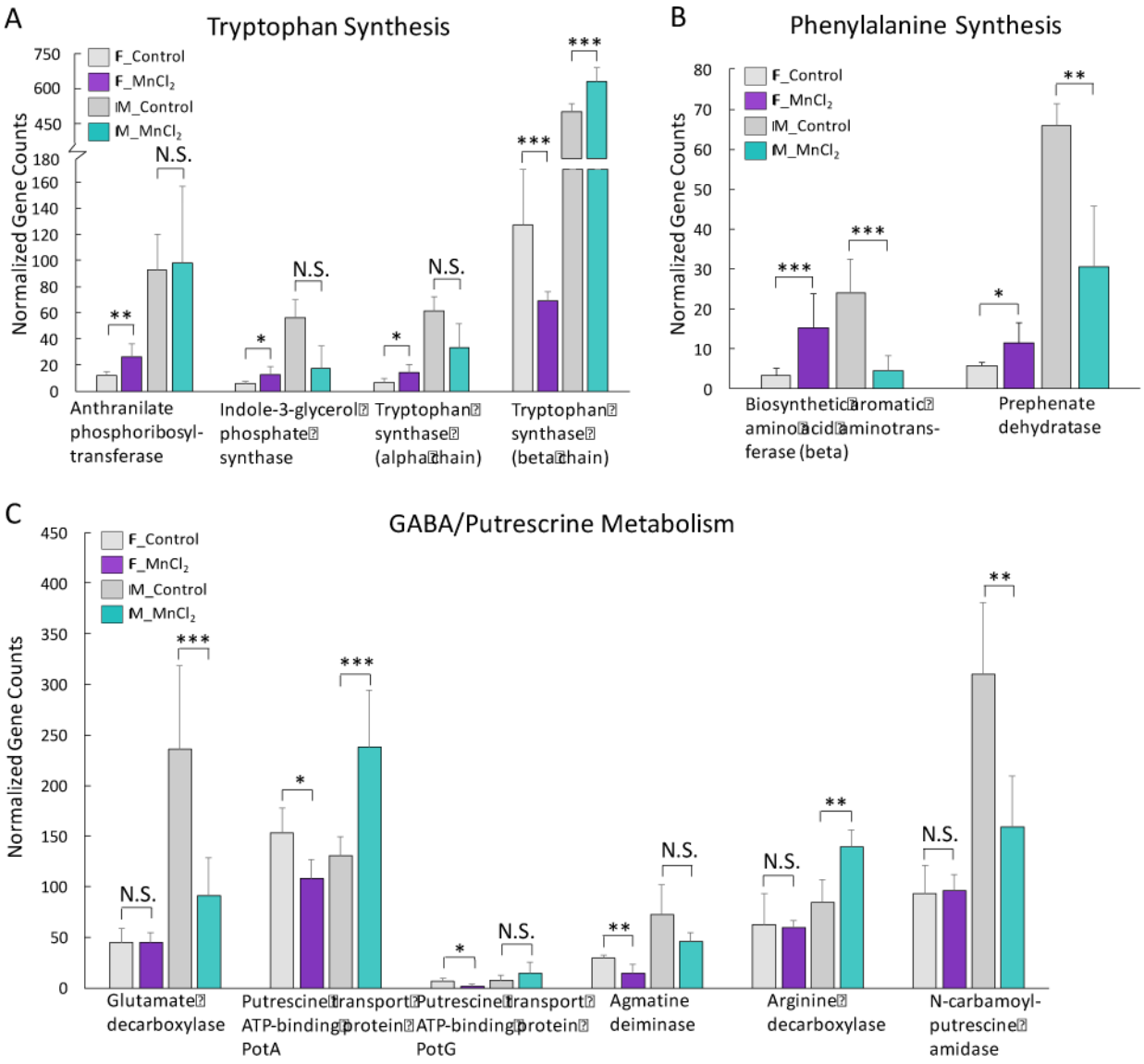

Fig. 3.

Sex-selective effects of Mn^{2+} on bacterial genes involved in neurotransmitter pathways. (A: tryptophan synthesis genes; B: phenylalanine synthesis genes; C: GABA/putrecrine metabolism genes) (***p<0.001, **p<0.01, *p<0.05, N.S., no statistically significant change)

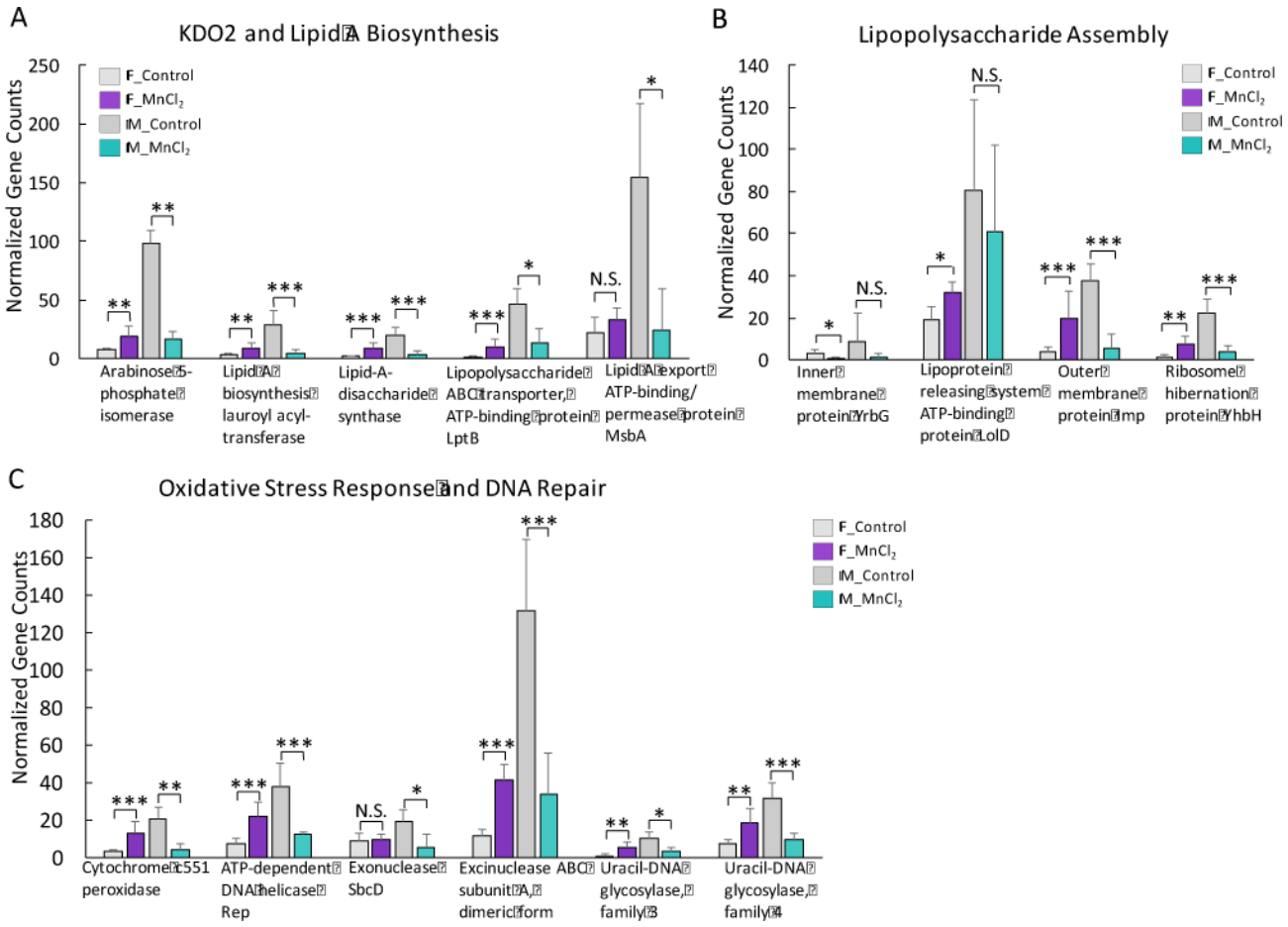

Fig. 4.

Gut bacterial genes involved in Kdo2 and lipid A synthesis (A), LPS assembly (B) and oxidative stress response and DNA repair (C) were significantly increased in Mn^{2+}-exposed female mice and decreased in male mice. (***p<0.001, **p<0.01, *p<0.05, N.S., no statistically significant change)

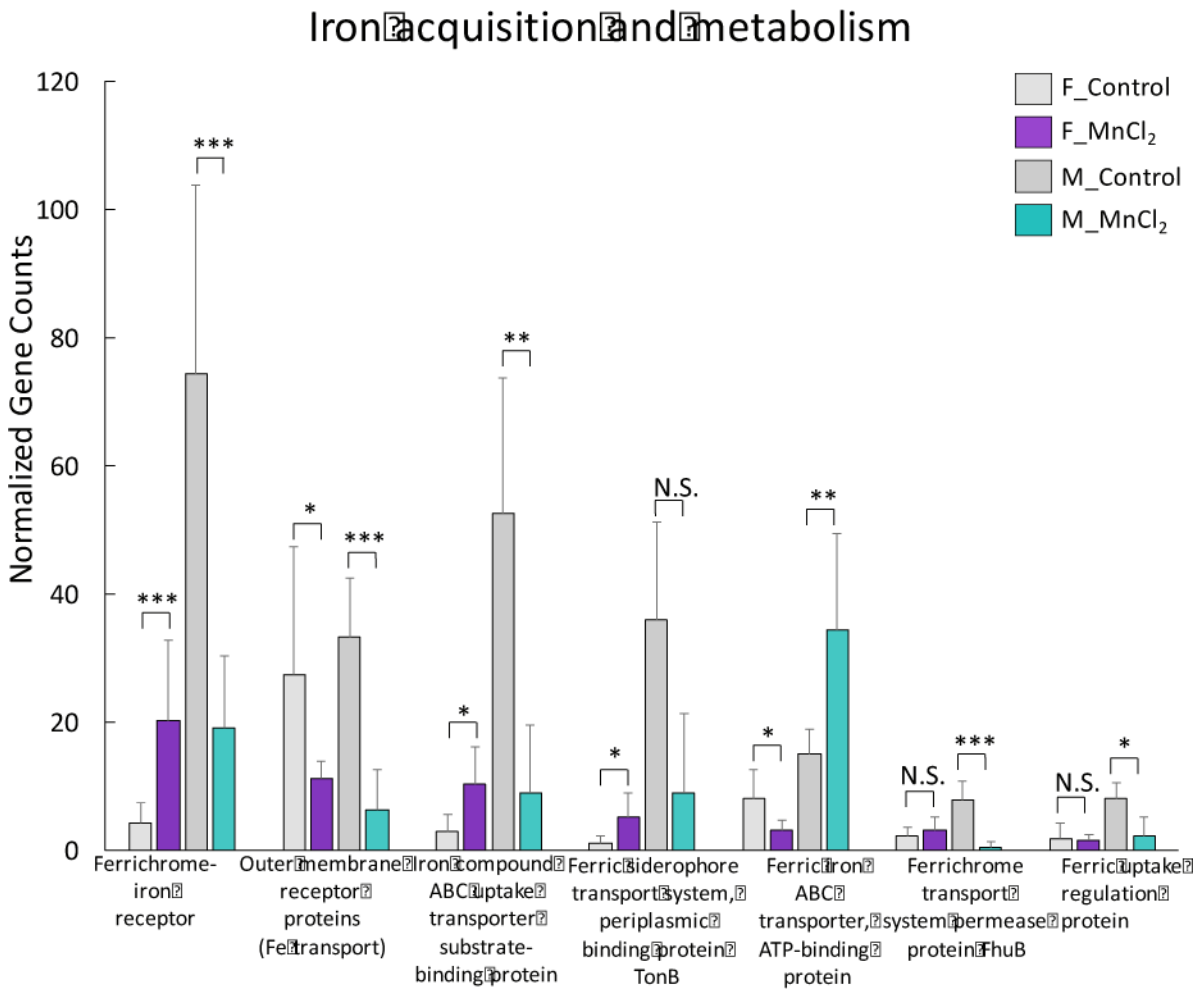

Fig 5.

Iron acquisition and metabolism related genes were widely altered in Mn^{2+}-exposed female and male mice. (***p<0.001, **p<0.01, *p<0.05, N.S., no statistically significant change)

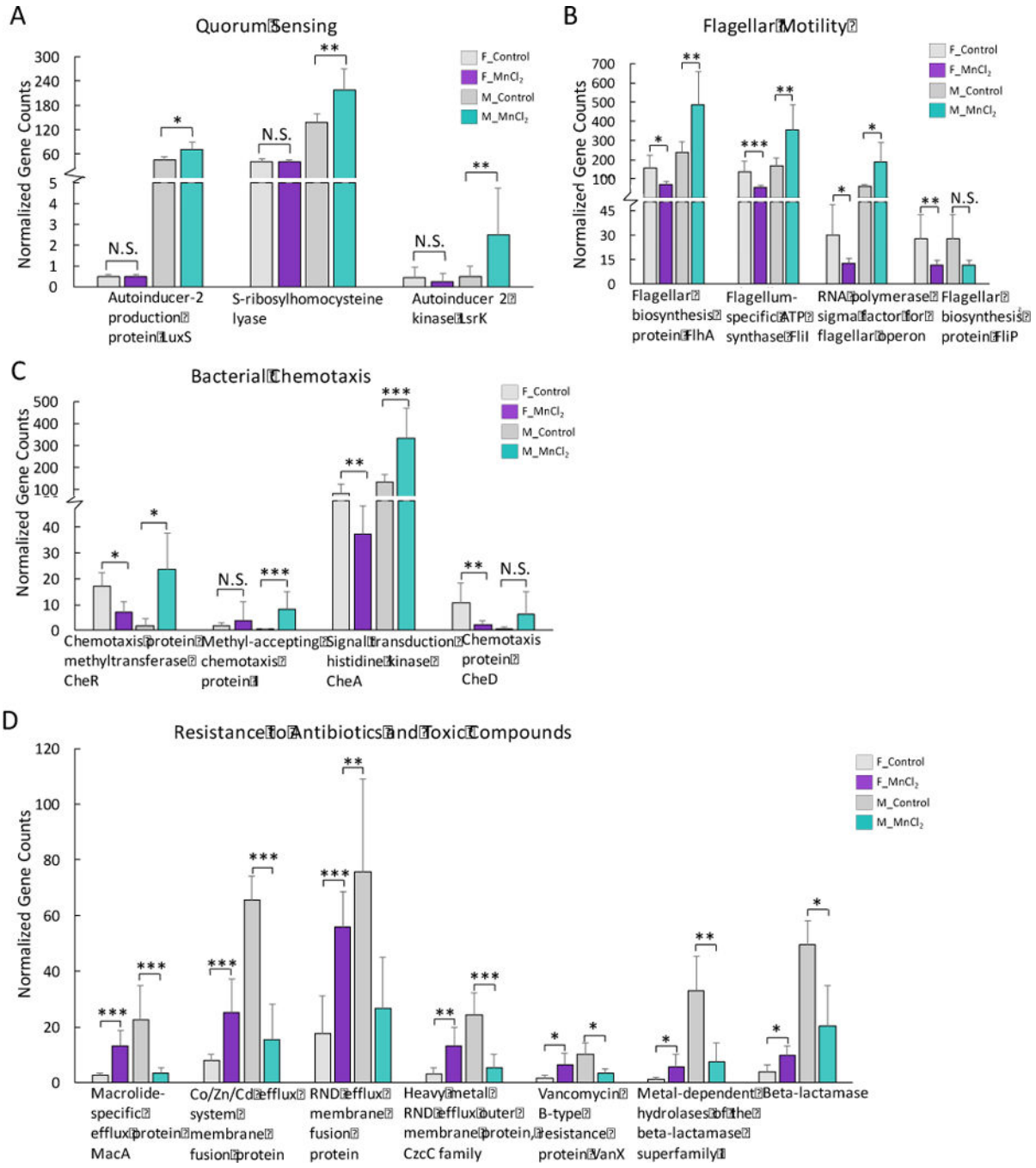

Fig. 6.

Quorum sensing genes specifically increased in Mn^{2+}-exposed male mice (A). Bacterial genes of flagellar motility (B) and chemotaxis (C) increased in Mn^{2+}-exposed male mice but significantly decreased in female mice. Heavy metal or antibiotic resistance genes decreased in the gut bacteria of Mn^{2+}-exposed male mice, but increased in Mn^{2+}-exposed female mice (D). (***p<0.001, **p<0.01, *p<0.05, N.S., no statistically significant change)

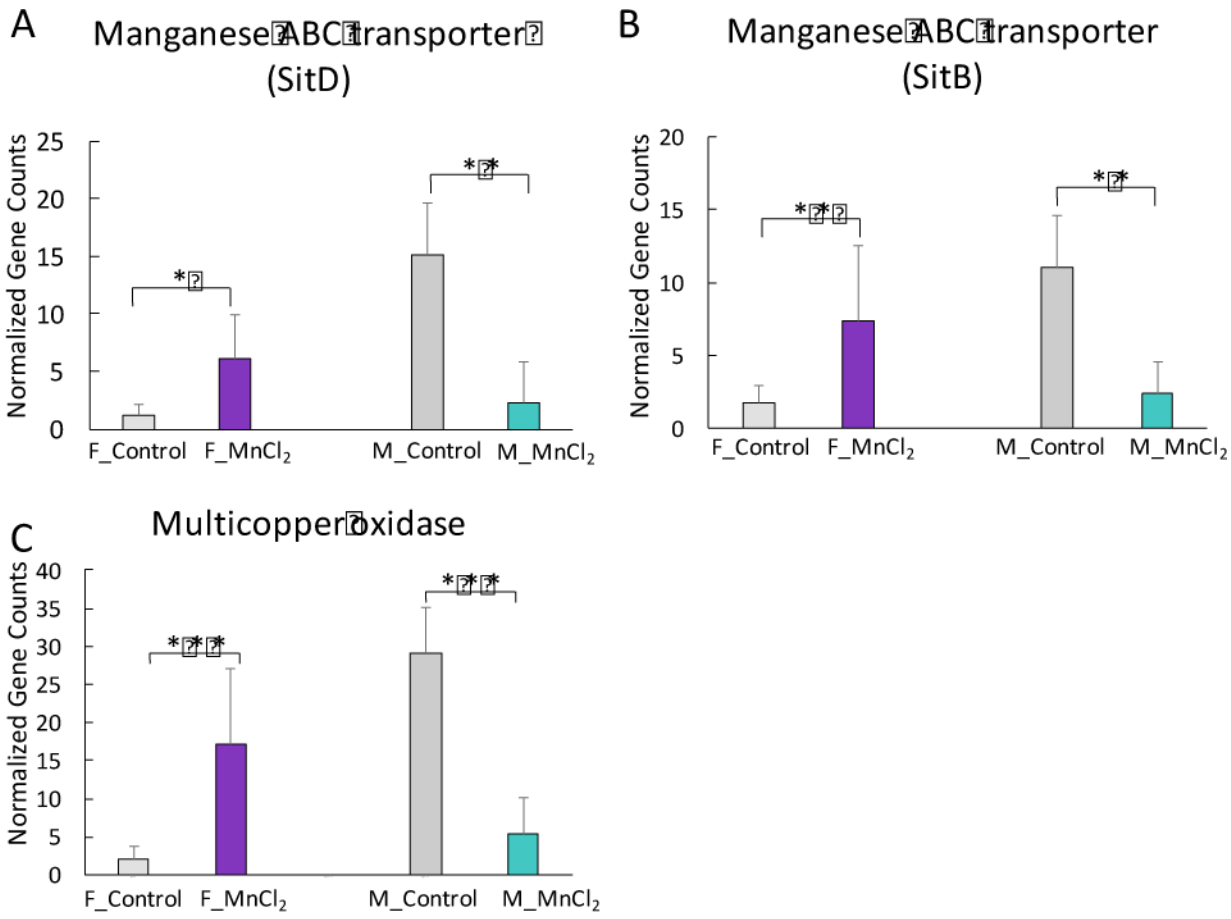

Fig. 7.

Sex-selective alterations of bacterial genes of Mn transportation and oxidation in mice exposed to Mn^{2+}. Mn ABC transporter genes (A and B) and Mn oxidation gene (C) were significantly increased in female mice but decreased in male mice after Mn^{2+} treatment. (***p<0.001, **p<0.01, *p<0.05)

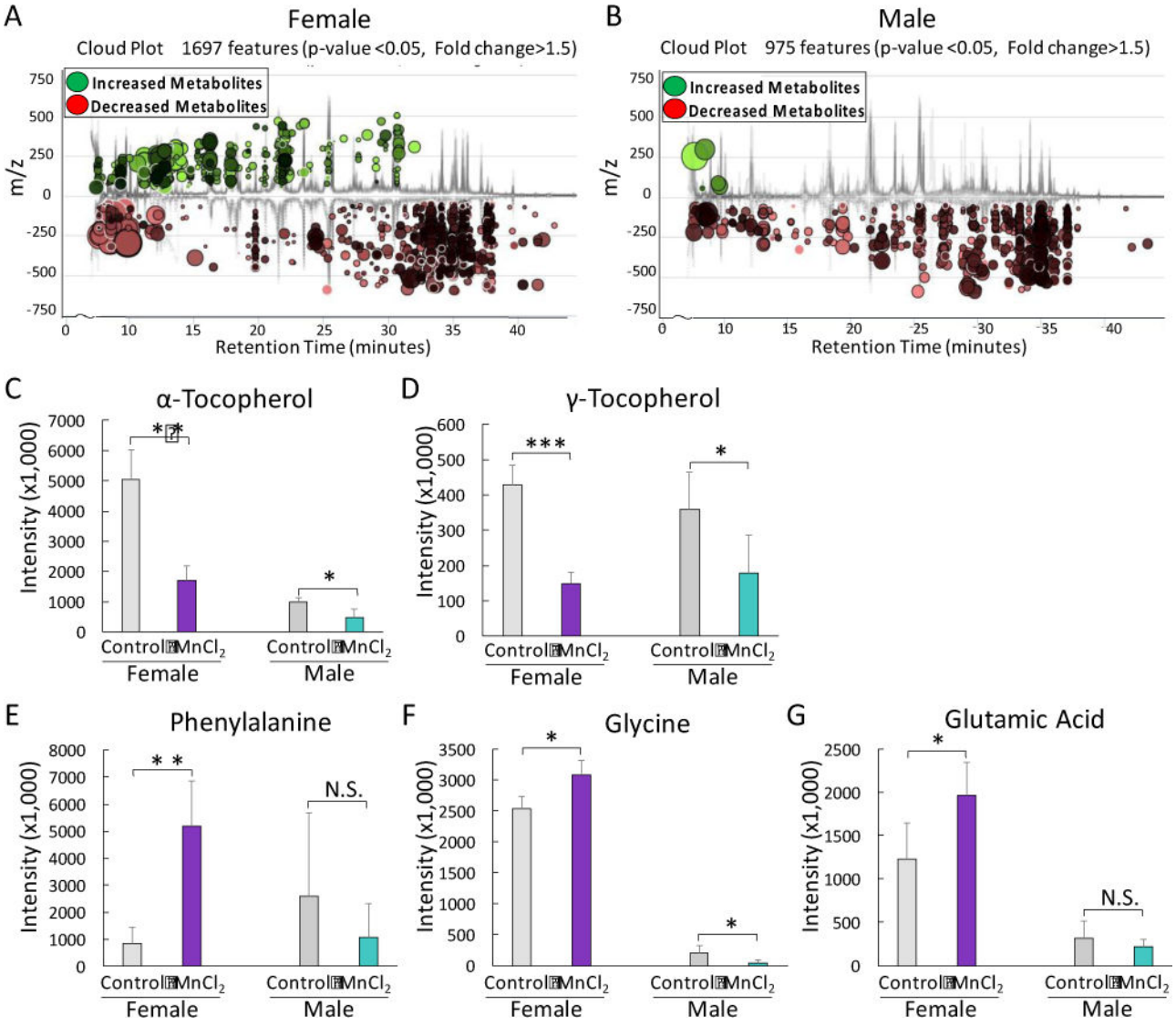

Fig. 8.

Mn exposure perturbed the metabolic profiles of fecal samples of female (A) and male mice (B). Antioxidants, such as α-tocopherol (C) and γ-tocopherol (D), were significantly decreased in the fecal samples of Mn^{2+}-exposed female and male mice, with a stronger effect being observed in female animals. Relative intensity of phenylalanine (E), glycine (F), and glutamic acid (G) was significantly increased and decreased in Mn^{2+}-exposed female mice and male mice, respectively. (***p<0.001, **p<0.01, *p<0.05, N.S., no statistically significant change)