Crosstalk of gut microbiota and serum/hippocampus metabolites in neurobehavioral impairments induced by oral zinc oxide nanoparticles exposure

jianjun Chen
Chongqing Medical University
Shanshan Zhang
Chongqing Medical University
Chang Chen
Chongqing Medical University
Xuejun Jiang
Chongqing Medical University
Jingfu Qiu
Chongqing Medical University
Yu Qiu
Chongqing Medical University
Yujia Zhang
Chongqing Medical University
Tianxiong Wang
Chongqing Medical University
Xia Qin
Chongqing Medical University
Zhen Zou
Chongqing Medical University
Chengzhi Chen (chengzhichen@cqmu.edu.cn)
Chongqing Medical University https://orcid.org/0000-0002-1087-4911

Research

Keywords: Zinc oxide nanoparticles, Gut microbiota, Gut-brain axis, Neurobehavioral impairment

Posted Date: April 6th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-20829/v1

Version of Record: A version of this preprint was published at Nanoscale on January 1st, 2020. See the published version at https://doi.org/10.1039/D0NR04563B.

Abstract

Background

Gut microbiome can be readily influenced by external factors, such as diet, antibiotic, bacterial/viral infection and environmental toxicants. Gut microbiota-mediated effects of engineered nanomaterials as such become the new frontiers in nanotoxicology.

Methods

An integrated approach combining 16S rRNA gene sequencing and liquid chromatography-mass spectrometry (LC-MS) metabolomics was used to determine the potential mechanistic pathway by which disturbed gut microbiota induced by ZnONPs might modulate host physiology and neurobehavior.

Results

Herein, we showed oral exposure to zinc oxide nanoparticles (ZnONPs), one typical kinds of nanomaterials used widely in the food industry, could cause the neurobehavioral dysfunctions in mice, manifested by spatial learning and memory deficits and locomotor activity inhibition. Our mechanistic results elucidated that ZnONPs exposure led to a marked disturbance of gut microbial composition but did not alter the microbiome α-diversity indexes. We also provided new evidence that neurobehavioral impairments induced by ZnONPs was closely associated with perturbation in the gut microbiota composition that were both specific to changes of neurobehavior-related genes (such as Bdnf and Dlg4) and correlated with serum and hippocampal metabolic disorders. Our data further identified a unique metabolite [DG(15:0/0:0/22:4n6)] that linked the relationships among gut microbiota, metabolites and neurobehavior-related genes.

Conclusions

ZnONPs exposure not only alters the gut microbiome community but also substantially disturbs its metabolic profiles, and therefore leading to neurobehavioral impairments vi gut-brain axis. These findings will provide a novel view for understanding the ZnONPs neurotoxicity through gut-brain axis and may lead to new potential prevention and treatment strategies.

1. Background

Nanotechnology refers to the application of materials with length scales in the nanometer range (typically 1-100 nm). Accumulated consumer products containing engineered nanomaterials have been launched to the market with the development of nanotechnology [1]. Particularly, nanotechnology is expected to bring a range of benefits to the food sector aiming at providing better quality and conservation [2]. Major metal oxide nanoparticles of zinc, titanium and silica are showing promise in food safety and technology [2]. Since zinc (Zn) is the most important essential trace element for human beings and that there are hundreds of functional enzymes containing Zn in the body, zinc oxide nanoparticles (ZnONPs) are used

for nutritional purposes in food based on the enhanced gastrointestinal Zn uptake [3]. Moreover, ZnONPs have been added into food packaging materials or coated onto dental implants due to the excellent antibacterial ability [4]. Although zinc oxide is currently listed as a generally recognized as safe (GRAS) material and approved to be used as a food additive by the food and drug administration, materials in nano-sizes will adopt unique properties that are not present when the materials are in their original form [3, 5]. Therefore, there is a considerable concern that potential adverse effects may be associated with the application of ZnONPs as food additive.

Gastrointestinal tract is the major target organ of ZnONPs due to their wide application in the food industry. Mounting evidence indicates microbiota within the gut has a variety of neurofunctions, such as learning, memory and neurodevelopment etc., because of the appreciation of the importance of a distinct microbiota-gut-brain axis [6, 7]. The composition of gut microbiome is highly diverse, and this diversity can be readily influenced by external factors, such as diet with food additive, antibiotic or drug use, bacterial/viral infection and environmental toxicants [8]. Perturbation of gut microbiota is capable to show a profound impact on the neurofunctions [9, 10]. This raises the possibility that exposure to toxic food additive/chemicals leads to gut microbiota disturbance as a mechanism by which external agents exert their deleterious effects on human health.

Increasing studies have focused on direct toxic effects of nanoparticles on the exposed cells or animals, but few investigations have detected potential effects of nanoparticles on the intestinal microbiota of host and the clinical consequences [11]. Recently, available studies suggest that several nanoparticles, including titanium dioxide, cerium dioxide, nano-silver and silica nanoparticles, may cause the detrimental effects on the gut microbiota and that clinical disorders such as immunological dysfunctions, colitis, metabolic and inflammatory diseases may follow [12, 13, 14, 15]. Gut microbiota-mediated effects of engineered nanomaterials as such become the new frontiers in nanotoxicology [11]. Since neurobehavioral disorders due to alterations of gut microbiota have been established for several years [7, 8], the aforementioned evidence indicates disturbance of the microbiota-gut-brain axis induced by ZnONPs may result in neurobehavioral impairment by affecting gut microbiota.

To address this issue, in this study, 16S ribosomal RNA (16S rRNA) gene sequence-based approach was applied to compare the gut microbial communities of ZnONPs-treated animals with vehicle controls to assess whether microbial dysbiosis-induced by ZnONPs was associated with the neurobehavioral dysfunction. To further capture functional readout of microbial activity, we applied an integrated approach combining 16S rRNA gene sequencing and liquid chromatography-mass spectrometry (LC-MS) metabolomics to determine the potential mechanistic pathway by which disturbed gut microbiota induced by ZnONPs might modulate host physiology and neurobehavior. Our results highlight the potential deleterious effects of nanomaterials as the new environmental risk factors for gut-brain axis-mediated neurobehavioral dysfunctions.

2. Results

2.1 Oral exposure to ZnONPs did not significantly alter the α-diversity of microbiota but change the gut compositions in mice

The workflow diagram for this study was showed in the Fig. 1. After orally exposed to ZnONPs by intragastric administration for 30 days, no significant alterations on the body weight in ZnONPs-treated mice in comparison to vehicle controls were found. Also, no mortality was observed during the treatment of ZnONPs (data not shown). The fecal pellets of two groups were then collected at the end of administration and subjected to the 16S rRNA sequencing immediately. The microbial community profiles were produced by clustering 16S rRNA sequences into operational taxonomic units (OUT, ≥ 97% sequence match). The coverage indexes of both two groups exceeded 99%, indicating a high level of diversity coverage in the samples. The fecal microbiota from the two groups showed a similar Shannon index (P = 0.73), Simpson index (P = 0.43), phylogenetic diversity (P = 0.52) and observed species (P = 0.75) (Fig. 2A-2D). These results indicated that there were no significant differences on within-sample (α) phylogenetic diversity analysis between the two groups. Intriguingly, the results of PCoA demonstrated ZnONPs-treatment obviously changed the gut microbial community compositions as compared with vehicle controls (Fig. 3A). We further observed the relative abundances of Actinobacteria were significantly increased in ZnONP-treated group in comparison to vehicle group (Fig. 3B). To identify the gut microbiota primarily responsible for the discrimination between the two groups, the linear discriminant analysis effect size (LEfSe) was performed. This analysis identified 15 different bacterial clades for the discrimination between two groups (Fig. 4A and 4B).

2.2 Oral exposure to ZnONPs caused the spatial learning and memory deficits and locomotor inhibition in mice

Morris water maze is a classic and widely used test for examining spatial learning and memory ability [16]. To determine whether ZnONPs exposure affect the spatial learning and memory ability or not, the Morris water maze was carried out. Our results clearly showed the escape latency and swim distance in ZnONPs-administrated animals were much higher than those of vehicle controls in the navigation task (Fig. 5A and 5B). No significant change was found on the swim speed between two groups (Fig. 5C). On the day 5 of probe trial without hidden platform, we found the number of platform crossings was sharply reduced by ZnONPs and similar trend was depicted in the time spent in target quadrant (Fig. 5D and 5E). The representative track maps of two groups in the hidden platform and probe trial tests were showed in Fig. 5F. These results together indicate that exposure to ZnONPs by oral administration leads to the impairments in the spatial learning and memory.

To further detect the impact of ZnONPs on the locomotor activity, the open field test was performed because it is a commonly used test for the measurement of exploratory behavior and general activity in animals [17]. As shown in Fig. 6A, a significant reduction on the total distance in ZnONPs group when

compared with vehicle group was found. Meanwhile, both the central square duration and the distance moved in the center were dramatically decreased in ZnONPs-treated mice as compared with vehicle ones (Fig. 6B and 6C). The representative track maps of two groups in the open field test were shown in Fig. 6D. These findings together indicate oral exposure to ZnONPs is capable to induce the inhibitory effects on locomotor activity.

To reveal the potential molecular mechanisms involved, the candidate genes associated with the functions of learning and memory and locomotor were determined by qPCR assay. Our results revealed that the mRNA expressions of Bdnf and Dlg4 were both enhanced by ZnONPs in the hippocampus as compared with vehicle controls (Fig. 7A and 7B). To our surprise, no apparent alteration was observed on the expression of Grin2a between two groups (Fig. 7C), implying that ZnONPs-induced neurobehavioral dysfunctions might be regulated by the specific genes.

2.3 Oral exposure to ZnONPs remarkably changed the levels of plasma and hippocampal metabolites in mice

Next, we carried out non-targeted metabolomics to investigate whether or which metabolisms modulated by gut microbiome were paralleled by an altered gut-brain axis. As shown in Fig. 8, 30 differentially expressed metabolites (n = 26 from the plasma and n = 4 from the hippocampus) were identified between the two groups. Among these metabolites, we found 18 metabolites were of particular relevance to lipids and lipid-like molecules. As compared to vehicle controls, QH(2), Cortisol, DG(22:5(7Z,10Z,13Z,16Z,19Z)/18:4(6Z,9Z,12Z,15Z)/0:0), Taurocholic acid, Turanose, Tauroursocholic acid, SM(d18:0/18:1(9Z)), Sphinganine, Camellianin A, sodium taurocholate, Sulfamerazine levels in ZnONP group were significantly elevated. On the contrary, the levels of 28-Galloylglucosylpomolate 3-arabinoside, 3-Oxocholic acid, Chenodeoxycholic acid disulfate, Cholic acid, DG(22:5(7Z,10Z,13Z,16Z,19Z)/15:0/0:0), Myzodendrone, LysoPE(22:1(13Z)/0:0), DG(15:0/0:0/22:4n6), PGP(18:3(6Z,9Z,12Z)/18:1(9Z)), PIP2(16:1(9Z)/16:0), (E,E,E)-Sylvatine, PG(16:0/16:1(9Z)), Octanoylglucuronide, PGP(18:0/18:0), erythromycin B(1+), LMST01070013, NPC, Piromidic acid and sodium globostellatate B in the ZnONPs were dramatically reduced in comparison to the vehicle group. Hierarchical clustering heat map constructed using these differential metabolites also showed a consistent clustering pattern within individual groups.

2.4 Relationships among metabolites and bacterium after oral exposure to ZnONPs

Increasing evidence indicates that perturbations of the gut microbiome and its influence on metabolic functions may play an essential role in the development of various disease [18, 19]. Therefore, to determine the relationships among metabolites and bacterium after oral exposure to ZnONPs, the correlation pairs of metabolite-bacterium were then analyzed. As shown in Fig. 9A, the loading values of each differential metabolite and bacterial clade were calculated by rCCA. The loading values of 14

differential metabolites (Cortisol, 3-Oxocholic acid, LMST01070013, (E,E,E)-Sylvatine, PGP(18:0/18:0), PGP#, LysoPE(22:1(13Z)/0:0), PG(16:0/16:1(9Z)), CAD, NPC, 28-G, DG(15:0/0:0/22:4n6), Octanoylglucuronide, Myzodendrone and seven differential bacterial clades (g_Sutterella, g_Adlercreutzia, c_Actinobacteria, p_Actinobacteria, f_Bifidobacteriaceae, g_Bifidobacterium and o_Bifidobacteriales) were higher than the others, indicating their greater contributions to the overall correlation between these differential metabolites and bacterial clades. The spearman correlation analysis also showed the similar results (Fig. 9B): these 14 differential metabolites and seven differential bacterial clades had greater contributions to the overall correlation. Our data also showed that four groups of “metabolite-bacterium” correlation pairs stood out, including: 1) cortisol with 13 differential bacterial clades; 2) LMST01070013 with 12 differential bacterial clades; 3) g_Sutterella with 26 differential metabolites; and 4) g_Adlercreutzia with 17 differential metabolites (Fig. 9B).

2.5 Relationships among Bdnf, Dlg4, metabolites and bacterium after oral exposure to ZnONPs

The spearman correlation analysis showed that Bdnf was significantly correlated with five differential bacterial clades (f_Bifidobacteriaceae, p_Actinobacteria, c_Actinobacteria, g_Bifidobacterium and o_Bifidobacteriales) and two differential metabolites ((E,E,E)-Sylvatine, DG(15:0/0:0/22:4n6)). In addition, we found Dlg4 was positively correlated with eight differential metabolites (DG(15:0/0:0/22:4n6), Taurocholic acid, Tauroursocholic acid, Piromidic acid, 3-Oxocholic acid, Turanose, erythromycin B(1+), DG(22:5(7Z,10Z,13Z,16Z,19Z)/18:4(6Z,9Z,12Z,15Z)/0:0)) (Fig. 10).

3. Discussion

Gut microbiota plays essential roles in the maintenance of human health and involves in the development of various diseases [20]. The large group of microbial microflorae inside the gastrointestinal tract can also form a host-microbiota co-metabolism structure that participates in a variety of metabolic processes in the body [21]. Herein, we elucidated the gut microbiota was perturbed by oral exposure to ZnONPs, and this microbiota dysbiosis was strongly associated with neurobehavioral dysfunction, mainly manifested by spatial learning and memory deficits and locomotor activity inhibition. By using metabolomics, our results illustrated animals treated with ZnONPs displayed disturbances of metabolites involved in lipid or lipid-like metabolism, including cortisol, which has been highly implicated in the pathological process of memory loss [22]. These findings support the possibility that alterations of gut microbiota induced by ZnONPs may engage in the onset and/or development of neurobehavioral impairments through modulating gut-brain metabolic pathways.

ZnONPs have been used as an anti-bacterial agent against pathogenic microorganisms for years [23]. To our surprise, we found the richness and evenness of gut microbiota in the animals treated with ZnONPs did not show apparent alterations on the α-diversity scores as compared with vehicle controls. Generally speaking, reduced α-diversity is usually thought to represent a marker of “bad” health status. For example, in response to nano-silver exposure, α-diversity were significantly reduced in a dose-dependent manner,

while the β-diversity did not alter enough to reach the statistical difference [24]. By disrupting the gut microbiome, nano-silver consequently caused an increase in anxiety-like and possibly stereotypical behaviors through the gut-microbiome-brain axis in mice [25]. Another kind of nanomaterials, titanium dioxide nanoparticles was capable to shift gut microbiota composition dynamically in a time-dependent manner, although they did not decrease gut microbiota diversity [26]. On the one hand, the above results imply that dysbiosis of gut microbiota in response to ZnONPs may be distinct from the microbiota characteristic of other types of nanomaterials. On the other hand, our data may provide a potential mechanism for explaining the neurotoxic effects of ZnONPs as follow: ingested ZnONPs affect the intestinal epithelial barrier that increases the probability that the metabolites derived from gut microbiota penetrate to enter the systemic circulation; whereupon, they may reach the brain and cause neurofunction impairment.

In this study, we demonstrated the relative abundances of Actinobacteria were significantly reduced by ZnONPs when compared with vehicle controls. The Actinobacteria are ubiquitous gram-positive bacteria and the dominant gut microbial phyla observed in the human gut microbiome [27]. They are also pivotal in the maintenance of gut health and are widely used as probiotic in the therapeutic role against many gastrointestinal and systemic diseases [27]. The decrease in this gut microbiota species suggest abnormal microbial status in ZnONPs-treated mice. However, the mechanisms underlying how Actinobacteria or other specific affect neurobehavioral impairments induced by ZnONPs remain undefined. Several previous studies reported an interesting phenomenon that treatment with Bifidobacteria, one main anaerobe family of Actinobacteria, attenuated the pro-inflammatory immune response and regarded them as probiotic in the therapy of depression [28]. In addition, Actinobacteria exhibited an improvement in the age-related long-term potentiation reduction [29] and involved in regulation of the level of BDNF in the hippocampus [30]. In the context of these findings, the scholars proposed the notion that dysbiosis of gut microbiota might increase the intestinal permeability and consequent up-regulated the systemic inflammation that might also affect the functions of central nervous system [27]. On the other side, gut microbiota produced a variety of neurochemicals and neurotoxic metabolites and therefore strengthening the possibility of the gut-brain axis [31].

Consistent with the neurobehavioral changes, the learning/memory and locomotor functions related-genes Bdnf and Dlg4 were significantly increased after ZnONPs treatment, while no apparent alteration was observed on the expression of Grin2a. As mentioned before, Actinobacteria plays a regulatory role in the expression of Bdnf [30]. It is unlikely a coincidence that increased Bdnf levels in the hippocampus may result from microbiota change after ZnONPs administration. Similar phenomenon was found in another animal model of duodenum-jejunum gastric bypass, showing decreased Actinobacteria led to the increased BDNF protein levels in the hippocampus [32]. Dlg4, also known as PSD-95, is a scaffolding molecule enriched at glutamatergic synapses that involves in the synapse formation and stabilization in plasticity [33]. Previous investigation has demonstrated increased level of PSD95 is highly linked to anxiety-like behavior in germ-free models [34], indicating dysbiosis of gut microbiota may cause the change of PSD95 level.

The metagenomic and metabolomic findings signify that alterations in gut microbiota may be associated with ZnONPs-induced neurobehavioral impairments through gut-brain axis by amino acid and lipid metabolic pathways. Significant disturbances in serum and brain lipids or lipid-related substances have been observed in animals with ZnONPs, including Cortisol, Turanose, Taurocholic acid and Tauroursocholic acid etc.

Intriguingly, some of these lipid-related substances, such as cortisol, play key roles in the formation of myelin and neuronal membranes [35, 36]. Our results showed that these lipids and lipid-related metabolites were typically decreased or increased in the hippocampus and serum of ZnONPs in comparison to vehicle controls. We also found cortisol, LMST01070013 were positively related to more than ten bacterial clades in the gut, while g_Sutterella and g_Adlercreutzia were highly associated with greater than ten metabolites. Whether these observed changes of microbiota were causal or consequent to, or independent of, the corresponding alterations in metabolites requires further studies. Regardless, our findings indicate that the disturbance of gut microbiome induced by ZnONPs resulted in changed content of metabolites in the serum and hippocampus, which may be linked to the neurobehavioral dysfunctions.

There are several limitations worth noting in current study. First, the tissue distribution of ZnONPs was recently shown to occur in a size-dependent manner, and the nanoparticles with size less than 100 nm distributed primarily to the liver, lung and kidney, but not to brain [37, 38, 39]. That means the neurotoxic effects induced by oral ZnONPs administration are probably due to the release of soluble zinc ions from nanoparticles being taken up by the cells in the gastrointestinal tract [39]. Second, the integrated metagenomics and metabolomics analysis was performed to screen the potential biomarkers involved in ZnONPs-neurotoxic effects. Although the findings will provide novel insights regarding perturbations of gut microbiome and its-related neurofunctions, the newly discovered potential targets should be verified in intervention experiment. Third, neurobehavioral phenotypes seen in the animal models may be vary substantially by manner of induction. More importantly, the neurobehavioral disorders are also composed of various behavioral impairments seen in association with neurological disease [40]. Therefore, whether ZnONPs-induced neurobehavioral dysfunctions ultimately leads to the neurological disease should be evaluated by using a battery of neurobehavioral tests in the future work.

4. Conclusions

In summary, in this study, we provide new evidence that neurobehavioral dysfunctions induced by ZnONPs is highly associated with perturbation in the gut microbiota composition that are both specific to changes of neurobehavior-related genes (such as Bdnf and Dlg4) and correlated with serum and hippocampal metabolic disorder. Our results also identify a unique metabolite [DG(15:0/0:0/22:4n6)] that plays in the central role in linking the relationships among gut microbiota, metabolites and neurobehavior-related genes. These findings will propose a novel framework for understanding the neurotoxic mechanisms of ZnONPs through gut-brain axis and may lead to new potential prevention and treatment strategies.

5. Materials And Methods

5.1 Animals and treatment of ZnONPs

Healthy male specific pathogen-free C57BL/6J mice, aged 4 weeks and weighed 8–12 g, were obtained from Experimental Animal Center of Chongqing Medical University [License numbers: SCXK(Yu)2012-001]. All the experimental procedures involving animals were approved by the Institutional Animal Care and Use Committee of Chongqing Medical University. Animals were fed with a standard rodent diet and tap water ad libitum, and they were kept in the controlled room with conditions at the temperature 23 ± 1 °C and humidity 55 ± 10% on a 12 h light/dark cycle. The mice were randomly subjected into vehicle group and ZnONPs group by the website https://www.randomizer.org, and treated with either vehicle solution or with 26 mg/kg ZnONPs suspension solution once a day by intragastric administration.

Zinc oxide nanoparticles (ZnONPs), < 50 nm particle size, were from Sigma Aldrich Chemical Co. (MO, USA). The characteristics of ZnONPs, such as the level of agglomeration, chemical elemental composition, size and zeta potential, had been described in our previous work [41]. The intragastric dosage of ZnONPs at 26 mg/kg were calculated based on Chinese National food safety standard, Infant formula (GB 10765 – 2010) and food and nutrition enhancer standard (GB14880-2012) [42]. The detailed calculation methods and the preparation of ZnONPs solution were described in previous work [41]. The treatment of ZnONPs carried out in the morning between 9:30 AM and 10:30 AM and lasted for 30 days.

5.2 Morris water maze

Morris water maze was performed to determine the spatial learning and memory function as described previously [43]. Before test, the animals were habituated to the water maze with a 60 sec free swim in the pool without a platform. The hidden platform test was carried out for four consecutive days. The swim path of each animal was recorded by tracking system with video camera during the trials. Escapes latency, swim distance and swim speed were recorded. At last day, the probe test was carried out without the platform. The time spent in the target quadrant and the numbers of platform crossing were recorded during the 60 sec test.

5.3 Open field test

Open-field test was used to evaluate the locomotor activity according to the procedures described previously [43]. Briefly, the animal was placed in the center of the open field apparatus in the same square facing the same direction. The activity of animal in the apparatus box were observed by tracking system with video camera. Each test lasted for five minutes, and the apparatus was cleaned with ethanol between tests. The total distance, distance moved in center and central square duration were recorded.

5.4 Quantitative PCR assay

The quantitative PCR assay was carried out to determine the mRNA expressions of target genes according to procedures described previously [44]. In brief, total RNA was isolated by using TRizol method. First strand cDNA was synthesized from total RNA using Perfect Real Time PrimeScript™ RT

Master Mix, and the quantitative RT-PCR was performed with the TBGREEN Premix Ex Taq™ II (TliRNaseH Plus) on CFXConnect™ Real-Time System (Bio-Rad, Hercules, CA, USA). PCR reaction was taken as follows: the initial step was 95 °C for 2 min, followed by 40 cycles at 95 °C for 5 sec, 15 sec at 60 °C and 20 sec at 72 °C. The specific primers of Bdnf, Dlg4 and Grin2a were shown in Supplementary Table. 1. β-actin was used as normalized gene.

5.5 16S rRNA gene sequencing

We collected the fecal samples and then stored them at -80 °C. In this study, we used the standard Power Soil kit protocol to extract bacterial genomic DNA. Firstly, we thawed the frozen sample on ice and then pulverized it with a pestle and mortar in liquid nitrogen. Secondly, we mixed MoBio lysis buffer and these samples. After centrifuging, we put the obtained supernatant into the MoBio Garnet bead tubes. Roche 454 sequencing (454 Life Sciences Roche, Branford, PA, USA) was used here to obtain the raw sequences. The V3-V5 regions of the gene were PCR-amplified with barcoded universal primers containing linker sequences for 454-pyrose-quencing [45].

5.6 16S rRNA gene sequencing analysis

To obtain the unique reads, the Mothur (version 1.31.2) was used to quality-filter the raw sequences [46]. The exclusion criteria of raw sequences included: i) the length less than 200 bp or greater than 1000 bp; ii) containing any barcode mismatches, ambiguous bases and primer mismatches; and iii) homopolymer runs exceeding six bases. The remaining sequences were assigned to operational taxonomic units (OTUs) (97% threshold), and then taxonomically classify using the RDP reference database [47]. At last, to calculate the relative abundances of gut microbiota at different levels, we used these taxonomies to construct summaries of the taxonomic distributions of OTUs. In this study, four different parameters (Shannon index, observed species, phylogenetic diversity and Simpson index) were used to assess the α-diversity. The β-diversity was reported according to principal coordinate analysis (PCoA). Furthermore, to obtain the dominant phylotypes responsible for differences between the two groups, the linear discriminant-analysis effect size (LEfSe) was used.

5.7 Metabolomic analysis

The 50 μL of plasma sample was mixed with 300 μL of acetonitrile, vortexed and centrifuged at 17,000 g for 5 min at 4 ºC. The supernatant was transferred and dried at 30ºC under vacuum using a CentriVap® concentrator (Labconco Corporation, Kansas City, MO, USA), followed by reconstitution in the mixture of 20 μL methanol and 80 μL water. Hippocampus was first homogenized with 50 μL of saline and then extracted in the same way as the serum samples. The 5 μL of the hippocampus or serum extract was injected for metabolome profiling. An ACQUITY I Class UPLC system (Waters, Milford, MA, USA) and a Waters ACQUITY UPLC HSS T3 column (2.1 × 100 mm, 1.8 μm) coupled to a Waters ACQUITY UPLC HSS T3 VanGuardTM pre-column (2.1 × 5 mm, 1.8 μm) were used for analyte separation. A 25.5-min gradient at 450 μL/min was used. 0.1% formic acid and 5 mmol/L ammonium formate (pH 9) were used as mobile phase A in positive and negative modes, respectively, while acetonitrile was used as mobile phase B in both positive and negative modes. The gradient was set as follows: 0–1 min: 2–5% B; 1–3 min: 5–

40% B; 3–17 min: 40–98% B; 17–23 min: 98% B; 23-23.1 min: 98 – 2% B, 23.1–25.5 min: 2% B. The UPLC system was coupled with a G2-S QTOF system (Waters, Milford, MA, USA) in MSE mode at the resolution of 30,000 and the scan rate of 0.2 s in the mass range of 50–2000, with 3 kV capillary voltage, 40 V cone voltage, 80 V source offset, 120 °C source temperature, 40 °C desolvation temperature, 5 h/L cone gas flow and 800 L/h desolvation gas flow for both positive and negative runs. Metabolite identification was performed using Progenesis QITM (Waters, Milford, MA, USA). The software allows peak alignment, peak picking, deconvolution and metabolite identification against the HMDB, ChemSpider, LipidBlast, METLIN, and CCS library databases. Only mass accuracy within ± 5 ppm and overall score ≥ 36 were considered as high confidence and retained for further investigation. Subsequently, the ions and corresponding peaks were exported into EZinfoTM (Umetrics, Umeå, Sweden) for a combinational analysis using ANOVA and multivariate statistics, including principal component analysis (PCA) and orthogonal partial least squares-discriminant analysis (OPLS-DA). Pareto scaling was used for data scaling. Variable importance for Projection (VIP) from OPLS-DA analysis was used to gauge the statistical significance of the metabolite features that contribute to the difference between the control and ZnONPs groups.

5.8 Statistical analysis

All experimental data were presented as mean ± standard error of the mean (S.E.M.). The Student t-test, non-parametric Mann-Whitney U test and chi square test were used when appropriate. The repeated measure analysis of variance (ANOVA) was applied to evaluate the statistical significance on the escape latency, swim distance and swim speed obtained from Morris water maze. The metabolites with variable importance in projection (VIP) > 1.0 and fold change > 2.0 were identified as differential metabolites. Heatmap with hierarchical clustering algorithm was used to show the metabolite difference between the two groups. The spearman correlation analysis and regularized canonical correlation analysis (rCCA) were used to identify the“metabolite-bacterium” correlation pairs. The statistical analysis was performed by using GraphPad Prism 8.0 (GraphPad Software, La Jolla, CA). A p-value less than 0.05 was statistically significant.

Declarations

Acknowledgments

We thank Professor Yan Tang (Southwest Medical University, School of Public Health) for her help on doing the neurobehavioral tests on animals.

Funding

This research was supported by Natural Science Foundation of China (81602820, 81703187, 81903358) and the Science and Technology Research Program of Chongqing Municipal Education Commission (KJQN201800434 and KJQN201900419).

Author contributions

J.C. analyzed the experimental data and wrote the draft manuscirpt. S.Z. performed the major experiments. J.Q. and Y.Q. provided the advice on the study design and revised the manuscript. X.J., X.Q. and C.C. planned some of the experiments and helped in the data analysis. Y.Z. and T.W were responsible for the animal treatment. Z.C.C. and Z.Z. conceived this project, designed the experiments, overseen the progress of work, wrote and corrected the manuscript with inputs from all authors.

Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Consent for publication

Not applicable.

Ethics approval and consent to participate

The study did not include human data. The animal study protocol was approved by the Ethics Committee of Chongqing Medical University.

References

1. Bhushan B. Springer handbook of nanotechnology. Springer; 2017.
2. Chaudhry Q, Watkins R, Castle L. Nanotechnologies in food: What, why and how? 2017.
3. Siddiqi KS, ur Rahman A, Husen AJNrl. Properties of zinc oxide nanoparticles and their activity against microbes. 2018;13 1:1-13.
4. Shi L-E, Li Z-H, Zheng W, Zhao Y-F, Jin Y-F, Tang Z-XJFA, et al. Synthesis, antibacterial activity, antibacterial mechanism and food applications of ZnO nanoparticles: a review. 2014;31 2:173-86.
5. Wang Y, Yuan L, Yao C, Ding L, Li C, Fang J, et al. A combined toxicity study of zinc oxide nanoparticles and vitamin C in food additives. 2014;6 24:15333-42.
6. Dinan TG, Cryan JFJNRG, Hepatology. Gut–brain axis in 2016: Brain–gut–microbiota axis—mood, metabolism and behaviour. 2017;14 2:69.
7. Liang S, Wu X, Jin F. Gut brain psychology: rethinking psychology from the microbiota–gut–brain axis. Frontiers in integrative neuroscience. 2018;12:33.
8. Cryan JF, O'Riordan KJ, Cowan CSM, Sandhu KV, Bastiaanssen TFS, Boehme M, et al. The Microbiota-Gut-Brain Axis. Physiological reviews. 2019;99 4:1877-2013; doi: 10.1152/physrev.00018.2018.

9. Zheng P, Zeng B, Liu M, Chen J, Pan J, Han Y, et al. The gut microbiome from patients with schizophrenia modulates the glutamate-glutamine-GABA cycle and schizophrenia-relevant behaviors in mice. 2019;5 2:eaau8317.
10. Kundu P, Lee HU, Garcia-Perez I, Tay EXY, Kim H, Faylon LE, et al. Neurogenesis and prolongevity signaling in young germ-free mice transplanted with the gut microbiota of old mice. 2019;11 518.
11. Pietroiusti A, Magrini A, Campagnolo LJT, pharmacology a. New frontiers in nanotoxicology: gut microbiota/microbiome-mediated effects of engineered nanomaterials. 2016;299:90-5.
12. Taylor AA, Marcus IM, Guysi RL, Walker SLJEES. Metal oxide nanoparticles induce minimal phenotypic changes in a model colon gut microbiota. 2015;32 7:602-12.
13. Williams K, Milner J, Boudreau MD, Gokulan K, Cerniglia CE, Khare SJN. Effects of subchronic exposure of silver nanoparticles on intestinal microbiota and gut-associated immune responses in the ileum of Sprague-Dawley rats. 2015;9 3:279-89.
14. Chen H, Zhao R, Wang B, Cai C, Zheng L, Wang H, et al. The effects of orally administered Ag, TiO2 and SiO2 nanoparticles on gut microbiota composition and colitis induction in mice. 2017;8:80-8.
15. Chen L, Guo Y, Hu C, Lam PK, Lam JC, Zhou BJEp. Dysbiosis of gut microbiota by chronic coexposure to titanium dioxide nanoparticles and bisphenol A: Implications for host health in zebrafish. 2018;234:307-17.
16. Vorhees CV, Williams MT. Morris water maze: procedures for assessing spatial and related forms of learning and memory. Nature protocols. 2006;1 2:848.
17. Seibenhener ML, Wooten MCJJ. Use of the open field maze to measure locomotor and anxiety-like behavior in mice. 2015; 96:e52434.
18. Zheng P, Zeng B, Zhou C, Liu M, Fang Z, Xu X, et al. Gut microbiome remodeling induces depressive-like behaviors through a pathway mediated by the host's metabolism. 2016;21 6:786-96.
19. Mathewson ND, Jenq R, Mathew AV, Koenigsknecht M, Hanash A, Toubai T, et al. Gut microbiome–derived metabolites modulate intestinal epithelial cell damage and mitigate graft-versus-host disease. 2016;17 5:505.
20. Valdes AM, Walter J, Segal E, Spector TDJB. Role of the gut microbiota in nutrition and health. 2018;361:k2179.
21. Wang X-q, Zhang A-h, Miao J-h, Sun H, Yan G-l, Wu F-f, et al. Gut microbiota as important modulator of metabolism in health and disease. 2018;8 74:42380-9.
22. Echouffo-Tcheugui JB, Conner SC, Himali JJ, Maillard P, DeCarli CS, Beiser AS, et al. Circulating cortisol and cognitive and structural brain measures: The Framingham Heart Study. 2018;91 21:e1961-e70.
23. Sirelkhatim A, Mahmud S, Seeni A, Kaus NHM, Ann LC, Bakhori SKM, et al. Review on zinc oxide nanoparticles: antibacterial activity and toxicity mechanism. 2015;7 3:219-42.
24. Van Den Brûle S, Ambroise J, Lecloux H, Levard C, Soulas R, De Temmerman P-J, et al. Dietary silver nanoparticles can disturb the gut microbiota in mice. 2015;13 1:38.

25. Javurek AB, Suresh D, Spollen WG, Hart ML, Hansen SA, Ellersieck MR, et al. Gut dysbiosis and neurobehavioral alterations in rats exposed to silver nanoparticles. 2017;7 1:1-15.
26. Li J, Yang S, Lei R, Gu W, Qin Y, Ma S, et al. Oral administration of rutile and anatase TiO 2 nanoparticles shifts mouse gut microbiota structure. 2018;10 16:7736-45.
27. Binda C, Lopetuso LR, Rizzatti G, Gibiino G, Cennamo V, Gasbarrini AJD, et al. Actinobacteria: A relevant minority for the maintenance of gut homeostasis. 2018;50 5:421-8.
28. Desbonnet L, Garrett L, Clarke G, Bienenstock J, Dinan TGJJopr. The probiotic Bifidobacteria infantis: an assessment of potential antidepressant properties in the rat. 2008;43 2:164-74.
29. Distrutti E, O'Reilly J-A, McDonald C, Cipriani S, Renga B, Lynch MA, et al. Modulation of intestinal microbiota by the probiotic VSL# 3 resets brain gene expression and ameliorates the age-related deficit in LTP. 2014;9 9.
30. O'Sullivan E, Barrett E, Grenham S, Fitzgerald P, Stanton C, Ross R, et al. BDNF expression in the hippocampus of maternally separated rats: does Bifidobacterium breve 6330 alter BDNF levels? 2011;2 3:199-207.
31. Mayer EA, Tillisch K, Gupta AJTJoci. Gut/brain axis and the microbiota. 2015;125 3:926-38.
32. Jiang S, Wang Q, Huang Z, Song A, Peng Y, Hou S, et al. Gastric bypass surgery reverses diabetic phenotypes in Bdnf-deficient mice. 2016;186 8:2117-28.
33. Chen X, Nelson CD, Li X, Winters CA, Azzam R, Sousa AA, et al. PSD-95 is required to sustain the molecular organization of the postsynaptic density. 2011;31 17:6329-38.
34. Heijtz RD, Wang S, Anuar F, Qian Y, Björkholm B, Samuelsson A, et al. Normal gut microbiota modulates brain development and behavior. 2011;108 7:3047-52.
35. Chari DM. How do corticosteroids influence myelin genesis in the central nervous system? Neural regeneration research. 2014;9 9:909.
36. Choi GE, Lee S-J, Lee HJ, Ko SH, Chae CW, Han HJ. Membrane-associated effects of glucocorticoid on BACE1 upregulation and Aβ generation: involvement of lipid raft-mediated CREB activation. Journal of Neuroscience. 2017;37 35:8459-76.
37. Choi J, Kim H, Kim P, Jo E, Kim H-M, Lee M-Y, et al. Toxicity of zinc oxide nanoparticles in rats treated by two different routes: single intravenous injection and single oral administration. 2015;78 4:226-43.
38. Li C-H, Shen C-C, Cheng Y-W, Huang S-H, Wu C-C, Kao C-C, et al. Organ biodistribution, clearance, and genotoxicity of orally administered zinc oxide nanoparticles in mice. 2012;6 7:746-56.
39. Vandebriel RJ, De Jong WHJN, science, applications. A review of mammalian toxicity of ZnO nanoparticles. 2012;5:61.
40. Zasler ND, Martelli MF, Jacobs HE. Neurobehavioral disorders. Handbook of clinical neurology. 2013;110:377-88; doi: 10.1016/b978-0-444-52901-5.00032-0.
41. Jiang X, Tang Q, Zhang J, Wang H, Bai L, Meng P, et al. Autophagy-dependent release of zinc ions is critical for acute lung injury triggered by zinc oxide nanoparticles. Nanotoxicology. 2018;12 9:1068-91.

42. Wang Y, Yuan L, Yao C, Ding L, Li C, Fang J, et al. A combined toxicity study of zinc oxide nanoparticles and vitamin C in food additives. Nanoscale. 2014;6 24:15333-42.
43. Chen C, Tang Y, Jiang X, Qi Y, Cheng S, Qiu C, et al. Early postnatal benzo (a) pyrene exposure in Sprague-Dawley rats causes persistent neurobehavioral impairments that emerge postnatally and continue into adolescence and adulthood. Toxicological Sciences. 2012;125 1:248-61.
44. Zhang Y, Tu B, Jiang X, Xu G, Liu X, Tang Q, et al. Exposure to carbon black nanoparticles during pregnancy persistently damages the cerebrovascular function in female mice. Toxicology. 2019;422:44-52.
45. Tamaki H, Wright CL, Li X, Lin Q, Hwang C, Wang S, et al. Analysis of 16S rRNA amplicon sequencing options on the Roche/454 next-generation titanium sequencing platform. PloS one. 2011;6 9.
46. Yang S, Liebner S, Alawi M, Ebenhöh O, Wagner D. Taxonomic database and cut-off value for processing mcrA gene 454 pyrosequencing data by MOTHUR. Journal of microbiological methods. 2014;103:3-5.
47. Cole JR, Wang Q, Fish JA, Chai B, McGarrell DM, Sun Y, et al. Ribosomal Database Project: data and tools for high throughput rRNA analysis. Nucleic acids research. 2014;42 D1:D633-D42.

Figures

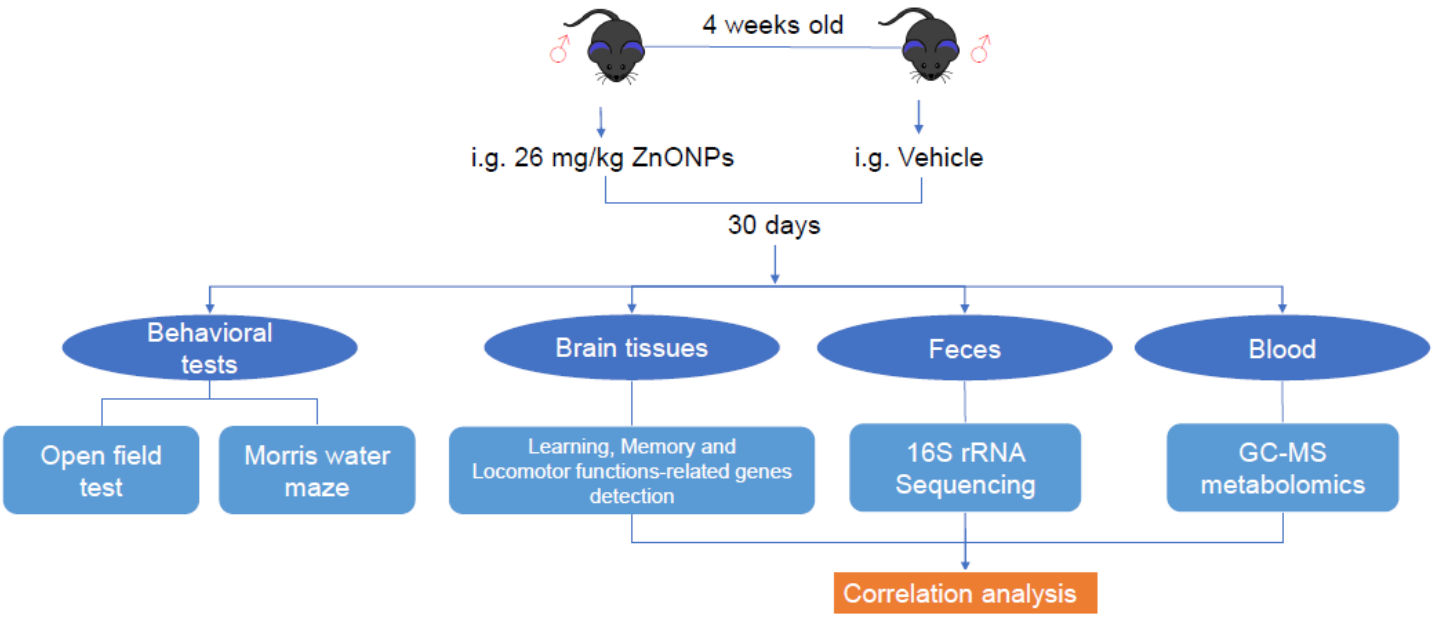

Figure 1

The workflow diagram for this study

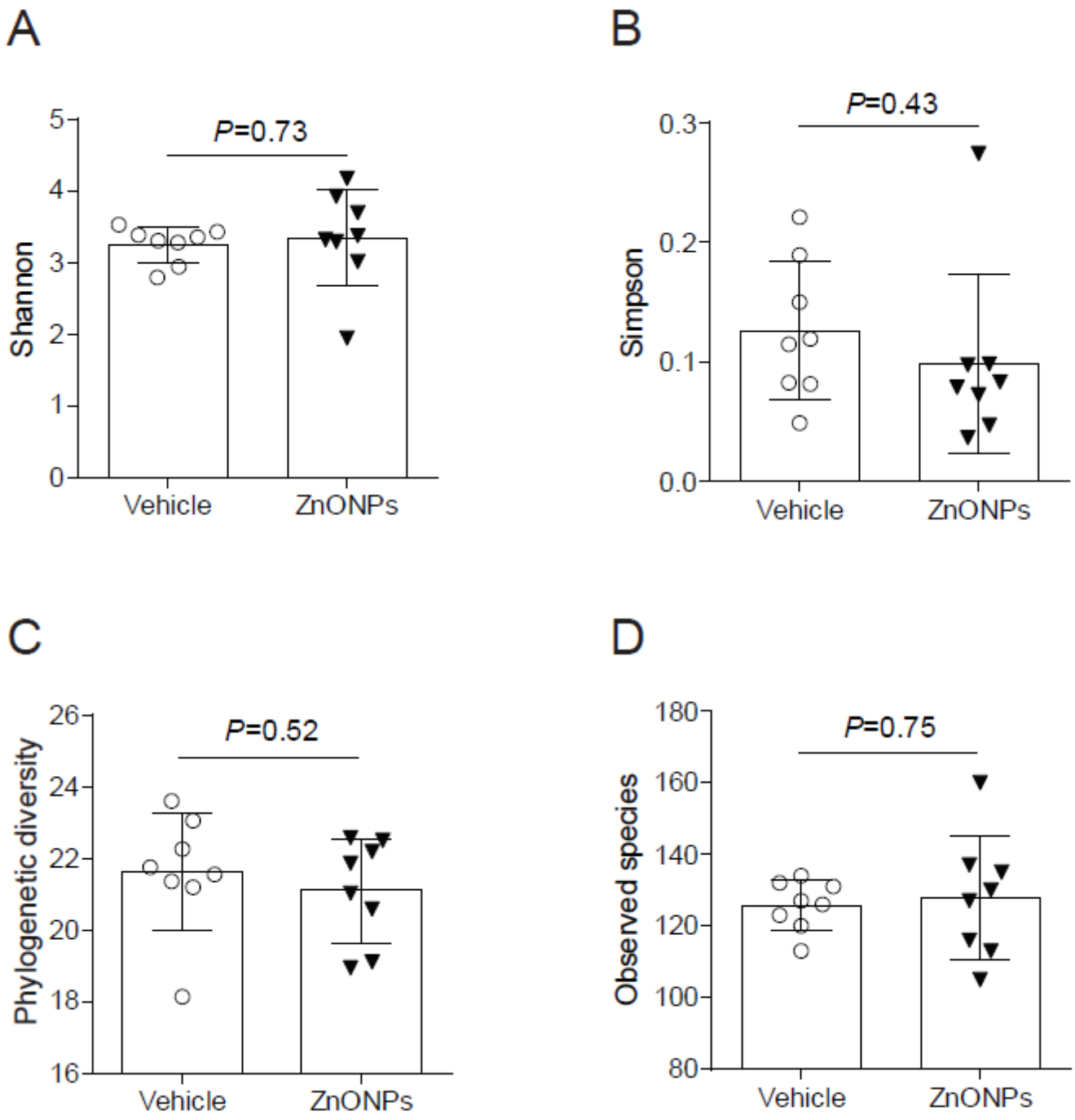

Figure 2

Effects of ZnONPs on the richness and evenness of gut microbiota. After indicated treatment of ZnONPs for 30 days by oral administration, the indexes of richness and evenness of gut microbiota were evaluated. Effects of ZnONPs on the (A) Shannon, (B) Simpson, (C) Phylogenetic diversity were showed. Data were derived were reported as mean ± S.E.M. (n=8).

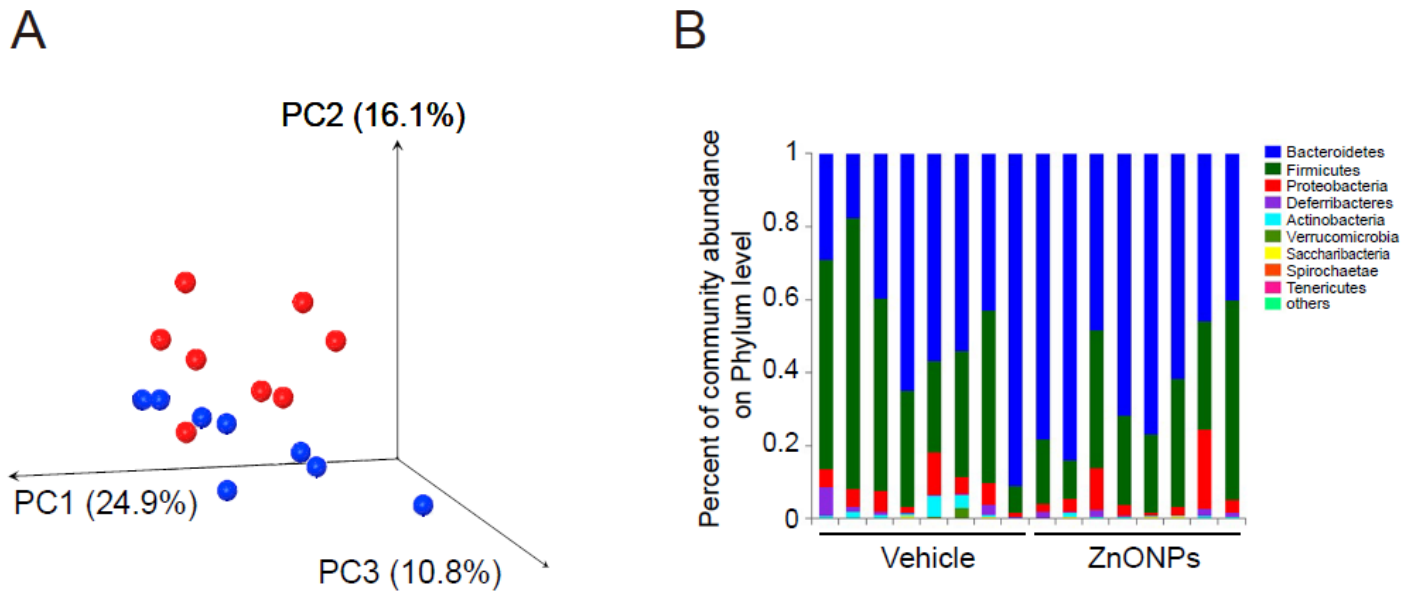

Figure 3

Effects of ZnONPs on the composition of gut microbiota. (A) The results of PCoA showed ZnONPs-treatment significantly changed the gut microbial community in comparison to vehicle controls. The relative abundances of gut microbiota composition were showed in (B). (n=8).

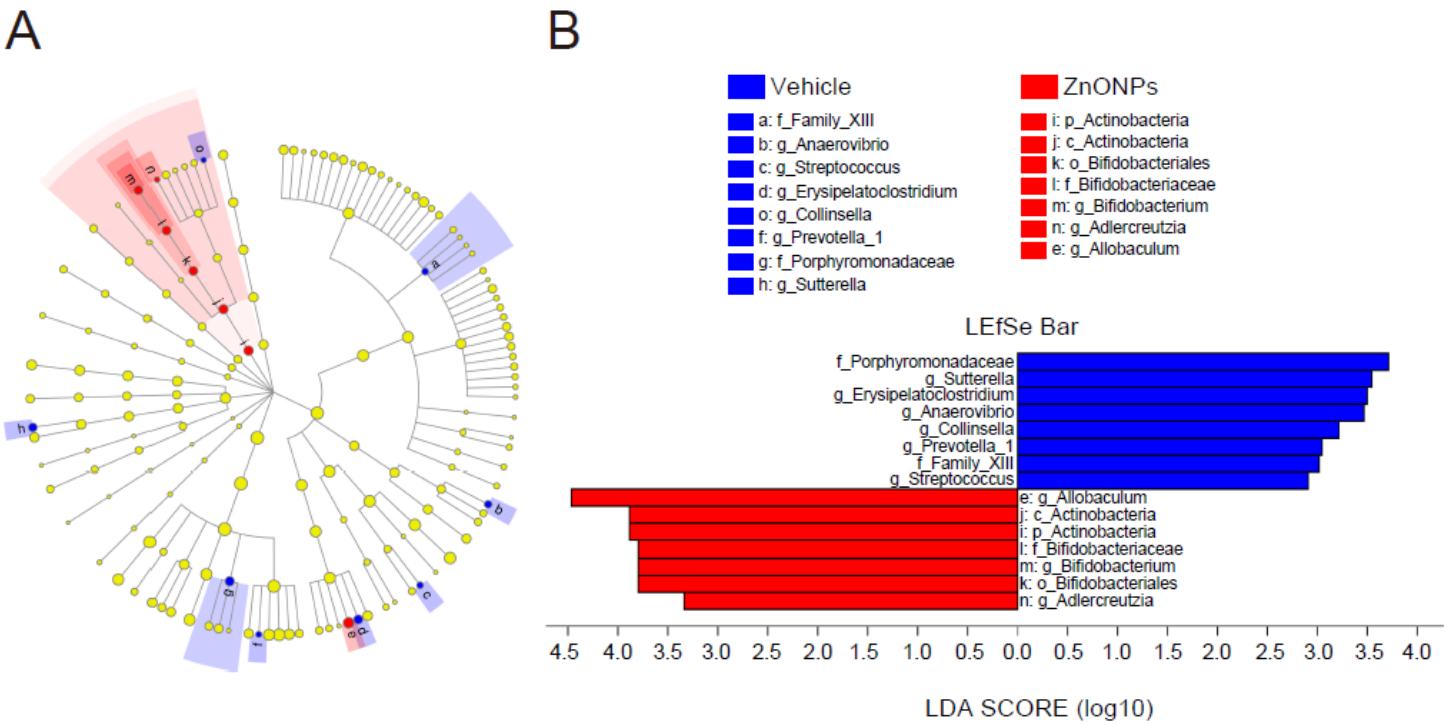

Figure 4

Identification of gut microbiota responsible for the discrimination after oral exposure to ZnONPs. (A) Linear discriminant analysis Effect Size (LEfSe) was performed to identify the gut microbiota primarily responsible for the discrimination between the two groups, and this analysis identified 15 different bacterial clades for the discrimination between two groups (B).

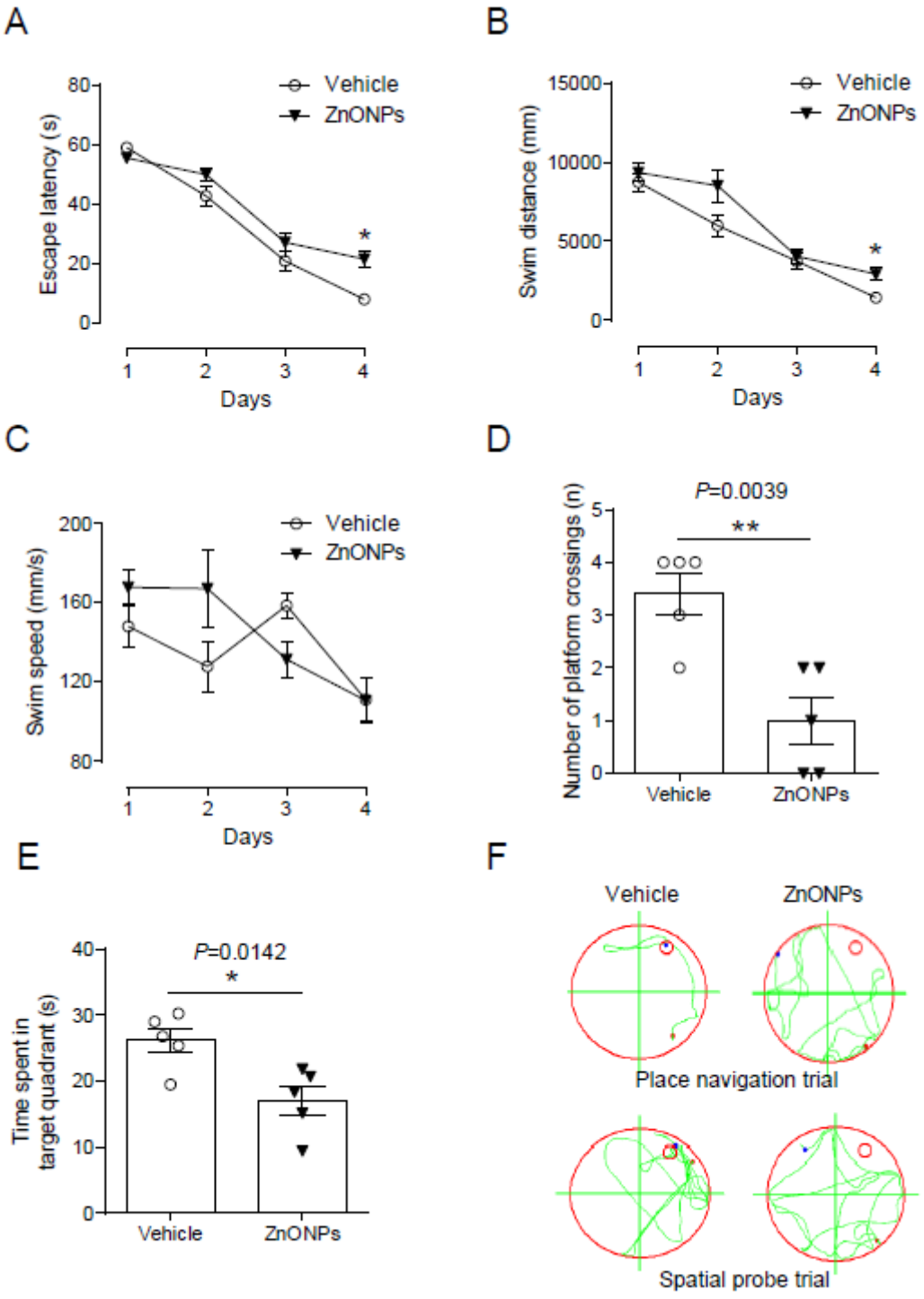

Figure 5

Effects of ZnONPs on the spatial learning and memory function in the mice. Morris water maze was applied to detect the spatial learning and memory ability of mice after ZnONPs exposure. (A) Effects of ZnONPs on the escape latency. (B) Effects of ZnONPs on swim distance. (C) Effects of ZnONPs on swim speed. (D) Effects of ZnONPs on the number of platform crossings. (E) Effects of ZnONPs on the time spent in the target quadrant. (F) Representative track map of two group during the test. Data were derived were reported as mean ± S.E.M. (n=5).

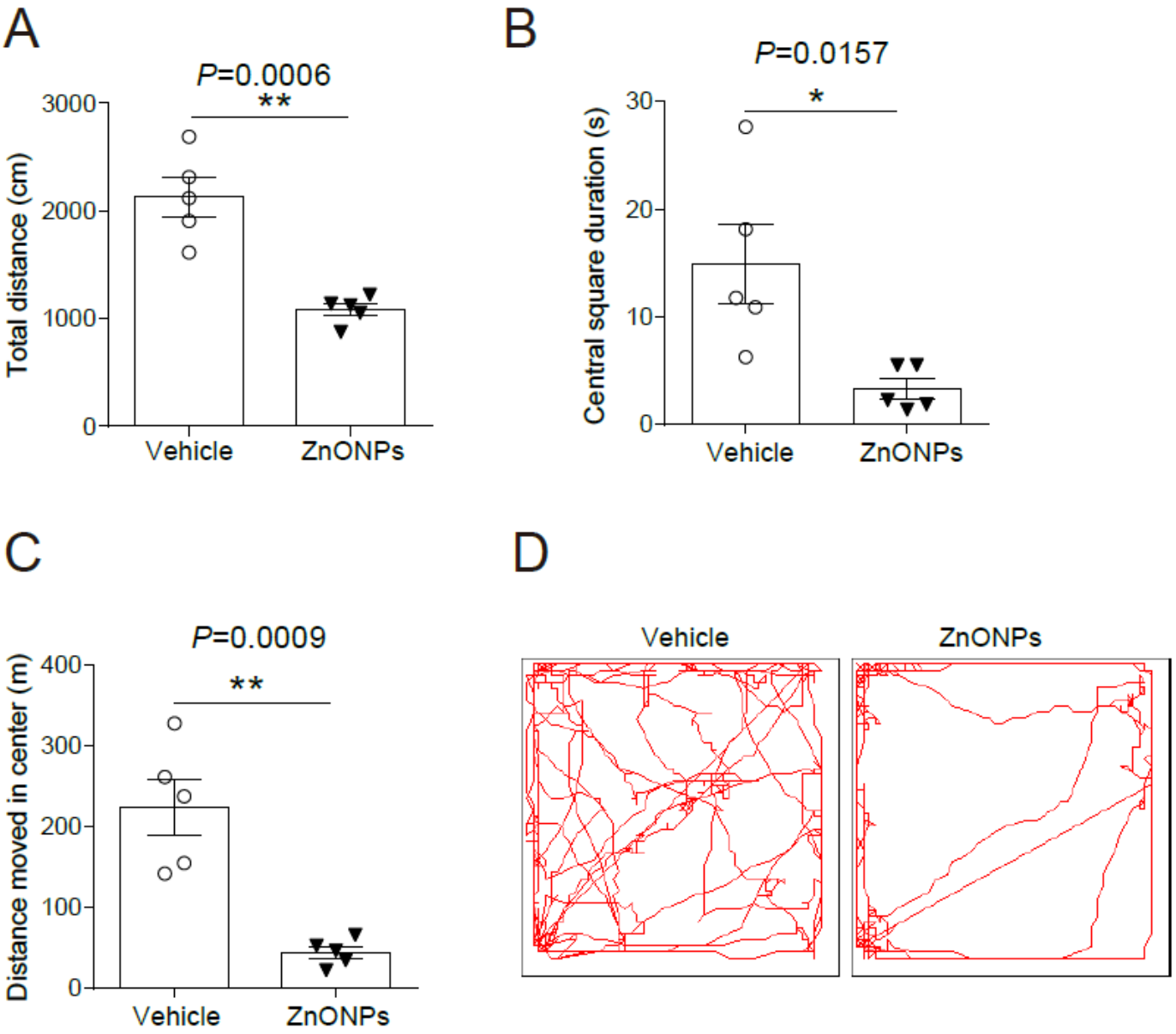

Figure 6

Effects of ZnONPs on the locomotor function in the mice. The open field test was used to evaluate the locomotor activity after ZnONPs exposure. (A) Effects of ZnONPs on the total distance in the test box. (B) Effects of ZnONPs on the central square duration. (C) Effects of ZnONPs on the distance moved in the center. (D) Representative track map of two group during the test. Data were derived were reported as mean ± S.E.M. (n=5).

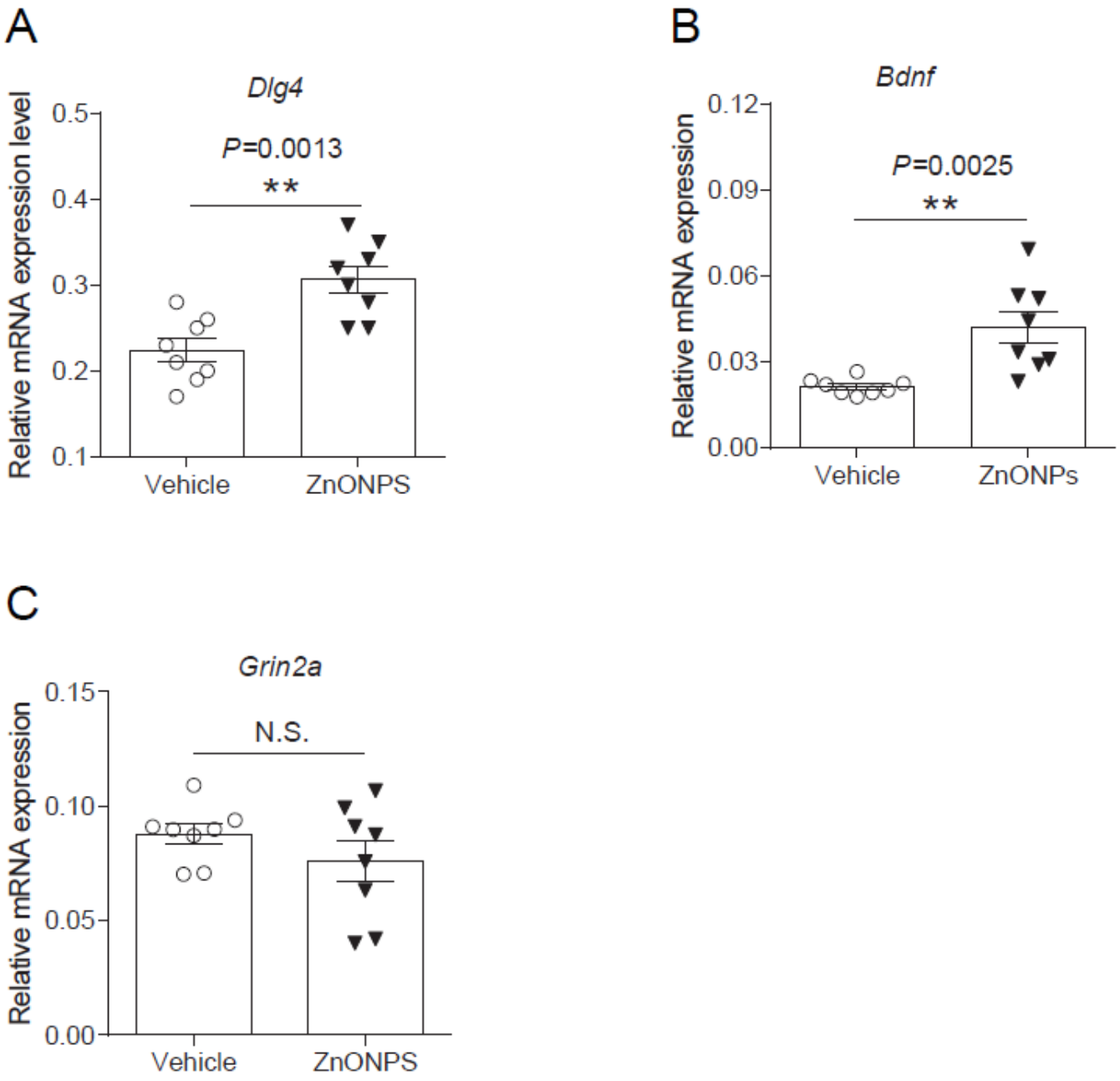

Figure 7

Effects of ZnONPs on the mRNA expressions of Dlg4, Bdnf and Grin2a. The effects of ZnONPs on the mRNA expressions of spatial learning and memory and locomotor functions associated genes in the hippocampus were detected by qPCR assay. The effects of ZnONPs on the expression of Dlg4 (A), Bdnf (B) and Grin2a (C) were showed. Data were derived were reported as mean ± S.E.M. (n=8).

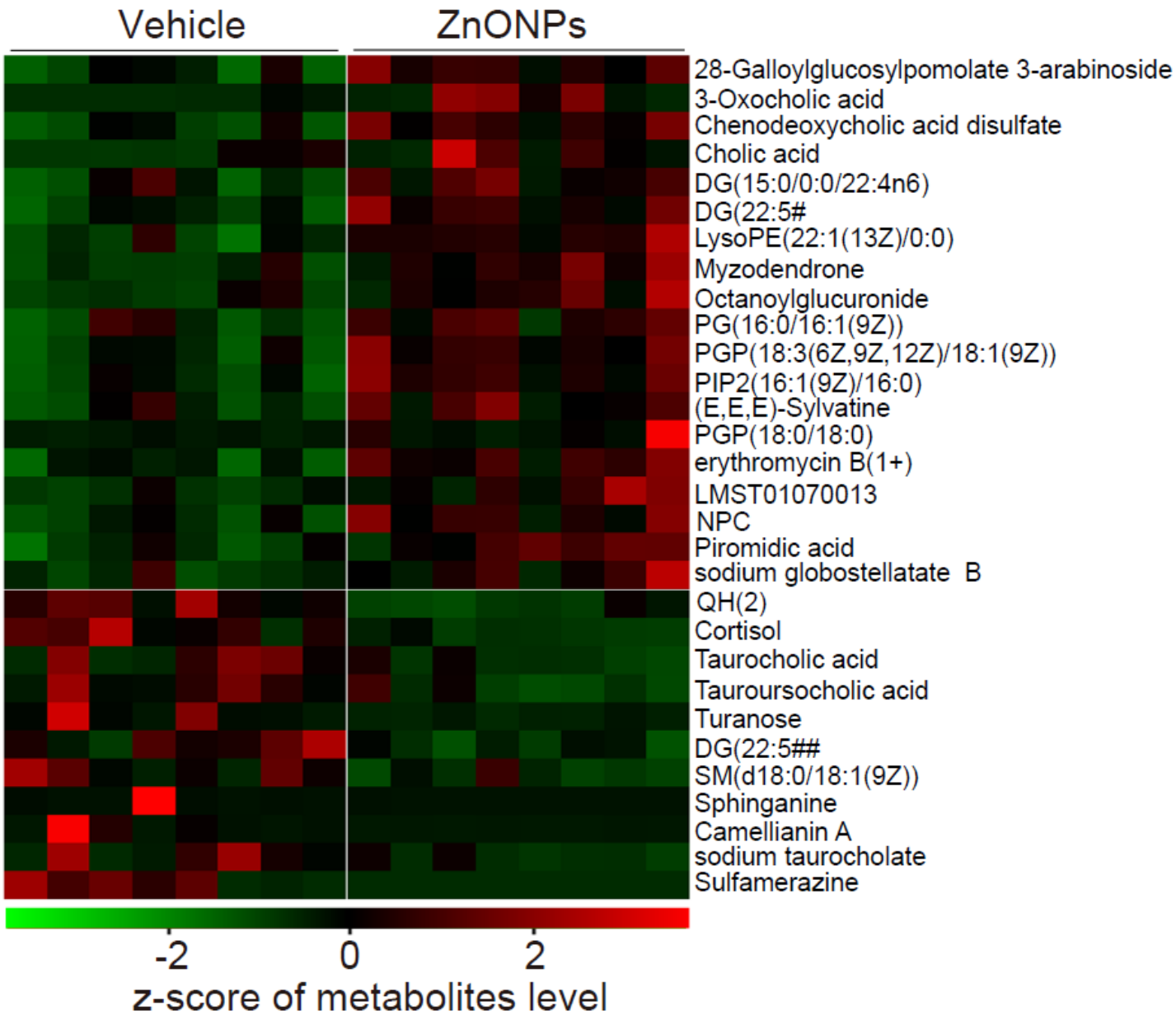

Figure 8

Effects of ZnONPs on the metabolites in the serum and hippocampus. The heat map showed the significant changed metabolites between ZnONPs-treated mice and vehicle ones (n=8).

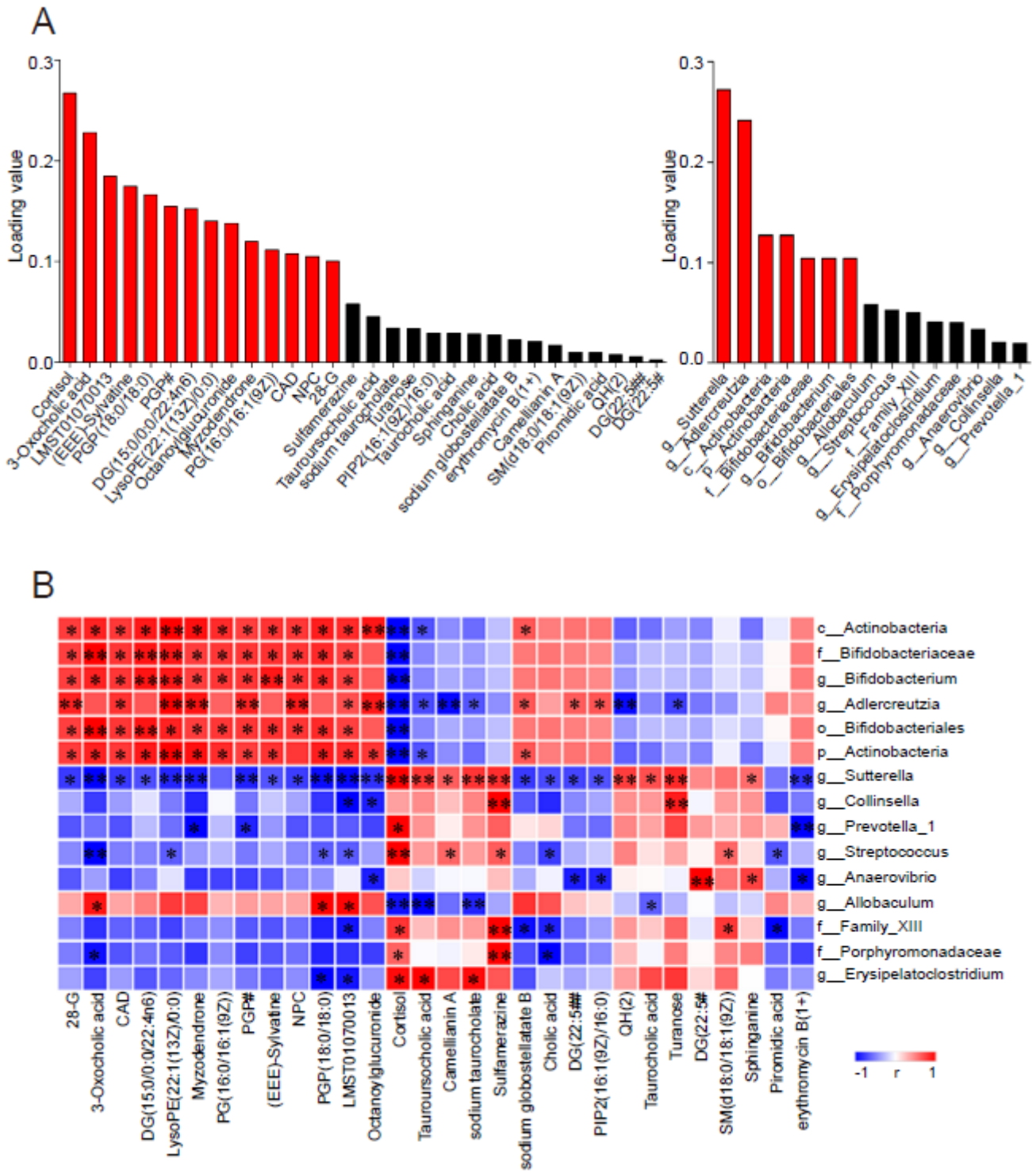

Figure 9

Relationships among metabolites and bacterium in response to oral ZnONPs exposure. The correlation pairs of metabolite-bacterium were analyzed after ZnONPs exposure. The loading values of each differential metabolite and bacterial clade were calculated by rCCA (A) and spearman correlation analysis (B), respectively. “*” denoted the significant difference in the statistical analysis.

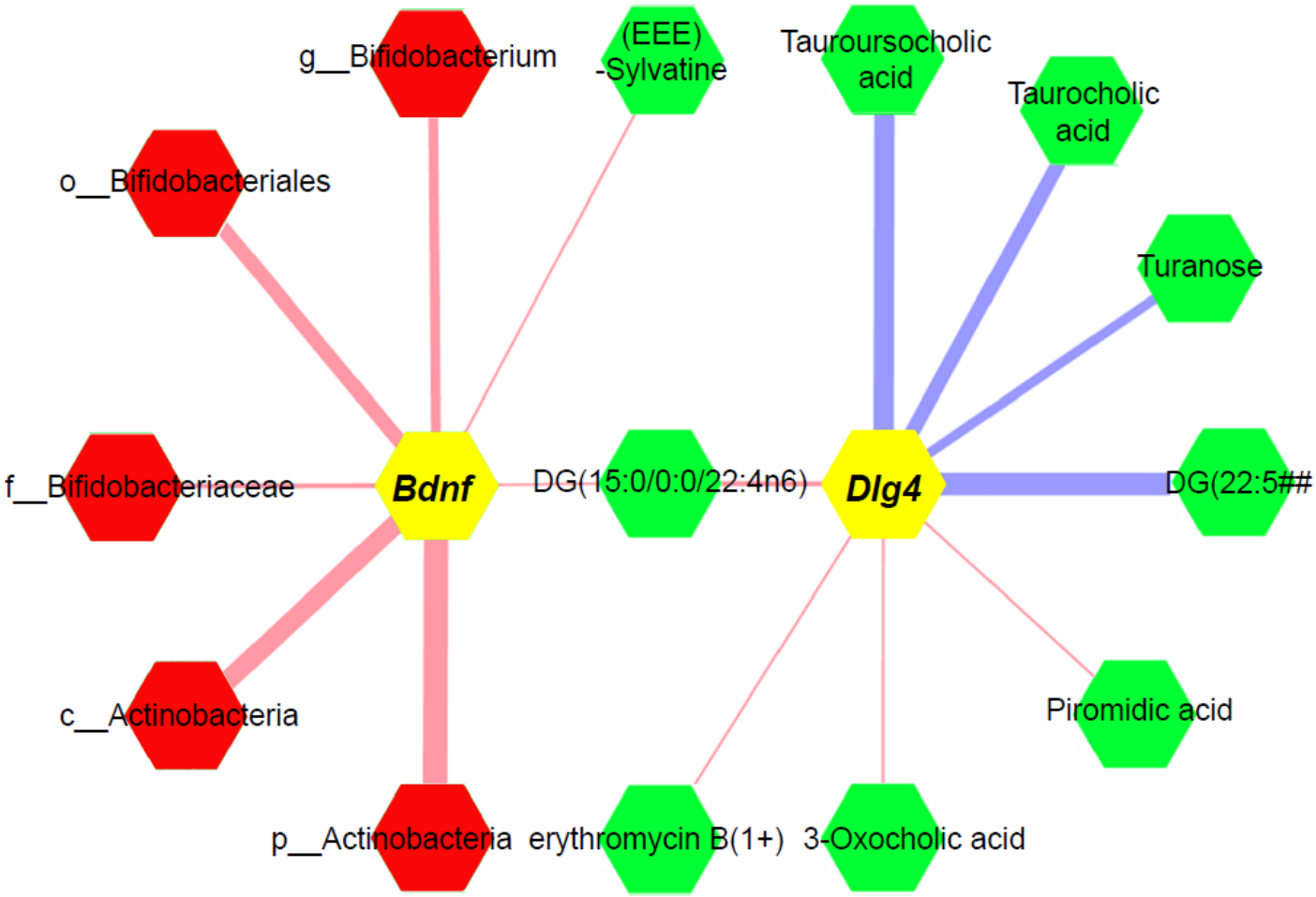

Figure 10

Relationships among gut microbiota, metabolites, Bdnf and Dlg4 in response to ZnONPs exposure. Relationships among Bdnf, Dlg4, metabolites and bacterium in response to oral ZnONPs exposure were analyzed by using the spearman correlation analysis.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryTableJianjunChen.doc