

D-methionine alleviates cisplatin-induced mucositis by restoring the gut microbiota structure and improving intestinal inflammation

Cheng-Hsi Wu*, Jiunn-Liang Ko*, Jiuan-Miaw Liao, Shiang-Suo Huang, Meei-Yn Lin, Ling-Hui Lee, Li-Yu Chang and Chu-Chyn Ou

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Abstract

Background: There are close links between chemotherapy-induced intestinal mucositis and microbiota dysbiosis. Previous studies indicated that D-methionine was an excellent candidate for a chemopreventive agent. Here, we investigated the effects of D-methionine on cisplatin-induced mucositis.

Materials and methods: Male Wistar rats (176–200 g, 6 weeks old) were given cisplatin (5 mg/kg) and treated with D-methionine (300 mg/kg). Histopathological, digestive enzymes activity, oxidative/antioxidant status, proinflammatory/anti-inflammatory cytokines in intestinal tissues were measured. Next-generation sequencing technologies were also performed to investigate the gut microbial ecology.

Results: D-methionine administration increased villus length and crypt depth and improved digestive enzyme (leucine aminopeptidase, sucrose and alkaline phosphatase) activities in the brush-border membrane of cisplatin-treated rats ($p < 0.05$). Furthermore, D-methionine significantly attenuated oxidative stress and inflammatory reaction and increased interleukin-10 levels in cisplatin-induced intestinal mucositis ($p < 0.05$). Cisplatin administration resulted in high relative abundances of Deferribacteres and Proteobacteria and a low diversity of the microbiota when compared with control groups, D-methionine only and cisplatin plus D-methionine. Cisplatin markedly increased comparative abundances of *Bacteroides caccae*, *Escherichia coli*, *Mucispirillum schaedleri*, *Bacteroides uniformis* and *Desulfovibrio C21-c20*, while *Lactobacillus* was almost completely depleted, compared with the control group. There were higher abundances of *Lactobacillus*, Lachnospiraceae, and *Clostridium butyrium* in cisplatin plus D-methionine rats than in cisplatin rats. D-methionine treatment alone significantly increased the number of *Lactobacillus reuteri*.

Conclusion: D-methionine protects against cisplatin-induced intestinal damage through antioxidative and anti-inflammatory effects. By enhancing growth of beneficial bacteria (Lachnospiraceae and *Lactobacillus*), D-methionine attenuates gut microbiome imbalance caused by cisplatin and maintains gut homeostasis.

Keywords: cisplatin, D-methionine, gastrointestinal mucositis, *Lactobacillus*, next-generation sequencing

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Introduction

There is mounting evidence of a close relationship between gastrointestinal (GI) mucositis and

dysbiosis of the gut microbiota.^{1,2} Therapeutic strategies for intestinal disorders can be developed based on the etiologies of these events.

Correspondence to:

Chu-Chyn Ou
School of Nutrition, Chung
Shan Medical University,
110, Sec. 1, Chien-Kuo
N. Road, Taichung 40203,
Taiwan

occ@csmu.edu.tw

Cheng-Hsi Wu
Department of Family
Medicine, Jen-Ai Hospital,
Dali, Taichung, Taiwan

Jiunn-Liang Ko
Institute of Medicine,
Chung Shan Medical
University, Taichung,
Taiwan

Jiuan-Miaw Liao
Department of Physiology,
School of Medicine, Chung
Shan Medical University,
Taichung, Taiwan

Shiang-Suo Huang
Department of
Pharmacology and Institute
of Medicine, Chung
Shan Medical University,
Taichung, Taiwan

Meei-Yn Lin
Ling-Hui Lee
Department of
Food Science and
Biotechnology, National
Chung Hsing University,
Taichung, Taiwan

Li-Yu Chang
Department of Nursing,
Jen-Ai Hospital, Dali,
Taichung, Taiwan

*These authors
contributed to the work
equally and should be
regarded as co-first
authors.

Chemotherapeutic agents effectively kill neoplastic cells, but also damage various organs, including the GI tract, due to their inability to differentiate between normal and neoplastic cells. GI mucositis, a chemotherapy-induced serious side effect, leads to functional and structural damage to the GI tract, such as shortening of intestinal villi, intestinal crypt ablation, local accumulation of inflammatory cells, impaired intestinal barrier function^{3,4} and decreased intestinal digestive enzyme activities.⁵ Clinical symptoms include nausea, vomiting, bloating, anorexia and subsequent body weight loss. Approximately 40% of chemotherapy patients⁶ suffer from GI mucositis which can result in further devastating effects, such as decreased quality of life, longer hospitalizations, higher health care costs, lower dosages of chemotherapeutics and even cessation of treatment.

Five important biological steps in the process of mucositis have been proposed by Sonis.⁷ They are initiation, primary damage response, signal amplification, ulceration and wound healing. Based on the pathobiology of mucositis, the inflammatory response plays a major role in the development of chemotherapy-induced mucositis with atrophy, thinning and ulceration of the mucosal epithelium. The loss of mucosal integrity following chemotherapy treatment results in an increase in intestinal permeability, which enables the entry of numerous microorganisms (especially pathogenic microorganisms). These microorganisms translocate into the mucosa, rapidly reproducing and releasing proinflammatory cytokines.⁷

The abundance and diversity of gut microbiota have been shown to be significantly altered by chemotherapeutic agents.^{1–3} Intestinal microbial dysbiosis is considered to be involved in the pathogenesis of GI mucositis.¹ Moreover, chemotherapy efficacy and toxicity are affected by gut microbiota.^{8,9} It remains unknown whether intestinal microbial dysbiosis directly results in GI mucositis or merely creates a disturbance in the environment of the GI tract.^{3,10}

Cisplatin is effective and widely used in cancer treatment, but has been demonstrated to induce various adverse effects,^{11–13} such as intestinal mucositis.^{14,15} Cisplatin-induced intestinal mucositis, characterized by epithelial sloughing and mucosal ulceration of villous tips, has been reported^{14,16} and the imperfection of mucus

barrier is considered a ‘cause’ of cisplatin-induced mucositis.¹⁷ The intestinal mucosa acts as a barrier against invasion by pathogenic microorganisms. Loss of barrier function enables bacterial translocation and triggers the proinflammatory cascade to initiate intestinal mucositis.⁴ However, a causal relationship between cisplatin-induced GI mucositis and cisplatin-induced changes in the gut microbiota remains limited.¹⁸ D-methionine is a sulfur-containing essential amino acid. D-methionine protection against cisplatin-induced ototoxicity in rats was first reported by Campbell and colleagues.¹⁹ The mechanisms of D-methionine’s otoprotective action were associated with its specific antioxidant enzyme activities and its ability to counteract oxidative stress.^{20,21} In addition, D-methionine had a hepatoprotective effect by the prevention of the decrease of glutathione (GSH) levels in liver²² and antineurotoxicity.²³ The latest double-blind placebo-controlled multicenter phase II trial revealed that oral D-methionine exhibited a protective effect against radiation and cisplatin-induced mucositis²⁴ and did not alter tumor response to cisplatin.^{25,26}

It remains unknown whether D-methionine exerts protective effects on cisplatin-induced GI mucositis and gut microbiota dysbiosis. To test the hypothesis that treatment with D-methionine attenuates cisplatin-induced intestinal mucositis through antioxidative and anti-inflammatory activities, as well as regulation of microbial community composition, we investigated the effects of D-methionine on oxidative stress, inflammatory markers and gut microbiota modification in cisplatin-induced intestinal mucositis. The results revealed that oral administration of D-methionine alleviates cisplatin-induced GI mucositis by suppressing oxidative stress and inflammatory mediators. Therefore, we suggest that D-methionine helps to markedly decrease lipopolysaccharide (LPS)-producing and proinflammatory bacteria (e.g. *Deferribacteres* and *Proteobacteria*) and increase anti-inflammatory bacteria (e.g. *Lachnospiraceae* and *Lactobacillus*).

Materials and methods

Reagents

Cisplatin and D-methionine were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). All other chemicals and reagents used in this study were of analytical grade.

Animals

Male Wistar rats (6 weeks old), weighing 176–200 g, were purchased from BioLASCO Taiwan Co., Ltd. and randomly divided into four groups of eight animals each. Animals were housed in cages with a maximum of four rats per cage on a 12-h light/dark cycle and allowed free access to rodent chow (LabDiet, 5001) and sterile water during the study. The experimental protocols were approved by Affidavit of Approval of Animal Use Protocol, Chung Shan Medical University Experimental Animal Center, Taichung, Taiwan (approval no: 1752).

Experimental protocol

Following 1 week of acclimatization, male Wistar rats were divided into four groups and toxicity was induced by intraperitoneal administration of cisplatin at the dose of 5 mg/kg body weight once a week for 3 weeks. In the control group, rats were given 0.9% (w/v) NaCl injection on the 1st, 8th, and 15th days and oral sterile water daily by gavage. The cisplatin group received cisplatin treatment on the 1st, 8th, and 15th days and oral sterile water daily by gavage. The cisplatin combined with D-methionine group was given cisplatin treatment on the 1st, 8th, and 15th days and D-methionine (300 mg/kg/day) daily by gavage for 3 days before the first cisplatin injection and then until the end of the experiment. D-methionine was administered at least 30 min before cisplatin injection. D-methionine group received a single intraperitoneal injection of 0.9% (w/v) NaCl solution on the 1st, 8th, and 15th days and oral D-methionine (300 mg/kg/day) by gavage 3 days before the first 0.9% NaCl injection and then until the end of the experiment. In current study, the dose of either cisplatin or D-methionine was cited from the previous published literature.^{19,20}

Sample collection and treatment

Body weight, food and water consumption were monitored daily before cisplatin injection. Feeding efficiency was measured weekly, and calculated using the following formula: Feeding efficiency (%) = [body weight change (g)/food intake (g)] × 100%. On day 17, stool samples were collected on the grid floor, placed on a stainless-steel wire mesh, and dried in an oven to measure moisture content. Fecal pH was determined by a pH meter. At the end of the study (day 19), animals were sacrificed by CO₂ asphyxiation and the cecum contents were collected immediately under

laminar flow for 16S DNA (next-generation sequencing; NGS) analysis. Half of each section of duodenum, jejunum and ileum was snap-frozen in liquid nitrogen prior to storage at –80°C for biochemical analysis.

Histological analysis

The remaining intestinal segments were fixed in 10% buffered formalin for 24 h, embedded in paraffin, and cross-sectioned. Then 3- μ m thick transverse sections were stained with hematoxylin and eosin (H&E) to observe morphology changes. For the evaluation of intestinal tissue damage quantitatively, photographs were taken using an Olympus BX60 fluorescence microscope (Olympus America, Melville, NY, USA; original magnification, 40 \times).

Assessment of brush-border membrane enzyme activity

Freshly excised intestines were slit and washed with ice-cold phosphate-buffered saline (PBS) solution, followed by gentle scraping of the mucosa with a glass slide. Iced saline (containing protease inhibitor) was added to the mucosa for homogenization, followed by centrifugation at 10,000 rpm at 4°C for 10 min. The supernatants were used for brush-border membrane (BBM) assays. Aliquots of mucosal homogenates were quickly frozen until further analysis. The activity of alkaline phosphatase (AP) was determined by the yellow color that appeared upon hydrolysis of p-nitrophenyl phosphate. Leucine aminopeptidase (LAP) was assayed using leucine p-nitroanilide as a substrate. Sucrase was assayed by the reduced sugars formed upon the hydrolysis of sucrose. The activities of LAP and sucrase were measured using a Randox assay kit (Randox Laboratories, Crumlin, UK) and AP activity was measured using a BioVision ALP activity colorimetric assay kit (BioVision, Inc., Milpitas, CA, USA).

Determinations of lipid peroxide, antioxidant status and inflammation in intestinal tissues

Intestinal tissue samples were frozen in liquid nitrogen and mixed in a stainless-steel mortar and pestle homogenizer. Ice-cold PBS solution was used to homogenize parts of the frozen crushed intestinal samples to determine the malondialdehyde (MDA) level, reduced GSH level, and glutathione peroxidase (GPx) activity. The MDA level was determined in intestinal homogenates

after reaction with thiobarbituric acid. Total GSH was measured with a modified 5-5V-dithiobis 2-nitrobenzoic acid (DTNB)-GSSG reductase recycling assay. The GPx assay was based on the oxidation of GSH coupled to nicotinamide adenine dinucleotide phosphate by glutathione reductase (GSR). These biochemical parameters were assayed as described by Wen and colleagues.²⁷ A solution of 2% Triton X-100 containing 0.32M sucrose was used to homogenize other parts of the frozen crushed intestinal samples for superoxide dismutase (SOD) analysis. SOD activity in intestinal tissue was measured using colorimetric assay kit (Cayman Chemical, Ann Arbor, MI, USA). Another part of the intestinal tissue samples was homogenized in 1 ml ice-cold PBS solution and then centrifuged at 12,000 *g* at 4°C for 30 min. Supernatants were stored at -80°C for cytokine assays. The levels of interleukin (IL)-1 β , IL-6 and IL-10 in intestinal tissues were measured by specific enzyme-linked immunosorbent assay (ELISA) kits (R & D Systems, Minneapolis, MN, USA). The measurements of IL-1 β , IL-6, and IL-10 were performed step by step based on the manufacturer's standard protocol. The concentration of tumor necrosis factor (TNF)- α was measured using a commercial assay kit according to the manufacturer's instructions (rat TNF- α , ELISA kit, BioLegend, San Diego, CA, USA).

Gut microbiota analysis

Gut bacterial DNA from the rat cecum content was extracted with commercial DNA Stool Mini extraction kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. Library sequencing was performed on Illumina HiSeq 2500 platform. On alpha diversity analysis, gut microbial composition diversities of the groups were evaluated, and various indicators were calculated using Qiime software (version 1.9.1). These indicators included Chaol (community richness), Shannon (community diversity) and Observed Species [estimated operational taxonomic unit (OTU) amounts]. Alpha diversity was also estimated using the phylogenetic diversity metric. Beta diversity analysis was used to compare gut microbiota compositions among the groups and was performed using the unweighted pair group method with arithmetic mean (UPGMA) clustering method based on weighted and unweighted UniFrac distances. Principal coordinates analysis (PCoA) was based on distance matrix. Weighted UniFrac and Unweighted

UniFrac were calculated to assist PCoA. PCoA was conducted and displayed using Qiime software (version 1.7.0).

Statistical analysis

IBM SPSS Statistics 19 was used for all statistical analyses. All data are presented as mean \pm standard error of the mean (SEM). Statistical comparisons were carried out by one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test to measure variations between different groups; $p < 0.05$ was considered statistically significant.

Results

D-methionine effectively alleviates body weight loss and increases food intake and stool output

Loss of body weight and decrease in food intake are common phenomena after cisplatin treatment. They are also basic indicators of cisplatin toxicity. During the adaptation period, there were no differences in body weight or food intake among the four groups. As expected, cisplatin-treated rats and cisplatin combined with D-methionine treated rats showed decreases of 28% and 13% in body weight, respectively, compared with control animals ($p < 0.05$) (Table 1). In cisplatin-treated rats, there was a 95% reduction in food intake compared with the control group ($p < 0.05$). In contrast, co-administration of D-methionine resulted in a decline of 34% compared with the control group ($p < 0.05$). Body weight and food intake were unaffected in the D-methionine alone group when compared with the control group. During the experimental period, we also observed that the severity of anorexia (reduction in food intake) is associated with accumulated dose of cisplatin (data not shown).

To understand whether digestion is altered by cisplatin, feeding efficiency was evaluated and calculated weekly. Supplementary Figure 1(a) shows that, in the second week, the feeding efficiency of rats treated with cisplatin was negative and decreased to -114.6% in the third week ($p < 0.05$). These results implied that cisplatin completely obstructs digestion. However, the decline in feeding efficiency was inhibited by D-methionine supplement, indicating that D-methionine improves digestion. Feeding efficiencies were almost the same in D-methionine alone and control groups. Cisplatin contributes to

Table 1. Effects of D-methionine on body weight, body weight gain and food intake in cisplatin-treated rats.

	Control	D-methionine	Cisplatin	Cisplatin + D-methionine
Initial body weight (g)	304.13 ± 9.09	301.60 ± 10.95	308.63 ± 11.78	305.75 ± 10.79
Final body weight (g)	378.88 ± 9.85	370.80 ± 10.66	266.0 ± 11.66*	331.63 ± 11.30*#
Initial food intake (g)	28.75 ± 2.18	26.67 ± 0.47	29.75 ± 0.94	28.63 ± 1.19
Final food intake (g)	26.92 ± 1.40	25.67 ± 0.47	2.90 ± 2.29*	13.78 ± 6.05*#

Data are presented as mean ± SEM, $n = 5-8$. Differences were analyzed by one-way ANOVA.
 *represents a significant difference when compared with the control group;
 #represents a significant difference when compared with the cisplatin group ($p < 0.05$).
 ANOVA, analysis of variance; SEM, standard error of the mean.

lowered food intake and feeding efficiency. Stool samples also showed reduced stool production and watery cecum content [Supplementary Figure 1(b)]. Fecal water content and pH level were not influenced by cisplatin (Supplementary Table 1). In the cisplatin + D-methionine group, stool output increased with solid cecum content compared with the cisplatin group. Nevertheless, the stool output in the cisplatin + D-methionine group was still lower than in the control and D-methionine alone groups. In the D-methionine alone group, the wet weight, dry matter, fecal water content and pH level of stool remained almost unchanged in comparison with the control group.

D-methionine improves digestive enzyme activities

LAP, sucrase and AP were measured to evaluate digestive function and to determine whether decreases in food intake and feeding efficiency correlate with decreases in digestive enzyme activities. Cisplatin led to reductions in activities of LAP, sucrase and AP, while supplementation of D-methionine in cisplatin-treated rats significantly improved the activities of LAP, sucrase and AP ($p < 0.05$; Figure 1). Administration of D-methionine alone had no effect on digestive enzyme activities when compared with the control group.

D-methionine attenuates cisplatin-induced oxidative stress and inflammation

The high antioxidant efficiencies of D-methionine, also referred to as sulfur antioxidants, are well known. Several studies have revealed that the

protective effect of D-methionine against cisplatin-induced ototoxicity is associated with its potent antioxidant activity.^{19,21,28} MDA, lipid peroxidation product, is the major biochemical consequence of oxidative attack on cell membranes and is an oxidative stress biomarker. In the present study, cisplatin elevated the levels of MDA, decreased GSH content, and reduced GPx and SOD activities in intestinal tissue ($p < 0.05$; Figure 2). D-methionine co-administration resulted in decreased MDA concentrations, elevated GSH levels, and increased enzyme activities of GPx and SOD after cisplatin injection. Interestingly, D-methionine alone not only diminished MDA levels, but also increased antioxidative capacity, by increasing GSH and SOD in the intestinal tissues. Due to the antioxidative property of D-methionine, we next investigated if D-methionine mitigates the production of proinflammatory cytokines in cisplatin-mediated intestinal mucositis. We also observed that D-methionine alone treatment increased IL-10 levels in the intestinal tissues. After cisplatin injection, D-methionine demonstrated beneficial effects, such as decreasing levels of TNF- α , IL-6 and IL-1 β and increasing levels of IL-10, on intestinal tissues compared with cisplatin alone group ($p < 0.05$; Figure 3).

Histological examinations of intestine

Transparency was observed in the harvested intestinal tissues of cisplatin-treated rats. Histopathological examinations of sections of duodenum, jejunum and ileum were carried out with H&E staining [Figure 4(a)]. Intact finger-like elongated structures and compactly arrayed epithelium were observed in the control and

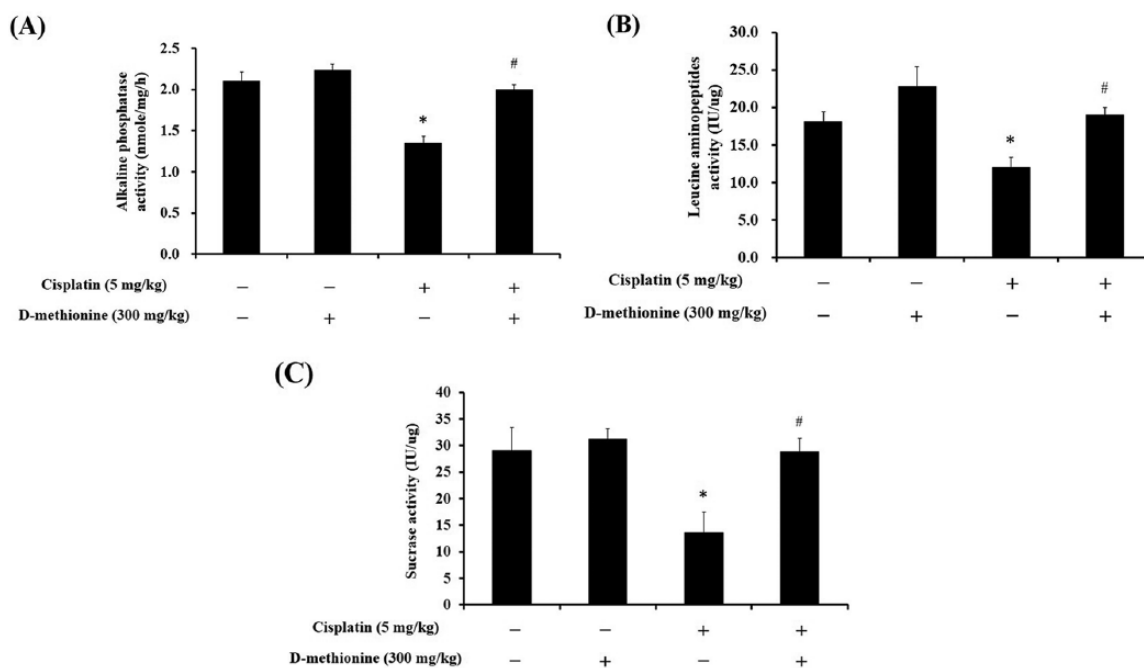


Figure 1. Effects of D-methionine treatment on BBM enzymes: AP, LAP and sucrase in cisplatin-treated rats. Data are presented as mean \pm SEM, $n = 5-8$.

*represents a significant difference when compared with the control group;

#represents a significant difference when compared with the cisplatin group ($p < 0.05$).

AP, alkaline phosphatase; BBM, brush-border membrane; LAP, leucine aminopeptidase; SEM, standard error of the mean.

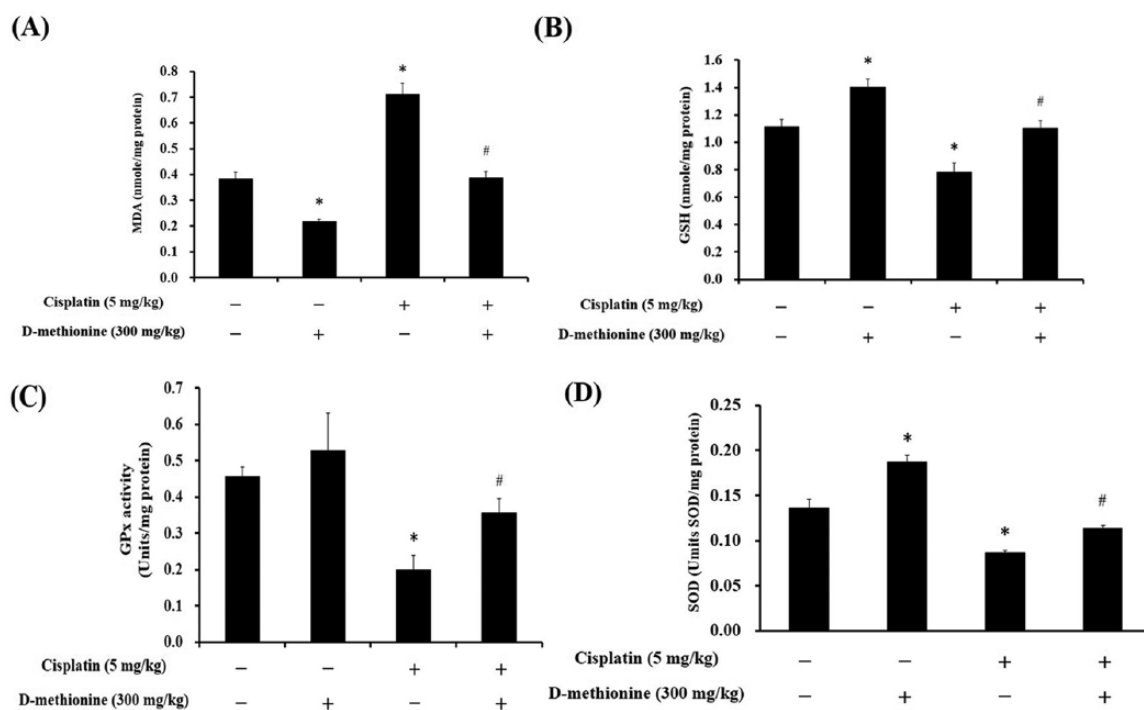


Figure 2. Effects of cisplatin and D-methionine administration on MDA and GSH concentrations and GPx and SOD activities in intestinal tissue homogenates. Data are presented as mean \pm SEM, $n = 5-8$.

*represents a significant difference when compared with the control group;

#represents a significant difference when compared with the cisplatin group ($p < 0.05$).

GPx, glutathione peroxidase; GSH, glutathione; MDA, malondialdehyde; SEM, standard error of the mean.

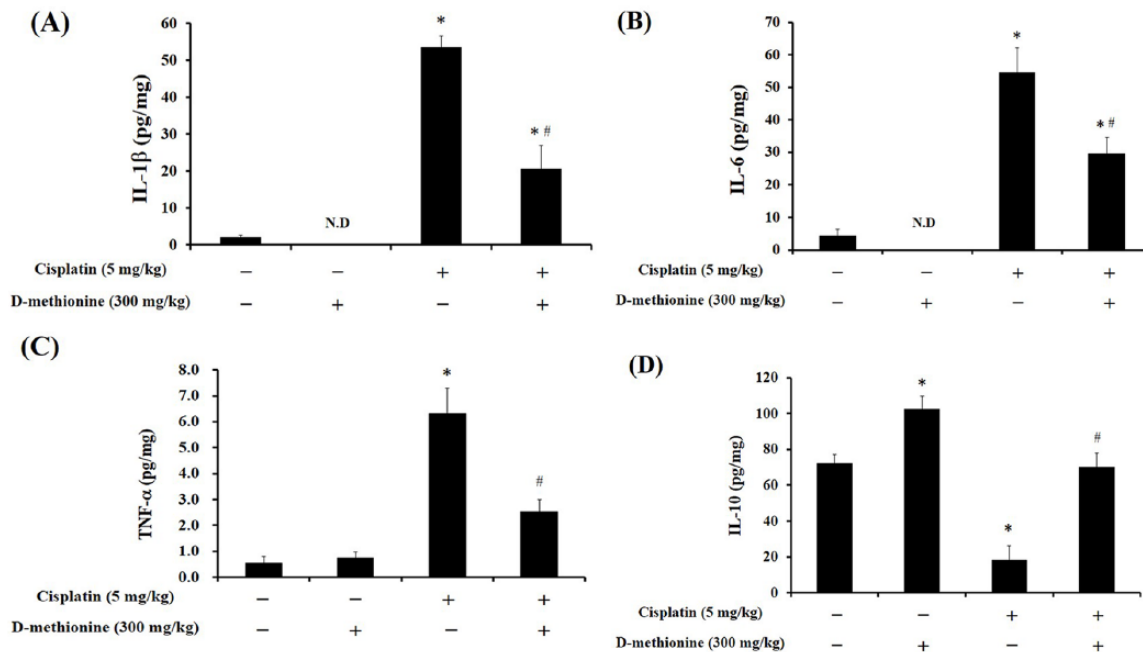


Figure 3. Effects of D-methionine on inflammation parameters in small intestinal homogenates after treatment with cisplatin. (a) IL-1 β , (b) IL-6, (c) TNF- α and (d) IL-10. Data are presented as mean \pm SEM, $n = 5-8$.

*represents a significant difference when compared with the control group; #represents a significant difference when compared with the cisplatin group ($p < 0.05$). IL, interleukin; SEM, standard error of the mean; TNF, tumor necrosis factor.

D-methionine-alone groups. However, cisplatin caused severe intestinal mucositis histologically characterized by shortened intestinal villi, shortened crypt depth and finger-like villi with broken and incomplete shapes. The most serious damage was found in the ileum. These histopathological abnormalities were restored by D-methionine supplementation. Next, villus length and crypt depth were measured, as shown in Figure 4(b). The villi of duodenum, jejunum and ileum in rats treated with cisplatin were shortened by 38.8%, 31.5% and 30.4%, respectively, compared with the control group ($p < 0.05$). In cisplatin combined with D-methionine treated rats, villi were shortened by 15.5%, 8.6% and 10.5%, respectively, compared with the control group ($p < 0.05$), which reveals significant recoveries of villus length and crypt depth. Next, quantitative histological scoring of intestinal damage was used to evaluate injury, as shown in Figure 4(c). The highest score for intestinal lesion was found in the cisplatin-treated group. This score was significantly reduced by D-methionine co-administration ($p < 0.05$). Together, these results demonstrated that D-methionine pretreatment protects rats from cisplatin-induced intestinal mucosal injury.

D-methionine improves gut microbiota dysbiosis in cisplatin-induced intestinal mucositis

Cisplatin caused variation in gut flora that could be prevented by D-methionine administration in cisplatin-treated rats

On PCoA of the caeca of 26 rats [Figure 5(a)], there were significant distances between cisplatin group and each of the other three groups, suggesting that gut microbial structures of control, D-methionine alone and cisplatin + D-methionine groups are much closer. We next used UPGMA to estimate the differences by constructing clustering trees on the basis of group phylogenetic data. From our results, the phylogenetic relationship (UPGMA clustering tree) of the cisplatin group was relatively distant from the other three groups on weighted UniFrac analysis [Figure 5(b)]. The evolutionary distances of species were quite short among control, D-methionine and cisplatin + D-methionine groups, suggesting similar microbial compositions and abundances among these three groups. These results indicated that cisplatin causes variation in gut flora.

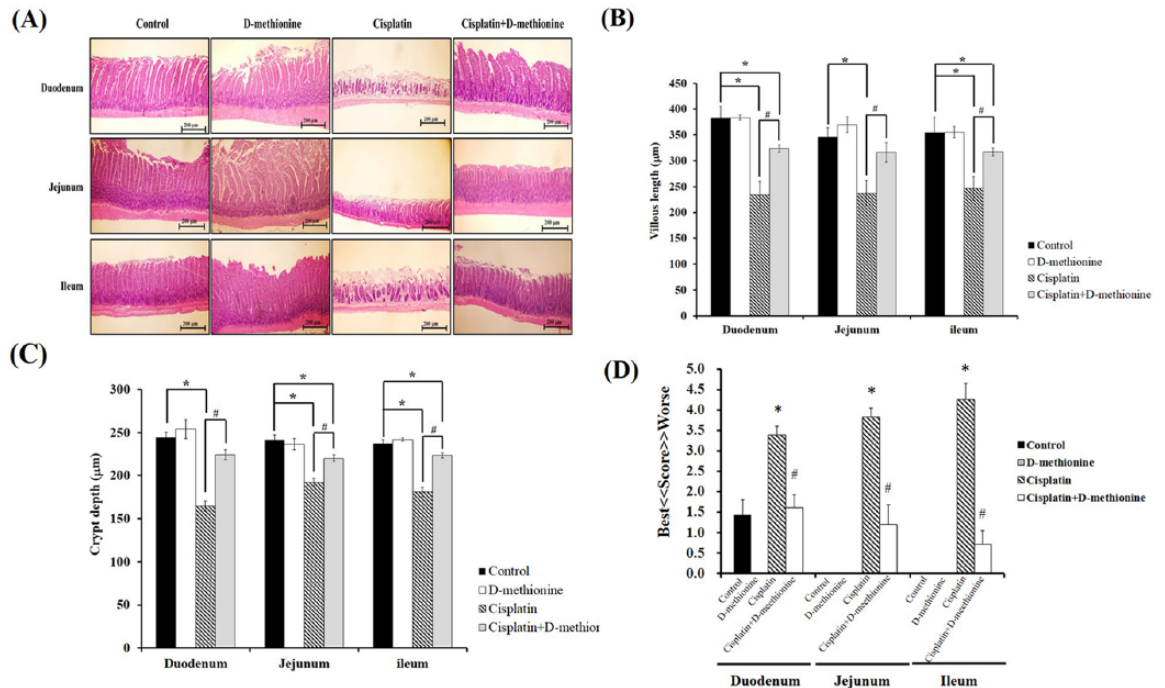


Figure 4. Effects of D-methionine on small intestinal damage after cisplatin treatment. (a) Histological staining of representative intestine, (b) villus length, (c) crypt depth and (d) grading score of intestinal tissue damage. Data are presented as mean \pm SEM, $n = 5-8$.

*represents a significant difference when compared with the control group; #represents a significant difference when compared with the cisplatin group ($p < 0.05$). SEM, standard error of the mean.

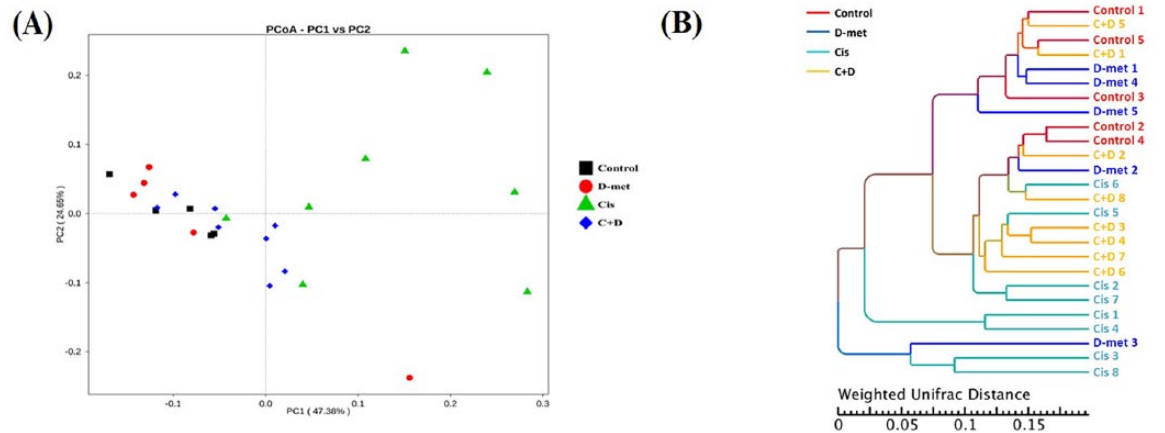


Figure 5. Beta diversity comparisons of gut microbiomes. PCoA analysis (a) and UPGMA (b). UPGMA clustering tree based on weighted UniFrac distance. Weighted UniFrac considers both composition and abundance of microbiomes. On PCoA, each point represents a sample, plotted by a principal component on the x-axis and another principal component on the y-axis, with each group represented by a different color. The percentage on each axis indicates the contribution value to discrepancy among samples. Different colored symbols represent rats receiving different treatments and every symbol represents individual animals. The black squares indicate control group. The red circles indicate D-methionine group. The green triangles indicate treatment with cisplatin only. The blue diamonds indicate combined treatment of cisplatin and D-methionine. PCoA, principal coordinates analysis; UPGMA, unweighted pair group method with arithmetic mean.

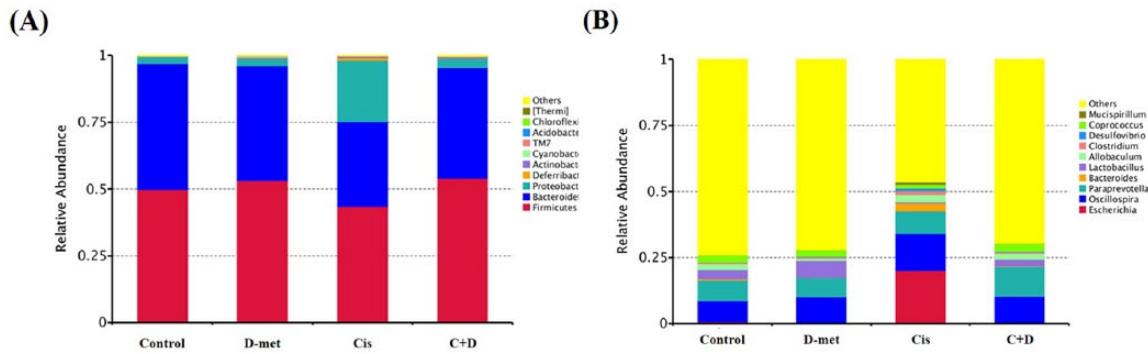


Figure 6. Phylum and genus distributions of experimental groups. The relative abundances of gut microbiota phyla (a) and genera (b) were analyzed by next-generation sequencing of bacterial 16S DNA. The y-axis and x-axis represent relative abundance and group, respectively. On the right, 'others' represents total relative abundances of the phyla and genera not included in the top 10. At the phylum level, abundance changes in Bacteroidetes, Proteobacteria and Firmicutes were observed.

D-methionine enhanced *Lactobacillus* and decreased *Bacteroides* relative abundance in cisplatin-treated rats

According to species annotation, the statistical numbers of sequences for each taxonomic classification level (kingdom, phylum, class, order, family, genus, species) were calculated. Accordingly, the top 10 species in the classification levels of phylum and genus were selected, and the distribution histograms of relative abundances of species were developed, as shown in Figure 6(a) for phylum and Figure 6(b) for genus. In our study, rat gut composition was dominated by three bacterial phyla: Firmicutes, Bacteroidetes and Proteobacteria. The relative abundances at the phylum level in all four groups followed the sequence Firmicutes > Bacteroidetes > Proteobacteria. In cisplatin-treated rats, the relative abundances of Bacteroidetes and Firmicutes significantly decreased, while those of Proteobacteria and Deferribacteres increased, when compared with the other three groups. However, in cisplatin + D-methionine-treated rats, the four phylum levels displayed almost the same abundances as in control rats. There was no significant change in phylum levels between control and D-methionine groups. Next, the levels at genus were compared among the four groups. Notably, the cisplatin-injected rats demonstrated greater relative abundances of *Escherichia*, *Oscillospira*, *Paraprevotella*, *Bacteroides*, *Clostridium*, *Desulfovibrio* and *Mucispirillum*, but lower relative abundances of *Lactobacillus* and *Coprococcus* than in the other three groups. The changes in relative abundances of *Escherichia*, *Bacteroides* and *Lactobacillus* were

obvious, implying that cisplatin significantly alters microbiota composition. Surprisingly, in the cisplatin + D-methionine group, *Escherichia* and *Mucispirillum* were hardly rare. The relative abundance of *Lactobacillus* increased and the relative abundance of *Bacteroides* decreased. Interestingly, D-methionine alone enhanced the production of *Lactobacillus* compared with the control group.

D-methionine co-treatment can increase microbiota diversity and enhance the growth of Lachnospiraceae and *Lactobacillus* after cisplatin treatment.

We used Chao-1, Observed species and Shannon as alpha diversity indices to represent in-group microbial community diversities. Higher index level represented higher microbial community diversity. Figure 7 shows that cisplatin led to marked decline in gut microbial diversity. Diversity increased under D-methionine co-administration. There were no significant differences in alpha diversity indices between control and D-methionine groups, suggesting that D-methionine alone does not alter gut microbiota structure.

Species of interest (top 10 genera in each group by default) were selected to draw the classification tree, as shown in Figure 8. Gut bacterial communities in cisplatin-treated rats were predominantly comprised of Proteobacteria and Deferribacteres. The phylum Deferribacteres, class Deferribacteres, order Deferribacterales, family Deferribacteraceae, genus *Mucispirillum* and species *schsedieri* increased in cisplatin-treated

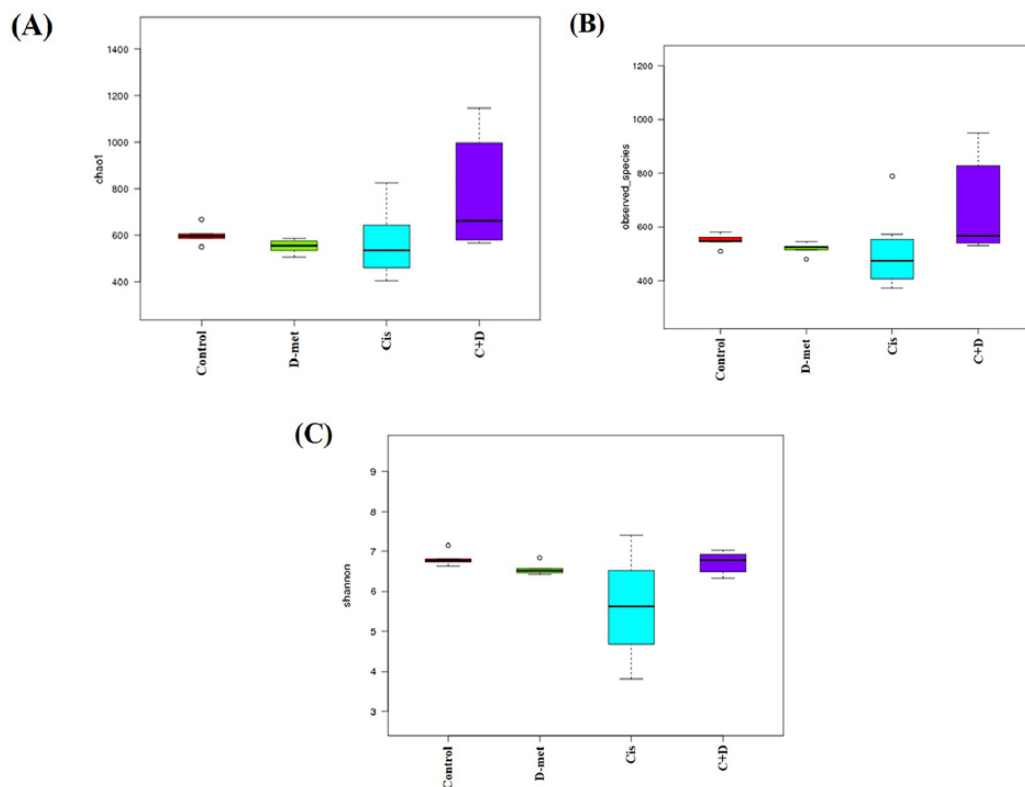


Figure 7. Alpha diversity indexes. Chao-1 (a), Observed species (b) and Shannon (c). The microbiota composition samples from four groups were clustered based on PCA, Pearson clustering and Shannon diversity index. Circles represent outliers. Group size is 5–8 individuals; results are shown as means \pm SEM. PCA, principal component analysis; SEM, standard error of the mean.

rats. Furthermore, we observed the highest abundances of phylum Proteobacteria, class Gammaproteobacteria, order Enterobacteriales, family Enterobacteriaceae, genus *Escherichia* and species *coli*, as well as the phylum Proteobacteria, class Deltaproteobacteria, order Desulfovibrionales, family Desulfovibrionaceae, genus *Desulfovibrio* and species *C21_c20*, in cisplatin-treated rats. At the species level, *Bacteroides caccae*, *Bacteroides ovatus* and *Bacteroides uniformis*, *Mucispirillum schaedleri*, *Desulfovibrio C21_c20* and *Escherichia coli* were more abundant in cisplatin alone group than in the other three groups. Interestingly, in terms of quantities of *Lactobacillus reuteri*, D-methionine > control > cisplatin + D-methionine. In the cisplatin + D-methionine group, gut microbiota composition largely shifted to *Clostridium butyrium*, *Coprococcus catus* and *Coprococcus eutactus*.

Taxonomic biomarkers were investigated based on linear discriminative analysis effect size (LEfSe) and cladogram (circular hierarchical tree; Supplementary Figure 2). Biomarkers from

the four groups are shown in Figure 9. Potentially enteropathogenic bacteria, such as *Bacteroides caccae* of the phylum Bacteroidetes and *Escherichia coli* of the phylum Proteobacteria, were predominant biomarkers in cisplatin group. Family Lachnospiraceae was the predominant biomarker in cisplatin + D-methionine group. *Lactobacillus* was the predominant biomarker in D-methionine alone group. The abundance distributions of the 100 most dominant OTUs of the four groups are displayed on species abundance heat map and represented by color gradient. In the heat map (Supplementary Figure 3), we found obvious discrepancies in species abundance between the cisplatin alone and cisplatin + D-methionine groups.

Discussion

The latest clinical trial revealed that oral D-methionine exhibit a protective effect against radiation and cisplatin-induced mucositis.²⁴ D-methionine has been demonstrated to reduce cisplatin-induced ototoxicity and neurotoxicity

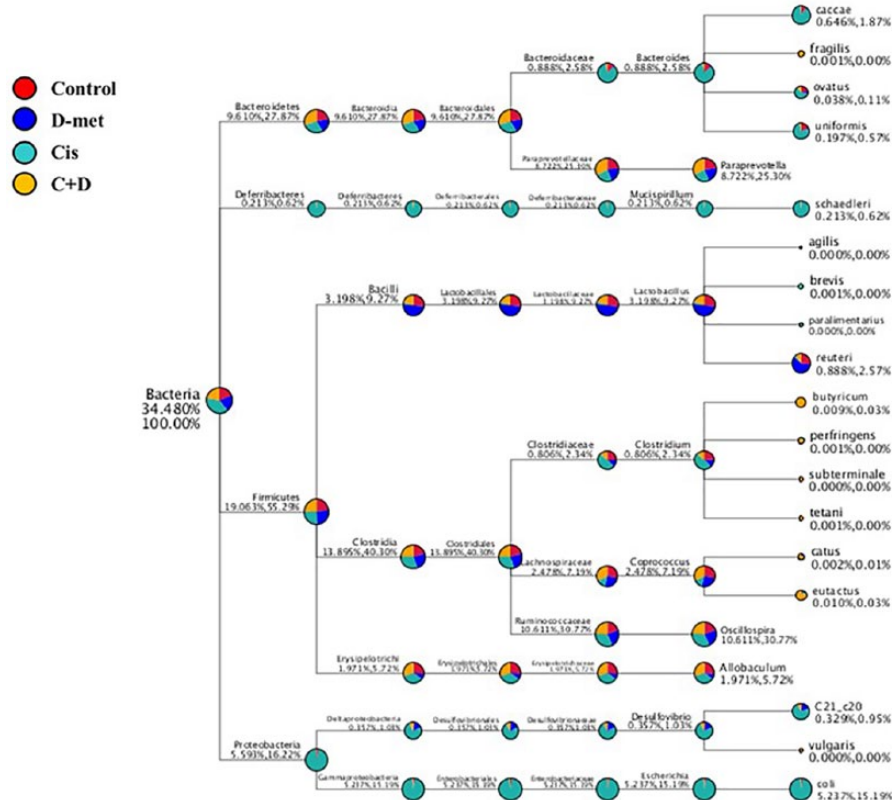


Figure 8. Classification tree. The first circle represents the kingdom level (bacteria). The second circle (in same column) represents the phylum level. The subsequent order is class, order, family, genus and species. The first number (after the taxonomic ranks) represents the relative abundance of the whole corresponding taxon, while the second number represents the relative abundance of the corresponding taxon. For example, for Firmicutes (19.05%, 55.29%), 19.05% represents the proportion of this phylum among all phylum levels; 55.29% represents the proportion of this phylum among the four phylum levels.

due to its antioxidative effect.^{21,28,29} However, the protective effect of D-methionine against cisplatin-induced GI mucositis has not been explored. In addition, the evidence that cisplatin affect gut microbiota composition remains limited.³⁰ In our study, both the antioxidant and anti-inflammatory effects of D-methionine were important mediators of cisplatin-induced GI mucositis. D-methionine exhibited gastroprotective effect partly through increased richness of Lachnospiraceae and *Lactobacillus* to modulate gut microbiota dysbiosis.

The common problems of cancer patients receiving chemotherapy were GI disorder and weight loss. Their occurrence was about 50% and 77% respectively.³¹ The incidence of anorexia was up to 46% after chemotherapy, which suggested that patients with a GI disorder and weight loss may closely associate with anorexia.³¹ Cancer patients with weight loss have also increased risks for

infection, life threat and malnutrition. Our results are consistent with those of another study³² in which cisplatin, the most potent emesis agent, caused significant weight loss and anorexia. Another study revealed that longer D-methionine pretreatment time is associated with better protective effects against cisplatin toxicity.²⁹ Our study confirmed that pretreatment with D-methionine effectively attenuates cisplatin-induced weight loss and anorexia and increases stool output. Campbell and colleagues¹⁹ and Ekborn and colleagues²⁸ also found that D-methionine supplementation results in less weight loss after cisplatin treatment. Thus, D-methionine potentially improves promotes GI function.

We attributed cisplatin-induced intestinal mucositis to the interaction between oxidative stress and inflammation, which is consistent with the findings of a previous study.^{33–35} Oral supplementation with D-methionine suppressed the

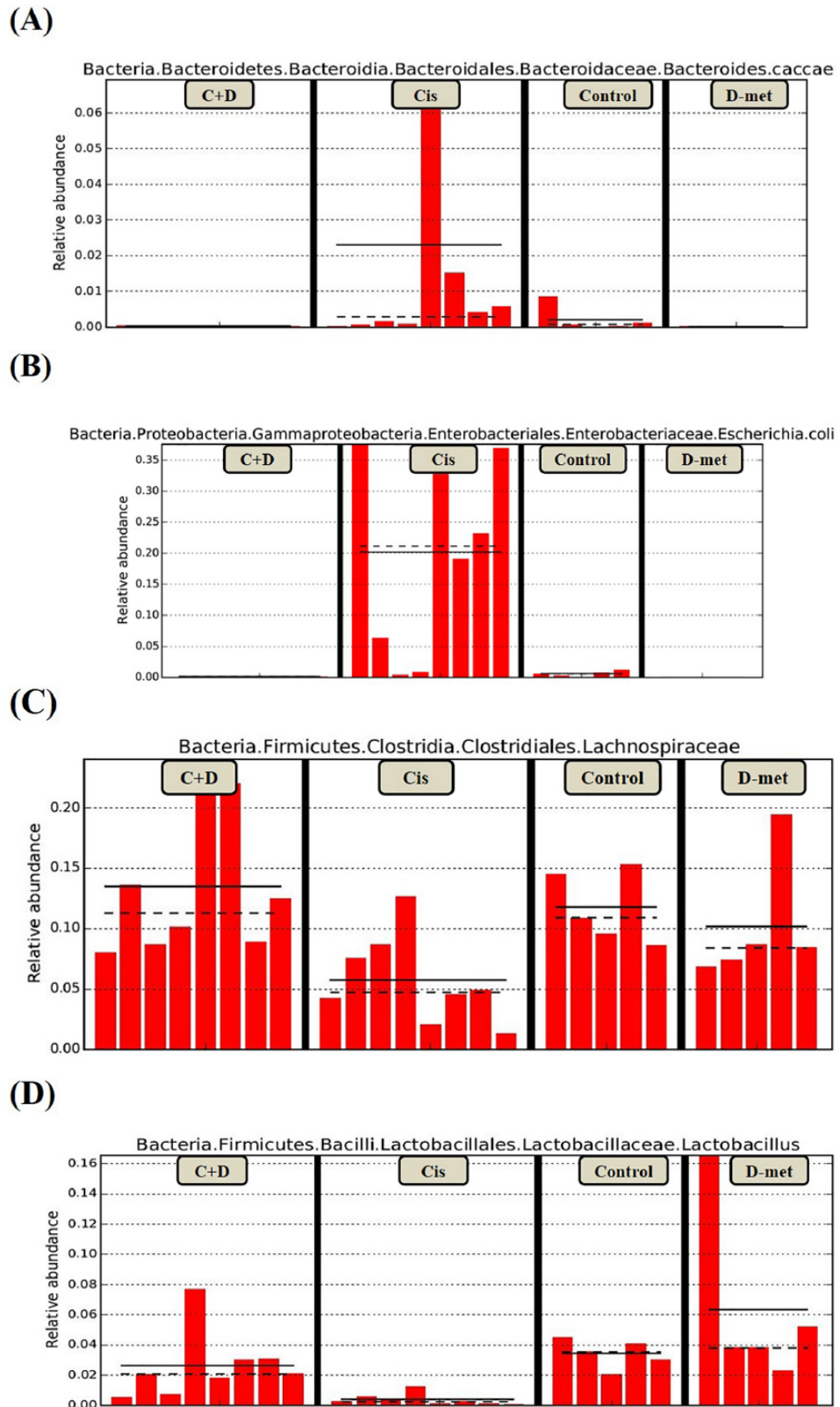


Figure 9. Biomarkers are represented by bar chart. The relative abundance with statistical differences biomarker (genus and species level) were compared among groups. *Bacteroides caccae* (a), *Escherichia coli* (b), *Lactobacillus* (c), Lachnospiraceae (d). The present study shows the proportion of *Bacteroides caccae*, *Escherichia coli* in cisplatin-treated rats were elevated than that other three groups, reflect chronic exposure to cisplatin results in a more proinflammatory microbiota growth.

oxidative stress in cisplatin-induced GI mucositis, characterized by decreased MDA levels, increased GSH concentration and enhanced SOD, CAT and GPx activities in the intestinal tissues (Figure 2).

The brush-border enzyme is located in the epithelial cells lining of the intestine and faces the lumen. It is part of digestive system which involves in digestion and absorption of nutrients. The brush-border enzyme was directly interaction with the luminal contents. These enzyme's activity were modified when intestine luminal exposure to ingested harmful materials. It has been shown that administration of cisplatin inhibits these brush-border enzyme activities by the cause of the production of lipid peroxidation products and reactive oxygen species (ROS), which affects membrane structure and function and leads to inactivation of these enzymes.³⁶ In addition, cisplatin also decreases the activity of the antioxidant enzymes in the rat intestine, indicating that cisplatin can exert direct toxic effects on the intestinal mucosa by increasing the formation of ROS to disrupt the intestinal function.³⁶ Antioxidant administration is able to restore the activities of these enzymes after exposure to hazardous substances including cisplatin.^{36,37} We speculated that the restoration of brush-border enzyme activity by D-methionine is in part due to its ability to increase SOD activity and GSH availability (Figure 2). The protective effect of D-methionine has also been reported to be due directly to its SOD promoting activity and through the metabolic product, GSH, which acts as a thiol-containing reducing agent to scavenge ROS.^{21,25} It is well known that through methionine cycle and trans-sulfuration pathway D-methionine induces cysteine and GSH production,³⁸ and acts as an antioxidant to reduce oxidative stress. It is worth noting that administration of D-methionine alone significantly increased GSH levels [Figure 2(b)] and activity of SOD [Figure 2(d)]. Hence, the antioxidant effect of D-methionine helps to improve digestive disorders induced by cisplatin.

There was evidence that repeated cisplatin treatment induced a significant increase in the size of inflammatory nodules and Ki-67-positive cell proliferation, which implies that cisplatin increased the synthesis of proinflammatory cytokines, contributing to inflammation.³⁹ The results of this study also revealed that cisplatin-induced inflammation is attenuated by administration of D-methionine, which inhibits cisplatin-induced generation of IL1 β , IL-6 and

TNF- α (Figure 3). Although the pathogenic mechanisms of chemotherapy-induced GI mucositis are still unknown, Hamouda and colleagues proposed a possible mechanism of 5-fluorouracil (5-FU)-induced intestinal mucositis in which TNF- α induces the destruction of intestinal epithelial barrier and triggers dysbiosis with increases in abundances of mostly Gram-negative bacteria to elicit secondary inflammation, ultimately resulting in intestinal mucositis.⁴⁰

A recent review has indicated that chemotherapeutic agents result in gut microbiota community imbalances by depressing the growth of beneficial bacteria and promoting the growth of harmful bacteria associated with GI mucositis.¹ For instance, methotrexate induces substantial decreases in lactobacilli and enterococci, but relative increases in potentially enteropathogenic bacteria *Bacteroides*, Enterococci and Enterobacteriaceae.⁴¹ Irinotecan regimen leads to decreases in the abundances of *Clostridium* cluster XIVa, *Lactobacillus* group and *Bifidobacterium* spp., and increases in the abundances of *Clostridium* cluster XI and Enterobacteriaceae.⁴² Therefore, changes in abundance and diversity of gut microbiota are greatly influenced by chemotherapy.

Our results clearly demonstrated an imbalance in gut microbiota in cisplatin-treated rats due to drastic increases in abundances of the phyla Proteobacteria (mucosa-associated inflammation-promoting bacteria) and Deferribacteres, supporting the positive correlation between intestinal inflammation and abundance of gut Proteobacteria (particularly *Enterobacteriaceae*). Gut Proteobacteria abundance is a potential diagnostic criterion for dysbiosis and disease.⁴³ *B. caccae* leads to mucin depletion and thinner mucus layer. This contributes to the entry of pathogenic bacteria into lumen and enhances susceptibility to colitis.⁴⁴ *M. schaedleri* control oxidative stress during inflammation through specialized systems, inhabiting mucus layer (rich in mucin) and exhibiting proinflammatory properties through activation of nuclear factor kappa B (NF- κ B) and peroxisome proliferator-activated receptor (PPAR)-delta or the inhibition of epidermal growth factor.⁴⁵ Inflammation creates a favorable opportunity for *E. coli* to exhibit an adhere-invasive effect on the intestinal mucosa.⁴⁶ The levels of Proteobacteria, particularly Enterobacteriaceae (including *E. coli*), increase in the gut microbiota of patients with inflammatory bowel disease

(IBD).⁴⁶ Both *Desulfovibrios* and *E. coli*, Gram-negative bacteria, are able to produce LPS, an endotoxic component of cell walls of certain bacteria (such as Gram-negative bacteria) that is a key mediator of gut dysbiosis and inflammation. When gut bacteria generate huge amounts of LPS, LPS forms a complex which contains LPS binding proteins and pattern recognition receptor. This is recognized by Toll-like receptors to trigger the activation of NF- κ B and the production of IL-6, IL-1 β and TNF- α .⁴⁷ *Desulfovibrio* subspecies are sulfate-reducing anaerobic Gram-negative bacteria associated with increased gut permeability.⁴⁸ Additionally, the *ompW* gene is detected only in the *B. caccae* TonB-linked outer membrane protein, which may contribute to the pathogenesis of intestinal inflammatory-associated diseases.⁴⁹ Mucin, a major component of mucus, is considered to play an important defense role in inflamed intestine. Cisplatin decreases GI mucin content, suggesting that mucus barrier dysfunction is a cause of cisplatin-induced mucositis.¹⁷ Thus, we speculated that decreased mucin content correlates with *M. schaedleri* and *B. caccae* overgrowth. Chemotherapy agent-induced upregulation of TNF- α and IL-1 β may be due to gut dysbiosis.⁴⁰ To sum up, we speculated that inflammatory response in intestinal tissue caused by cisplatin is correlated with overgrowth of *M. schaedleri*, *B. caccae*, and *E. coli*.

Cisplatin caused significant increases in bacteria of the Bacteroidaceae and Erysipelotrichaceae families, as well as in *Bacteroides uniformis*. Cisplatin-induced dysbiosis is associated with disruption of the intestinal mucosa. Meanwhile, cisplatin treatment also causes the depletion of health-promoting species *Ruminococcus gnavus*.¹⁸ Tumor with/without cisplatin treatment mediated the structural and functional composition of gut microbiota which shifted toward more Bacteroidetes and less Firmicutes and Proteobacteria.³⁰ Cisplatin had the strong effect in reshaping the gut bacteria community structure in a different way found by the liver tumor-bearing mouse model.³⁰ The results showed cisplatin treatment was effective in reducing liver tumor growth but led to an imbalanced gut microbiota and decreased the ecological diversity in the gut.

Intestinal bacteria can interfere with damage to intestinal tissues *via* the following mechanisms: (1) modulation of inflammation and oxidative stress through a variety of mechanisms by beneficial members such as Clostridium cluster XIVa in

the Lachnospiraceae family; (2) attenuation of intestinal permeability by members such as *Bifidobacterium* spp. and *Lactobacilli* which increase tight junction expression; and (3) maintenance of the mucus layer, for example, by various *Lactobacillus* species, which upregulate mucin production.⁵⁰ Interestingly, our results demonstrated that oral daily supplementation of D-methionine inhibits the overgrowth of inflammation causing-bacteria (*M. schaedleri*, *B. caccae*, *E. coli*) and increases the growth of Lachnospiraceae (*Clostridium* cluster XIVa), *C. butyricum* and *L. reuteri* in cisplatin-induced mucositis.

Lachnospiraceae administration attenuates colitis in mice.⁵¹ Using lactate and acetate, Lachnospiraceae can produce butyrate,⁵² which decreases gut pH levels and prevents growth of pathogenic bacteria.⁵³ *Coprococcus eutactus* is the predominant butyrate-producing bacteria.⁵⁴ Acetic acid, propionic acid, and butyric acid are known as short-chain fatty acids (SCFAs). SCFAs maintain intestinal homeostasis by suppressing the growth of Gram-negative pathogens. They are also energy sources and anti-inflammatory agents and promote apoptosis of cancer cells.⁵⁵ Thus, prominent SCFA-producing bacteria can act as indicators of a stable and healthy gut. IL-10 protects intestinal health and is produced *via* T helper 2 (Th2) cells, regulatory T-cells, dendritic cells and macrophages. Recent research has indicated that maintenance of high levels of IL-10 contributes to a reduction in damage to the intestinal epithelium caused by cisplatin.³³ *C. butyricum*, known as a probiotic and classified in *Clostridium* cluster I, promotes IL-10 production by intestinal macrophages in inflamed mucosa, and has been shown to prevent experimental colitis in mice.⁵⁶ Our study showed that D-methionine supplementation prior to cisplatin treatment attenuates the decreased IL-10 level [Figure 3(d)], implying anti-inflammatory effects of IL-10 in the pathogenesis of GI mucositis.^{33,57}

Lactobacillus spp. can prevent overgrowth of potentially pathogenic bacteria⁵⁸ and possess antioxidative effects.⁵⁹ *L. reuteri* exerts anti-inflammatory effects on intestine through reduction of TLR4 signal, leading to transcriptional inhibition of NF- κ B genes in necrotizing enterocolitis model.⁶⁰ *L. reuteri* belongs to vitamin B₁₂ activity-producer strain,⁶⁰ of which cobalamin is involved in methionine metabolic pathways,⁶¹ helpful to GSH production and antioxidant activity.

A recent review has shown that imbalances in intestinal microbiota can be modulated by natural antioxidants through their antioxidant and anti-inflammatory activities.⁴⁷ Our results also confirmed that the antioxidant properties of D-methionine play important roles in gut microbiota composition modulation during cisplatin treatment. It is worth further investigating whether oral supplementation with Lachnospiraceae and *L. reuteri* can improve cisplatin-induced mucositis.

The first limitation of our study was lacking the quantitative assessment to calculate the number of bacteria/g of feces for microbiota compositional variation. The human/animal microbial communities were analyzed using NGS to represent the percentage of various bacteria, which was mostly described by relative microbiome profiling (RMP). However, the RMP analysis might probably underestimate the microbiota richness associated with disease. Furthermore, due to the limitation in the read length of sequences, primer biases, the quality of databases, leading to the level of genus and species classifications were also difficult to measure. In our study, the species level was too little to be determined, and this restricted a better understanding of the microbial ecology of cisplatin and D-methionine treatment. Another limitation of our study was that we did not perform a tumor-bearing model. The gut microbiota composition under tumor progression may differ from tumor-free animals. The present study was only performed in a nontumor-bearing model. Above all, the current results demonstrated that D-methionine effectively alleviated cisplatin-induced mucositis and gut dysbiosis, which are provided as a reference for further research.

In conclusion, our data suggest that D-methionine dually mediates cisplatin-induced GI mucositis and microbiota community dysbiosis. D-methionine protects against cisplatin-induced gastrointestinal damage through its antioxidant and anti-inflammatory activities and enhances growth of beneficial bacteria (Lachnospiraceae and *Lactobacillus*), thereby regulating gut microbiome imbalance induced by cisplatin.

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Conflict of interest statement

The authors declare that there is no conflict of interest.

Supplemental material

Supplemental material for this article is available online.

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