Probiotic Lactobacillus rhamnosus Attenuates Cisplatin-Induced Intestinal Mucositis in Mice via Modulating the Gut Microbiota and Improving Intestinal Inflammation

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Abstract

*Lactobacillus rhamnosus* (LBS) is well documented probiotic strain in oncology and has a pivotal role in clinical applications. Here we investigated the Intervention effect of *Lactobacillus rhamnosus* on intestinal mucositis induced by cisplatin (CP). BALB/c mice were pre-treated with or without LBS via oral gavage, followed by induction of mucositis with cisplatin. Our results revealed that LBS-treated groups significantly attenuated proinflammatory cytokine levels (IL-1β, IL-6, and TNF-α) compared to the CP group. Furthermore, LBS mitigated the damaged tight junction integrity caused by CP via up-regulating levels of Claudin, Occludin, ZO-1, and Mucin-2 protein (MUC-2). Finally, the 16S rRNA fecal microbiome genomic analysis showed that LBS administration enhances the growth of beneficial bacteria, i.e., *Firmicutes, and Lachnospiraceae*, while the relative abundance of the opportunistic bacteria *Bacteroides* and *proteobacteria decreased*. Collectively, LBS found to beneficially modulate microbial composition structure and functions and enrichment the ecological diversity in the gut.

1. Introduction

The gut microbiome plays a pivotal role in maintaining the host’s health, potentially through regulating immune stability and protecting from gastrointestinal diseases [1, 2]. A healthy gut microbiome is characterized by bacterial richness and ecological diversity [3], which enhance the integrity of the mucosal epithelium and invade against pathogenic microbes [4]

Disturbed gut flora has been linked to various diseases including inflammatory bowel disease (IBD) [5], depression [6], obesity[7] and type 2 diabetes [8]. Maintaining the balance in the abundance and diversity of the microbial communities appears to assist in the restoration of various disorders.

Previous studies have shown that bio-diversity and richness of the gut microbiome changed significantly by chemotherapeutic drugs [9, 10]. The structure and function of the intestinal barrier may impair through changes in host physiology, resulting in the pathogenesis of Intestinal mucositis [11].

Intestinal mucositis represents one of the most frequent side effects in oncology patients undergoing chemotherapy, it is characterized by increased intestinal permeability, reduction of mucin levels, and oxidative damage [12]. Cisplatin is one of the most potent platinum chemotherapeutic agents widely used as an effective therapy against various types of malignancies. For over four decades, CP is commonly used for the management of cervical cancer, testicular cancer, and bladder cancer with cure rates of over 90% [13–15]. CP is able to exert strong cytotoxic effects by blocking the DNA repair mechanism in tumor cells, preventing replication and consequently triggering apoptosis [16]. However, the clinical application of CP is still limited due to its toxicity. Moreover, the adverse effect of CP is well recognized with high-dose therapy which includes hepatotoxicity, renal damage, nephrotoxicity, and damage to the intestinal epithelium [17]. Therefore, it is necessary to find an effective way to mitigate intestinal barrier damage caused by cisplatin.
Cisplatin-induced alterations to the gut microbiome is yet to be conducted minutely. Furthermore, restoration of the intestinal flora through probiotics has been clinically promising therapeutic option for gut-associated disorders [18]. Intestinal injury is closely linked to inflammation, treatment with probiotics has been found to have the potential to activate anti-inflammatory compounds such as IL-10, and it has been reported to upregulate the expression of anti-inflammatory cytokines including Interferon-γ, tumor necrosis factor-α (TNF-α) and inhibiting the proinflammatory transcription factor NF-κB [19, 20].

*Lactobacillus rhamnosus* (LBS) is a member of the Lactic Acid Bacteria group (LAB), which is one of the most studied probiotic strains in oncology, it exerts numerous beneficial effects [21, 22]. In clinical application, LBS is a bacterium resident in the gut which can recover disturbed gut microbiota and demonstrate anti-inflammatory effects as well as boost the immune system that could accelerate the healing of intestinal epithelial homeostasis [23–27]. Also, it has been used in a wide range of other illnesses, reduce of severe diarrhea, inflammatory bowel disease, ulcerative colitis, and yellow fever [28, 29]. Previous studies have shown the efficacy of several LABs in regulating microbiome dysbiosis [30], and maintaining the intestinal epithelium integrity of tight junction proteins [31].

Here, we aim to investigate the protective effects and safety of probiotic *Lactobacillus rhamnosus* on cisplatin-induced intestinal injury and explore the underlying mechanisms targeting inflammatory proteins as well as histological changes in the intestinal tissue of the BALB/c mice. In addition, we studied the bacterial strains that may be related to the health-enhancing properties by 16S rRNA sequencing.

### 2. Methods

#### 2.1 Probiotic strain

*Lactobacillus rhamnosus* LBS strain was obtained from (BeNa Culture Collection (Xinyang City, Henan province, China). LBS were cultured in anaerobic condition at 37°C in De Mann Rogosa Sharpe solid medium (MRS) for 24 h, after that, a single colony was inoculated into MRS liquid medium and cultured overnight. Before administration to mice, LBS cultures were centrifuged at 6,000 × g, 4°C for 10 min, then washed trice with physiological saline and resuspended to get the final concentration of CFU 1 × 10⁹ Lactobacilli/ml.

#### 2.2. Ethical statement and experimental animals

Approval for Animal Ethics and Experimental Design was obtained from Dalian Medical University. Forty-eight male BALB/c mice (5–6 weeks old, body weight 18 ± 2 g), were obtained from the Specific Pathogens Free animal house Facility (SPF) of Dalian Medical University, and Committee guidelines for the care and handling of animals were followed by the National Institutes of Health. Mice were kept in sterilized cages at room temperature (22 ± 2°C), with humidity of 65% ± 5%, 12 h cycle of light and darkness, with free access to food and distilled water.
2.3 Study Design

After one week of acclimation, the mice were randomly assigned into four groups (n = 12): Normal control group (control), control + probiotic (LBS), model group (CP), and model + probiotic group (CP.LBS). Saline (0.9% NaCl, wt/vol) was given to the Control and LBS groups once daily via oral gavage. Mice in the CP and CP.LBS groups were received by intraperitoneal injection of cisplatin at the dose of (6 mg/kg/d) once daily for three days to cause intestinal mucositis, while mice in the LBS and CP.LBS groups received LBS probiotics orally from day 1 to day 7 in addition to LBS pre-treatment one week prior to IM induction. Also, from day 1 to day 3, saline was intraperitoneally administered to the mice in the control and LBS groups. The body weight and food consumption were noted every day until one day before killing the animal. Additionally, daily assessments of the mice’s health, including their appetite, activity, fur, and feces, were observed. The methods used in animal studies are all based on previous research and the pre-experiments described in (Fig. 1A).

2.4 Stool output and diarrhea assessment

After induction of intestinal mucositis, stool samples of all the mice were checked daily and the severity of diarrhea was assessed by using Bowen's score system [32], to classify the stool consistency into four grades: 0. normal stool; 1. Soft, slightly wet stool indicating mild diarrhea; 2. wet and unformed stool indicating moderate diarrhea; and 3. watery stool indicating severe diarrhea

2.5 Measurement of organ indices

After the mice were sacrificed by cervical dislocation, the immunological organs thymus and spleen were harvested and weighed immediately. The spleen and thymus indices were calculated by this formula:

Spleen or thymus indices (mg/g) = weight of spleen or thymus (mg)/weight of mouse (g).

2.6 Pro-Inflammatory Cytokines analysis

The whole blood was drawn through eye orbit and serum was obtained in a 1.5 mL tube via centrifuged at 2000× g for 10 min then stored at −20 °C until further process. Cytokine concentrations (IL-1β, IL-6, TNF-α) were measured through the mouse ELISA kit (Shanghai Longton Biotechnology Co, Ltd, Beijing, China) according to the manufacturer’s guidelines.

2.7 Real-time quantitative PCR (RT-qPCR)

Total RNA was extracted from colonic tissues through TRIzol® Reagent Kit (life technology, USA). NanoDrop 2000 (Thermo Scientific) was used to check the quantity of cDNA, and using the commercial kit HiScript II Q RT SuperMix (Vazyme Biotech Co., Ltd.), 2 µg of RNA was reverse transcribed to cDNA. The ChamQ SYBR qPCR MasterMix kit was used to measure gene expression utilizing Bioer light gene 9600 analyzers (Hitech (Binjiang) District, Hangzhou, 310053, China). The following procedures were used for PCR cycling: 50°C for 2 minutes, 95°C for 10 minutes, and 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The 2 − ΔΔCt equation was used to measure the relative gene expression level for target genes through the instrument software gene 9660 as outlined by Livak et al. [33]. The kits for the
primers used were bought from Invitrogen (Table 1). The GAPDH served as a control gene and healthy control functioned as an endogenous calibrator.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>AACGACCCCTTCATTGAC</td>
<td>CCACGACATACTCAGCAC</td>
</tr>
<tr>
<td>IL-1β</td>
<td>CTCCATGAGCTTTGTACAAGG</td>
<td>TGCTGATGTACCAGTTGGGG</td>
</tr>
<tr>
<td>IL-6</td>
<td>TGTGCAATGGCAATTCTGAT</td>
<td>GGTACTCCAGAAGACCAGAGGA</td>
</tr>
<tr>
<td>TNF-α</td>
<td>CATCTTCTCAAATTTGAGTGACA</td>
<td>TGGGAGTAGACAAGGTACAACCC</td>
</tr>
<tr>
<td>Claudin1</td>
<td>ATCGCAATCTTTTGTGACCATT</td>
<td>ATTCTGTCTTCCATACCAGCTGTG</td>
</tr>
<tr>
<td>Occludin</td>
<td>ACTCCTCAAGGGACAAGTG</td>
<td>CCCACCTGTGTAGTTCT</td>
</tr>
<tr>
<td>ZO-1</td>
<td>AACCCGAAACTGATGCTATGGA</td>
<td>GCGCGCCTTGGAAATGTATGTG</td>
</tr>
<tr>
<td>Muc2</td>
<td>GATGGCACCTACCTCGTTGT</td>
<td>GTCCGGCACTTGGTTGAAT</td>
</tr>
</tbody>
</table>

**2.8 Histological examination**

Colon and ileum tissues were excised and washed with PBS. These tissues were fixed with 10% formalin and dehydrated in different concentrations of alcohol, then encased in paraffin wax. About 5 µm of embedded tissue was sectioned by microtome and stained with hematoxylin and eosin. Microscopic examination for histological alteration was examined by a light microscope.

**2.9 Immunohistochemistry**

Immunohistochemistry (IHC) was used to examine the expression of Claudin, Occludin, Zonula occludens-1 (ZO-1), and Mucin-2 (MUC-2) in colon and ileum tissues. A 5 µm section of colon and ileum tissue was carefully cut, deparaffinized with xylene, and rehydrated in ethanol at various gradients, followed by incubation with 3% H2O2 for 10 minutes. For antigen retrieval, the tissue slide was warmed in antigen retrieval buffer (Na + 2 EDTA, pH 8.0), then incubated overnight with primary antibodies followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibody for 1 hour. The slides were stained with 3,3-iaminobenzidine (DAB) as substrate and hematoxylin was used as counterstains. Then the slides were fixed and visualized under a light microscope at 20 × magnification, 100 µm scalebar.

**2.10 Western blot**

Colonic tissues were cut into small pieces and homogenized through a Tissue Grinding instrument on ice with RIPA lysis buffer supplemental with protease inhibitor cocktail (Transgene, Biotech, Beijing, China), following centrifugation at 4°C for 15 minutes at 12000g to obtain the supernatant. Protein concentration was quantified by BCA protein assay kit. Later, Protein in equal quantities was separated by 12% sodium
dodecyl sulphatepolyacrylamide gel (SDS-PAGE) and electrotransfered onto PVDF (polyvinylidene difluoride) membranes in wet conditions. After that PVDF membranes were blocked with 5% (w/v) non-fat milk for (1.5 hour) at room temperature. Then incubated overnight at 4°C with primary antibodies. Primary antibodies dilutions were as follows: Claudin-1 diluted (1:1000), (catalog no. 13050-1-AP), Occludin diluted (1:2000), (catalog no. 13409-1-AP), and β-actin diluted (1:5000), (catalog no. 20536-1-AP). and loaded as a control. The membranes were washed three times with Tris-buffered saline containing Tween (TBST), and then incubated with horseradish peroxidase-conjugated (HRP) secondary antibodies (1:5000), (catalog no. SA00001-2), at room temperature for 1.5 hours, then washed three times with TBST. To visualize the protein bands, the membrane exposed with Chemiluminescent (ECL) kit by using Automated Imaging System (Imager-Bio-Rad, Bio-Rad Laboratories, Inc., Hercules, CA, USA). The results were analyzed by Image J software.

2.11 Gut microbiome genomic DNA extraction and 16S rRNA Pyrosequencing

Total genomic DNA samples were extracted from fresh fecal samples using the Power Max (stool/soil) DNA isolation kit (MoBio Laboratories) following the manufacturer's instructions, then stored at −80°C pending further analysis. Obtained genomic DNA was measured using the NanoDropND-1000 (Thermo Fisher Scientific, Waltham, MA, USA) to verify the DNA's purity, also agarose gel electrophoresis was used to evaluate the DNA's quality. The forward primer 515F (5’GTGCCAGCMGCCGCGGTAA-3’) and reverse primer 806R (5’- GGACTACHVGGGTWTCTAAT-3’) were used to amplify the V4 region of the 16S rRNA gene from whole genomic DNA extracts as the following procedure: initial denaturation temperature at 98°C for 30s, followed by 25 cycles of denaturation at 98°C for 15s, annealing at 58°C for 15s, and extension at 72°C for 15s, with a final extension of 1min at 72°C. Samples sequencing analyze using the Illumina Hiseq4000 sequencer (2 × 150 bp paired-end). Microbial Ecology software (QIIME software version 1.9.0) was used for sequence read processing, pipeline, also, sequence read was analyzed, and OUT with low-quality was excluded. Alpha diversity, richness, Shannon, Simpson, and evenness index were assessed using QIIME and R packages (v3.2.0). Additionally, beta diversity was evaluated through many parameters, UniFrac distance metrics, principal coordinate analysis (PCoA), principal component analysis (PCA), and non-metric multidimensional scaling (NMDS). Furthermore, the key biomarkers of the different groups were performed using LEfSe (LDA) linear discriminant analysis effect size which analyzes the predominance and differences in the species.

2.12 Metagenomic functional analysis of the microbiome composition

The mice gut microbiome is closely similar to its human homolog, with almost 95.2% of its KEGG orthologous groups shared as reported by Xiao et al.[34] KEGG Pathways is a set of pathway maps that illustrates the molecular correlation between genetic information and metabolism. The 16S rRNA sequences were used to determine the functional diversity and abundance of the gut flora in the varied research groups. For the analysis of the abundance of gene families with quantifiable uncertainty, (PICRUSt) was used to predict the important discoveries from the human microbiome project. The resulting sequence file was further examined using the STAMP software package, version 2.1.3, as
previously studied by Parks et al. [35]. Moreover, FAPROTAX [36], and BugBase [37], were used to analyze a diagram of ecologically related metabolites and functions in prokaryotic clades.

2.13 Statistical Analysis

The statistical data were examined using the software GraphPad Prism (7.00) (La Jolla, CA, USA). One-way analysis of variance (ANOVA) and Tukey’s multiple comparison test were utilized to ascertain differences, and p-value < 0.05 was regarded as statistically significant. LEfSe was studied by Kruskal-Wallis and Wilcoxon. OUT and phenotype were statistically analyzed using the Mann-Whitney test.

3. Results

3.1 LBS treatment attenuates body weight loss, increases food intake and organ index

Body weight loss and anorexia (loss of appetite) are common symptoms that often occur during cisplatin treatment; they are also providing basic signs of cisplatin toxicity. Before the induction of intestinal barrier damage, body weight and food intake have not differed among the four groups. As expected, and observed in our experiments, cisplatin-treated mice and cisplatin combined with LBS-treated mice showed reduces of (31% ± 3.9) and (20% ± 2.5) approximately in body weight, respectively, compared with the control group (P < 0.0001) as shown in (Fig. 1B). Additionally, in cisplatin-treated mice, there was a severe reduction in food intake with 90% compared with Control group (P < 0.0001). While co-administration of LBS with cisplatin ameliorates the mice’s appetite by 40% reduced significantly compared to the Control group (P < 0.001) (Fig. 1C). No significant change in body weight and food intake in LBS alone group vs. the Control group.

Moreover, the general health state of mice was monitored daily, and our observation indicates that the animals in the cisplatin group noted a severe reduction in activity, and the fur started to fall off after cisplatin injections compared with the Control group. In contrast, mice in CPLBS group was more active and their fur was in good condition compared to the model group. (Data not shown).

Accordingly, the immunological organs thymus and spleen indices were reduced in the model group compared with the control group, while that’s restored significantly with LBS treatment in CPLBS group compared to the model group. As shown in (Fig. 1D,E). Liver organ weight showed no significant change in the model and treatment group compared with the Control group (Data not shown).

3.2 LBS increases stool output and reduces the severity of diarrhea

Fecal samples of the mice were monitored daily and the results of all groups were compared. We noted a decrease in stool production in the cisplatin group compared with the Control. Nevertheless, in the cisplatin + LBS group, the stool output remained lower than in the Control and LBS alone groups. Indeed,
no diarrhea was noted in saline groups (LBS alone and Control group), on the contrary, in cisplatin-treated mice, diarrhea started on day 5 after cisplatin injections and developed to moderate diarrhea on the day (6, 7) (P < 0.01) according to Bowen's score system [32]. However, diarrhea grade was significantly reduced in those mice treated with LBS in CPLBS group compared to model group (P < 0.05) (Fig. 1F).

3.3 LBS attenuates pro-Inflammatory cytokine levels in cisplatin-induced intestinal mucositis mice model

Serum levels of pro-Inflammatory cytokine (IL-1β, IL-6, TNF-α) were measured after mice were sacrificed, the mice in the CP group were significantly elevated proinflammatory cytokine levels than those in the Control group, however, the levels decreased significantly in CPLBS group compared with the model group. As shown in (Fig. 2A). A similar observation was noted in the colonic mRNA for that pro-Inflammatory cytokine (Fig. 2B).

3.4 Effects of LBS on histopathological examinations in the intestinal mucosal layer

H&E were examined to observe histopathological changes in the colon and ileum in treated mice (Fig. 3A,B). The colon histology of the mice in the Control group seemed normal and healthy and the epithelium and goblet cells were both intact and uniformly arranged. On the other hand, cisplatin caused the intestinal mucosal injury and elevated intestinal permeability in the CP group, which featured shortened intestinal villi, crypt depth disruption, surface epithelial abrasion combined with a reduction in the number of goblet cells. These morphological changes lead to a significant impact on the expression of tight junction proteins (TJs) in the colon epithelium. However, these damages partly recovered the overall features of the ileum and colon and restored the loss of epithelial cells by the administration of probiotic LBS.

Moreover, Colon length in the cisplatin-treated group was significantly shorter among the other groups (CP 8.07 ± 0.27 vs Con. 10.3 ± 0.2) (P < 0.0001) as shown in (Fig. 3C,D). Interestingly, treatment with LBS had noticeably protective effects on colon health, it could improve the colon length shortening compared to the model group ( CPLBS 9.31 ± 0.3 vs. CP 8.07 ± 0.27)(P < 0.01).

3.5 LBS modulates the tight junction proteins expression in the colon and ileum of CP-induced IM Mice

The expression of tight junction proteins was examined by immunohistochemistry (IHC), qPCR, and Western blotting techniques. The results showed that the model group demonstrates the lower expressions of Claudin-1, Occludin, ZO-1, and Mucin-2, compared to the normal mice, while enhancing the relative expression levels of those tight junction proteins in CPLBS-treated mice compared to the model group in IHC as shown in (Fig. 4A-D) in the colon and ileum tissue. Mucin-2 is considered to be the primary component of the intestinal mucosa released by goblet cells [38]. In our study, a reduction was clearly noted in the CP group, this drive to epithelial cells damaged, Inflammatory cell infiltration, and a decrease in the availability of goblet cells. On the contrary, probiotics LBS enhanced the mucus layer...
thickness and increased expression of MUC-2 by recovering the epithelial cells and regenerating the number of goblet cells. A similar observation was noted in the colonic mRNA expression for those tight junction proteins (Fig. 5A). The results prove that LBS pretreatment protects mice against cisplatin-induced intestinal mucosal damage.

Also, we studied the relative expression of tight junction proteins (Claudin-1 and Occludin) in the colonic tissue by western blot technique. The findings showed a decrease in expression in the model group in Claudin-1 and Occludin (P < 0.001), (P < 0.0001), respectively, compared to the Control group, while enhanced expression of Claudin-1 and Occludin in the LBS treatment group (P < 0.05) compared with CP group (Fig. 5B).

### 3.6 LBS treatment modulates the gut microbiota dysbiosis

Cisplatin caused unstable in the gut microbiome, which could be modulated by LBS administration in cisplatin-treated mice. To reveal this effect, pyrosequencing targeting the V4 region of the 16S rRNA was analyzed by Illumina NovaSeq6000, there was a 97 percent similarity level between operational taxonomic units (OTUs) within the range of 600–1000. According to the Venn diagram (Fig. 6A), 802 OTUs were shared between Control and experimental groups, in addition, we observed differences among the groups. Control group, LBS, and CPLBS groups noted significant elevation in OTUs, while decreased OTUs were shown in the CP-treated group.

BugeBase microbial phenotypes results indicate elevated facultative anaerobic relative abundance, and gram-negative bacteria in the CP group, while gram-positive relative abundance bacteria increased in the probiotic supplementation group. Notably, LBS reduced potential pathogens’ relative abundance and stress-tolerant microbes that increased in the CP group as shown in (Fig. 6B).

Also, alpha diversity has been analyzed to evaluate bacterial richness and diversity, Chao-1 and Shannon indexes were used to indices bacterial community diversities and richness, while the Simpson index measures the evenness in the community. A higher index level indicates a more diverse bacterial community. We found that CP decreased the α-diversity of intestinal bacteria (Fig. 6C), which was demonstrated by the decline in the Chao-1, Simpson, and Shannon indexes. However, the reduction in microbial community diversity and richness indexes was reversed by the LBS administration. These findings suggest that LBS enhances the ecological diversity in the gut.

Moreover, to demonstrate the gut microbial structures and to reveal the similarity or dissimilarity of samples in species compositions, the beta diversity pattern was analyzed by Principal component analysis PCoA (weighted UniFrac analysis) (Fig. 6D) and non-metric multidimensional scaling (NMDS) (Fig. 6E). The Anosim analysis is shown in (Fig. 6F) according to Bray-Curtis algorithm, the R-value > 0, indicating that the difference between groups is greater than the difference within the group. Our results exhibited that groups of Control, LBS, and CPLBS were closer to each other than CP alone group, suggesting cisplatin induces variation in the gut flora while LBS treatment was more similar to the Control group.
The taxonomic classification level (Phylum, Class, Family, Genus) was identified for the intestinal flora to study the specific changes caused by CP and LBS in all treatment groups. Moreover, the top 10 species were selected to draw the species tree; in our results, the bacterial composition showed variation at all levels in the CP group as compared to the Control group. Furthermore, the dominant three bacterial phyla in mice gut composition are Bacteroidetes, Firmicutes, and Proteobacteria. In cisplatin-treated mice, the relative abundances at the phylum level of three dominant bacteria respectively Bacteroidetes > Firmicutes > Proteobacteria. In the model group, the relative abundances of Firmicutes significantly decreased, while Bacteroidetes and Proteobacteria increased, compared with the other three groups (Fig. 7A). However, CP, LBS-treated mice mitigated the CP-induced phylum-level alteration. Moreover, there was no significant change in phylum levels between Control and LBS groups shown in Table 2.

Next, at the class level (Fig. 7B). Notably, the cisplatin group displayed a higher relative abundance of Bacteroides and Gammaproteobacteria, but a lower relative abundance of Clostridia and Bacilli compared to the other three groups. At family levels (Fig. 7C) reveals the changes in the abundances between the four groups, Lactobacillaceae, Lachnospiraceae, and Rikenellaceae were less abundant in the CP group as compared to Control, LBS, and CP.LBS, and greater abundance of Bacteroidaceae level.

Interestingly, at the genus level. Notably, the cisplatin-injected mice demonstrated a decline in the abundances of Lachnospiraceae NK4A136_group, Lactobacillus, Alistipes, and Rosburia and enriched the abundance of Bacteroides, as well as, these changes were ameliorated with LBS supplementation (Fig. 7D).

Inclusively, our finding revealed that at taxonomic levels, the bacterial community was altered by cisplatin drug in CP mice, while LBS treatment partially restored the gut dysbiosis. Heatmaps were used to assess relative abundances of bacterial genera; (Fig. 7E). Additionally, we also investigated taxonomic biomarkers using GraPhlAn and linear discriminative analysis effect size (LEfSe) (Fig. 7F,G). Potentially enteropathogenic bacteria phylum Proteobacteria, family Bacteroidaceae, and genus Bacteroids were predominant biomarkers in the cisplatin group. While genus Rikenellaceae was the predominant biomarker in CPLBS group. The beneficial bacteria family Lachnospiraceae were predominant biomarkers in LBS treated group. Genus Rosburia and Rikenellaceae were highlighted in the Control group.

### 3.7 LBS effect on the gut metabolic functional profile

The gut metagenome of microbial communities’ analysis from 16S rRNA using STAMP (version 2.1.3) and KEGG pathways showed differences between the among CP treated animal and Control group, Interestingly, the most enriched metabolic pathways among these were sulfate and nitrogen respiration, amino acid biosynthesis, metabolism, starch degradation, creatinine degradation, L-rhamnose degradation, glycolysis, citrate cycle (TCA), energy production, photorespiration, biotin biosynthesis, sucrose biosynthesis, L-tyrosine, L-phenylalanine biosynthesis, and pyridoxal 5-phosphate biosynthesis. Together, KEGG pathways were altered in different groups, proving that LBS treatment can boost immunity by modulating the gut microbiota’s metabolism (Fig. 8A,B).
Table 2
The percentages of bacterial phylum in different treatment groups

<table>
<thead>
<tr>
<th>Phylum</th>
<th>Control</th>
<th>LBS</th>
<th>CP</th>
<th>CPLBS</th>
</tr>
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<tbody>
<tr>
<td>P_Bacteroidota</td>
<td>59.33%</td>
<td>59.81%</td>
<td>66.22%</td>
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<td>P_Firmicutes</td>
<td>37.78%</td>
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<td>P_Proteobacteria</td>
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<td>3.20%</td>
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<td>P_Actinobacteriota</td>
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<td>P_Campilobacterota</td>
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<td>P_Verrucomicrobiota</td>
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4. Discussion

In the current study, we used a BALB/c mouse model to investigate the protective effect of commensal probiotic LBS on gut microbiota dysfunction in cisplatin-induced intestinal mucositis.

Cachexia (involuntary loss of weight > 5%), a typical side effect of chemotherapy that decreased survival in oncology patients is a significant health issue that affected cancer patients. Weight loss in cancer patients has also a detrimental effect on malnutrition as a result of anorexia, which can result in infection and life-threatening conditions. Our study is confirmed with those of another study, which reported that cisplatin caused dramatic weight loss and severe anorexia [39]. Additionally, pretreatment with LBS attenuates the loss of weight, improves food intake, and increases stool output. Interestingly, LBS could Improve gastrointestinal function and promotes intestinal health.

Moreover, the thymus and spleen are the main component of the immune organ in the body and play a vital role in nonspecific immunity, in addition, it considered the site of the proliferation of immunological cells [40]. Consequently, the immune organ index is usually used to indicate the growth of immune organs and assess the role of probiotics in immunoregulation [41, 42]. *Lactobacillus* has been reported previously that have an impact on activating nonspecific immunity [43, 44]. Meng et al. and Li et al. reported that some types of *lactobacilli* significantly enhanced the immune organ index[45] [46], and that is consistent with our study which revealed the thymus and spleen indexes in three groups of control and with LBS treatment were greater than in CP group. These results indicated that LBS could resist the influence of cisplatin in immunosuppression on immune organs.
Given that, Intestinal epithelial cells (IECs) play an essential role in regulating intestinal homeostasis and are considered part of the immune system by taking part in the transmission of the signal to the intestine through the secretion of cytokines and oxidative stress mediators [47]. Cytokines are low-molecular-weight glycoproteins that are produced by various cells in the body and have a crucial role in the progression of immune response and the pathogenesis of the inflammatory disease [48], the effect of LBS on the production of IL-2, IL-6, and IFN-γ was determined and suggested to play a significant role in intestinal mucositis [49]. Previous studies have reported that pro-inflammatory cytokines are significantly increased in the large intestine of rats following post-treatment with chemotherapy [50]. In addition, several studies have endeavored to target pro-inflammatory cytokines as a precautionary measure for intestinal barrier damage [51–54]. Our findings are confirmed with previous results and showed that (IL-1β, IL-6, and TNF-α) are significantly upregulated in the serum and/or colon tissue at mRNA levels following administration of cisplatin and decreased proinflammatory cytokine levels in mice receiving CP.LBS. This indicated that LBS could reduce the inflammation and therefore enhance those pro-inflammatory mediators involved in the progression of mucositis.

Also, tight junction proteins (TJs) offer a physical barrier to the intestine that contributes to maintaining intestinal barrier function, enhancing GI permeability, and maintaining the intestinal mucosal barrier [55]. TJs are comprised of two protein categories, integral transmembrane proteins that form a connection between neighboring cell membranes like Claudin and Occludin, and peripheral membranes like ZO-1, which connects Claudin and Occludin that may serve to maintain tight junctions intact [56]. Therefore, TJ integrity is dramatically maintained by the strong bond between the integral transmembrane and peripheral membrane protein in addition to the arrangement of the actin cytoskeleton.

Moreover, decreased in TJs levels always revealed an elevated permeability of the intestinal epithelial cell barrier [55–57]. Leocádio et al.[58] and Beutheu Youmba[59] showed that chemotherapeutic agents caused elevation in intestinal permeability that led to damage of the epithelial barrier through lowering protein expression level of TJs. Maintenance of the integrity of TJ suggests an important strategy to prevent and/or treat the pathogenesis of illness and intestinal damage. However, the mucin secreted by the goblet cells in the intestine is also important for creating the intestinal barrier [60]. Intestinal mucus could protect the intestinal epithelium against microbes by removing harmful bacteria [61].

In the CP model group staining results indicated intestinal and mucosal barrier alteration, and reduction in the goblet cells and tight junction proteins. The LBS restores the damaged TJs integrity caused by CP via restoring the goblet cell and improving tight junction stability. In keeping with these observations, an up-regulate level of Occludin has a role in further improving TJ integrity and preventing disorders of the TJ [62].

This drives us to explore further whether there is a difference in the gut microbiome structure or composition involved in the development of mucositis. The protective effect of LBS and restoration of microbiota was examined using microbiota 16S rRNA pyrosequencing. The intestine plays a significant role in homeostasis. The microbiota mainly interacts to enhance barrier integrity. However, diseases
associated with metabolic disorders and immune suppression led to an imbalance in the microbial ecology and reduce the diversity and richness of the gut microbiome.

About five to seven of 52 identified bacterial phyla on Earth are known to live in the mammalian gut, Bacteroidetes and Firmicutes typically are the most common and highest relative abundance in the GIT, whereas phyla of Proteobacteria, Actinobacteria, Verrucomicrobia are found the less frequently [63]. Moreover, our experimental groups noted variations in the intestinal microbiota. Indeed, the model group increased the abundance of pathogenic bacteria Bacteroidots and Proteobacteria (mucosa-associated inflammation-promoting bacteria) at the phylum level and decrease beneficial bacteria Firmicutes, when compared with the other three groups, as well as LBS treated groups reversed the alteration induced by cisplatin.

However, in a healthy intestine, the gut flora normally has a minor abundance of phylum Proteobacteria, but the increased appearance of these bacteria in the gut refers to an imbalanced microbial community (dysbiosis) and has been reported to be elevated in chemotherapy-induced mucositis [64–66].

Obligate anaerobic bacteria are responsible to convert many fermentation products into short-chain fatty acids [67, 68]. The gut microbiota of a healthy colon is dominated by obligate anaerobes, whereas dysbiosis is often characterized by an increase of facultative anaerobic bacteria, Thus, in the large intestine, the dominance of obligate anaerobic bacteria maintains gut stability via the production of metabolites. Indeed, our results support this hypothesis that an imbalanced gut microbial community is featured by the enrichment of those facultative anaerobic bacteria. To sum up, we suggested that intestinal inflammatory response caused by cisplatin is associated with the overgrowth of members of facultative anaerobic bacteria such as Proteobacteria, and Actinobacteria while CPLBS group showed less abundance in those bacteria and was more similar to the Control group.

Given that, a leaky gut is demonstrated by the raising of gram-negative bacteria, Proteobacteria is considered gram-negative bacteria, and their cell wall is mainly composed of lipopolysaccharide. Risk of disease associated with the secretion of LPS which is positively correlated and triggers inflammation [69]. Therefore, it may be hypothesized the microbes that are identified as a potential overgrowth in the CP group may relate to the inflammatory response in the intestine and induced mucositis.

Undoubtedly, at the genus level, the beneficial bacteria include: Lachnospiraceae, Lactobacillus, Alistipes, and Roseburia are observed in LBS groups and decreased dramatically in the cisplatin group. Those bacteria play a key role in the metabolism of undigested carbohydrates[70] and produce butyrate and other SCFAs through hydrolyzing starch and sugars that contribute to increasing the energy extracted from the diet [71–73]. Also, clinical studies have shown that Lachnospiraceae is vital in attenuating intestinal inflammation and repairing intestinal mucosal damage, serving as protective intestinal commensal bacteria [63]. Moreover, previous studies reported that Lactobacillus species upregulate mucin content that inhibits by cisplatin chemotherapy, it is suggested that dysfunction of the mucus barrier may contribute to cisplatin-induced mucositis [74].
We further studied the metabolome functions from 16S rRNA data by using the bioinformatics tools, the dysbiosis has side effects on metabolic and functional pathways and has an impact on the physiological processes of the organism, the host immune system, and nutrient biosynthesis as studied in the KEGG orthologous analysis. Ultimately, in our study, 16S rRNA sequencing analysis and a STAMP analysis were used to investigate the metabolome of the mice's gut microbiome. We observed that LBS may improve the method of utilizing energy, carbohydrate metabolism, and nutrient absorption. Implying that LBS may act as an immunoprotective agent.

In summary, these findings enhanced that pretreatment with probiotic *Lactobacillus rhamnosus* protects against cisplatin-induced mucosal barrier damage through maintenance of the barrier integrity and up-regulating of tight junction proteins and anti-inflammatory properties, in addition, enhance positively the microbiome diversity and increasing the beneficial bacteria (*Lachnospiraceae* and *Lactobacillus*). In turn, this regulates gut microbiota imbalances caused by cisplatin and provides great potential in mitigating intestinal mucositis as a dietary agent.

**Abbreviations**

IM, Intestinal mucositis; IECs, Intestinal epithelial cells; IL-1β, Interleukin 1-β; IL-6, Interleukin-6; TNF-α, Tumor necrosis factor-α; TJs, tight junction proteins; ZO-1, Zonula Occludins-1; OTU, Operational taxonomic unit; KEGG, Kyoto Encyclopedia of Genes and Genomes; PICRUSt, Phylogentic Investigation of Communities by Reconstruction Unobserved State; NMDS, Non-metric Multidimensional Scaling.

**Declarations**

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**Authors Contribution**

Conceptualization, Duaa Alsholi, Yi Xin and Liang Wang.; Methodology, Duaa Alsholi, Ghazi SuleimanYacoub, Maroua Elkharti, and Ting Deng.; Software, Duaa Alsholi, Maroua Elkharti, Asif Iqbal Khan and Yamina Alioui.; Validation, Yi Xin; Formal analysis, Duaa Alsholi, Ghazi SuleimanYacoub, Ata Ur Rehman and Yamina Alioui.; Investigation, Duaa Alsholi, Ghazi SuleimanYacoub, Ata Ur Rehman, Asif Iqbal Khan, and Maroua Elkharti.; Resources, Yi Xin.; Data curation, Duaa Alsholi, Ghazi SuleimanYacoub, Ata Ur Rehman, Asif Iqbal Khan, and Maroua Elkharti.; Writing – Original Draft Preparation, Duaa Alsholi.; Writing – Review & Editing, Duaa Alsholi, Yi Xin.; Visualization, Yi Xin.; Supervision, Yi Xin.; Project Administration, Yi Xin.; Funding Acquisition., Yi Xin and Liang Wang.
All authors have read and agreed to the published version of the manuscript.

**Conflicts of Interest:** The authors declare no conflict of interest.

**References**


42. 42.


Figures
Figure 1

Effects of probiotic LBS on cisplatin-induced mucositis in mice: (A) Animal experimental design (B) Change in the BW percentage % = BW on the specified day/the BW at day 0 × 100 (C) Food intake (g)/day/animal. (D) Thymus index. (E) Spleen index. (F) Diarrhea assessment. Results reflect the average of 3 separate trial ± SEM; *P < 0.05, **P < 0.01, ***P < 0.001: compared with the control group; #P < 0.05, ##p < 0.01 and ### p< 0.001 compared to the model group.
Figure 2

Measurement of Pro-inflammatory cytokine (IL-1β, IL-6, and TNF-α) A. Serum concentrations of cytokines by ELISA. B. Relative expression of mRNA in colonic tissue. #p < 0.05, ##p < 0.01 and ### p < 0.001, vs CP group. *p <0.05, **p < 0.01 and ***p < 0.001, vs control group. The results are presented as the mean ± SEM.
Figure 3

Effects of LBS on histology of (A) colon and (B) ileum after cisplatin treatment. Colon and ileum sections are stained with hematoxylin and eosin. The black arrow indicates inflammatory cells, the red arrow indicates epithelial and goblet cells, the green arrow indicates epithelial surface, and the yellow arrow indicates the shortening of the villi. (C) The morphology of the mice colon, (D) Measurement of colon length. Data are presented as mean ± SEM, *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 4

LBS enhanced the expression in each of: (A) Claudin, (B) Occludin, (C) Zonula occludens-1 (ZO-1), and (D) Mucin-2 (MUC-2) in cisplatin-induced intestinal injury in mice in colon and ileum tissue of different groups by immunohistochemistry staining. Protein expression in each of Claudin, Occluden, Zo-1, and Mucin (indicated by red arrows), inflammatory cells (indicated by black arrows), and epithelium surface (indicated by green arrows). Original magnification 10×, 20×, scale bar: 100 µm.
Figure 5

(A) mRNA expressions in colonic tissue of Claudin-1, Occludin, Zo-1, and Mucin-2. (B) Representative western blot images and relative protein expressions of Claudin-1 and Occludin, with β-actin as reference gene. #p< 0.05, ##p < 0.01 and ### p < 0.001, vs CP group. *p <0.05, **p < 0.01 and ***p < 0.001, vs control group. The results are presented as the mean ± SEM.
Figure 6

CP decreased the diversity and richness of gut microbiome in feces of (A) Venn diagram demonstrates bacterial OTUs that are shared across treatment groups. (B) BugBase analysis identifies microbiome phenotypes; that contain mobile elements; facultative anaerobic; gram-negative; gram-positive microorganisms; potentially pathogens and stress-tolerant (C) Alpha diversity indexes. Simpson to measures the evenness in the community, Shannon, and Chao-1 to indices bacterial community diversities and richness. (D-F) Gut microbiome beta diversity comparisons, (D) PCoA (weighted UniFrac analysis) with Bray–Curtis dissimilarity, different colored dots show mice receiving various treatments and each colored dot indicates a particular animal. (E) Non-metric multidimensional scaling (NMDS). (F) The Anosim analysis, the R value > 0, indicates that the difference between groups is greater than the difference within the group.
Figure 7

Impact of LBS on modifications of colon microbiome. (A-D) Phylum, class, family, and genus level comparison among different groups. (E) Heat map analysis of highly characterized bacterial level clusters the gut microbiome into hierarchical clusters. (F) GraPhlAn circular image of the phylogenic tree from the extensive collections of the 16S rRNA metadata groups. (G) LEfSe analysis bars showing the taxonomic biomarker from phylum to genus between the experimental groups.
Figure 8

Analysis of the Functional Metabolic Pathways in the Control, Model-CP, and treated groups (A) KEGG STAMP analysis. (B) Heatmap analysis by the FABROTAX database is demonstrated to identify metabolic functions and other ecologically significant activities in groups.