Probiotic *Lactobacillus rhamnosus GG* (LGG) restores intestinal dysbacteriosis to alleviate upregulated inflammatory cytokines triggered by femoral diaphyseal fracture in adolescent rodent model

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Abstract. – OBJECTIVE: The aim of the study was to examine the influence of femoral shaft fracture on systemic inflammation and gut microbiome in adolescent rats and evaluate the anti-inflammatory effect of *Lactobacillus rhamnosus* GG (LGG) and its regulation of intestinal flora, as well as illustrate the mechanism by which LGG ameliorates the inflammatory response and restores intestinal dysbacteriosis.

MATERIALS AND METHODS: Twenty-four male Sprague Dawley rats of 5 to 6 weeks of age were subjected to a standard femoral shaft fracture and internally fixed with LGG supplementation in advance or on the same day of injury or with saline solution for 1 week. The levels of TNF-α, IL-6, IL-10, and CRP were assessed using standard protocols. Furthermore, gut microbiota composition was analyzed in the fecal samples using 16S rDNA gene sequencing, and the relationship between gut microbiota variation and inflammatory response was tested.

RESULTS: The serum indices of the above-mentioned inflammatory cytokines were significantly increased, and the gut microbial balance was significantly disturbed in adolescent rats by diaphyseal fractures of the femur and surgery. Moreover, *L. rhamnosus* strains manipulated the gut microbiota by decreasing the relative abundance of Proteobacteria and increasing that of Firmicutes, Actinobacteria and Bacteroidetes, which in turn increased the levels of IL-10 and alleviated the levels of IL-6, CRP, and TNF-α.

CONCLUSIONS: LGG exhibited anti-inflammatory effects by alleviating the inflammatory response and regulating the gut microbiota in adolescent rats who underwent skeletal fracture and surgery. Our results suggested that the *L. rhamnosus* strains could be considered as an alternative dietary supplement for the prevention or treatment of skeletal injury and its associated complications.

Key Words: Intestinal microbiota, Inflammation, *L. rhamnosus*, Femoral shaft fracture, Pediatric.

Introduction

Diaphyseal fractures of the femur (DFF) are common long-bone injuries in children and adolescents. DFF represent approximately 1.5% of all bony injuries in childhood. DFF not only cause pain and soft-tissue injury but also cause a hyper-inflammatory reaction of the immune system. Moreover, surgical intervention is a posttraumatic immune stimulus that can further augment the inflammatory reaction. These over-exuberant inflammatory cytokines can result in delayed fracture healing and elevate the risk of nonunion and infection. Bone remodeling is also regulated by the elements of the immune cytokines. Therefore, elucidation of the specific posttraumatic regulation of the inflammatory response has been considered a tempting research area to help improve posttraumatic therapeutic strategies and treatment concepts. Although the impact of traumatic insults on the immune system has been widely studied in adults or aged patients, little data exist concerning posttraumatic inflammatory changes in pediatric patients.

In recent years, the gut microbiota has received massive attention as a potential modifiable risk factor with regard to disease development. Probiotic supplementation is a common approach to confer health benefits. Probiotics are non-pathogenic microorganisms that confer several health benefits by modulating the gut microbiota and regulating the secretion of inflammatory cytokines.
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nes when administered in adequate amounts, and hence, could be used in inflammatory bowel disease\textsuperscript{9,10}, irritable bowel syndrome\textsuperscript{11}, and gastrointestinal cancers\textsuperscript{12}. \textit{Lactobacillus rhamnosus} GG (LGG) is one of the most common microorganisms used as probiotics\textsuperscript{13-16}, which confers beneficial effects such as the maintenance of immune homeostasis\textsuperscript{15,17}, management of children with inflammation-related diseases\textsuperscript{4,15} and modulation of intestinal microbiota composition\textsuperscript{13,18}. However, the dynamic changes in the gut microbiome throughout the progression of skeletal fractures are unclear. Furthermore, the effect of LGG on the modulation of intestinal microbial homeostasis and amelioration of the injury-induced inflammatory response in pediatric patients with skeletal fractures remains to be elucidated.

In this study, we aimed to evaluate the development of the inflammatory response following DFF injury in an adolescent rodent model. Next, we examined the influence of LGG on the modulation of intestinal dysbacteriosis and homeostasis of the systemic inflammatory cytokines following DFF injury. We hypothesized that systemic inflammatory cytokines would be elevated and intestinal microflora would be aberrant after DFF injury and the surgical procedure and that LGG intake would modify the state of gut microbiome thus attenuating the expression level of these markers.

**Materials and Methods**

**Experimental Design**

Experiments were performed according to the guidelines of the Experimental Animal Care and Use Committee approved by Qilu Hospital of Shandong University. Twenty-four male Sprague Dawley (SD) rats aged 5-6 weeks old were used. Profiling of a recent catalog showed that the rats attain puberty at about 42 days, and humans at about 11.5 years\textsuperscript{19}. Furthermore, the gut microbiome of SD rats is similar to that of humans\textsuperscript{20}. Animals were maintained in a general housing environment and fed sterilized food and autoclaved water ad libitum and were maintained on a 12:12 h light/dark cycle for 1 week before the experiments were conducted for acclimation. The rats were randomized into a sham model and two experimental models. Animals in the sham group (n = 8) received gavage saline solution. Animals in the two experimental groups were fed with probiotic supplements (LGG, 2x10\textsuperscript{9} colony-forming units [CFU]/day)\textsuperscript{13,15,21} daily starting from either 1 wk in advance (n = 8, LGG prevention, LGG Pre) or on the same day (n = 8, LGG treatment, LGG Tre) of DFF and surgery. Animals were killed at 7 d after injury by CO\textsubscript{2} inhalation and decapitation. The time points of the serum and stool specimens collected are shown in Figure 1. The number of animals used in this study was predetermined before the initiation of the investigation. The number was estimated based on prior data from our laboratory.

**Fracture Creation and Animal Care**

After anesthesia (intraperitoneal injection of 10% chloral hydrate [3 ml/100 g]), the rats were placed in a prone position, and the right femoral shaft was fractured with the use of a blunt guillotine three-point bending apparatus. This resulted in a closed femoral fracture with soft-tissue trauma consistent with a blunt force injury (Figure 2). Then, a medial parapatellar incision was created. A Dremel Moto-Tool drill (Synthes, Monument,
Colorado, USA) with a 0.5 mm Kirschner wire (Aap Implantate AG, Berlin, Germany) was inserted through the distal fracture fragment, crossing just lateral to the proximal fragment and engaging the medial femoral cortex. Finally, the skin was closed with 4-0 Vicryl resorbable sutures. Pinning fractures at the time of injury were established by the Institutional Animal Care and Use Committee to protect animal welfare. Besides, this model has been established as an accepted closed femoral fracture model within the literature22. Animals were allowed to drink freely from their water bottles following injury. Food was placed on the floor of the cage to facilitate normal feeding behavior.

Serum Assay

Whole blood was collected at each time point, allowed to clot, and spun at 250 g for 10 min at 4°C. Serum was collected and stored in aliquots at -80°C. Serum levels of four inflammatory markers, namely, interleukin(IL)-6, IL-10, tumor necrosis factor (TNF)-α and C-reactive protein (CRP) were analyzed by an in-house enzyme-linked immunosorbent assay (ELISA) (MDL, Biomedical Technology Co. Ltd, Beijing, China).

Stool Samples Collection, DNA Extraction, and 16S rDNA Sequencing

Stool samples were collected at each time point with sterilized 2 ml tubes containing pure ethanol, aliquoted, and frozen at -80°C until DNA extraction. Total DNA extraction was performed using an E.Z.N.A.® Stool DNA Kit (D4015, Omega Inc., Norwalk, CT, USA) according to the manufacturer’s instructions. For each DNA sample, the bacterial 16S rDNA genes were amplified using a primer set specific for the V3-V4 variable region of 16S rDNA gene: 341F (5’-CCTACGGGNGGCWGGCAG-3’) and 805R (5’-GACTACHVGGGTATCTAAATCCT-3’)23. The PCR products were purified using AMPure XT beads (Beckman Coulter Genomics, Danvers, MA, USA) and quantified by Qubit Fluorometer (Invitrogen, Carlsbad, CA, USA). The amplicon pools were prepared for sequencing, and the size and quantity of the amplicon libraries were assessed on an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA) and with the Library Quantification Kit for Illumina (Kapa Biosciences, Woburn, MA, USA), respectively.

Statistical Analysis

Inflammatory Marker Analysis

Statistical tests for significance were performed using the Statistical Package for the Social Sciences software (SPSS Statistics, version 22.0, IBM, Armonk, NY, USA). The constant variables were analyzed using an unpaired t-test. Pearson’s correlation test was used to calculate the correlation coefficients. Differences were considered statistically significant when the p-value was <0.05.

Fold Change Analysis

Alpha and beta diversities were calculated using QIIME2, and the relative abundance of the bacterial taxonomic groups was assessed. Alpha and beta diversities were analyzed by the QIIME2 workflow, and graphs were plotted using R (v3.5.2). Multiple sequence alignment for species annotation was performed by BLAST against the databases SILVA and NT-16S.

Results

DFF Injury and Fixation Induces a Systemic Inflammatory Response

To determine whether DFF and fixation were associated with changes in the expression of inflammatory markers, the levels of CRP, IL-6, IL-
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10, and TNF-α were quantified. Figures 3A-6A illustrate the changes in the expression of the inflammatory markers. The concentrations of CRP, IL-6, IL-10, and TNF-α were significantly elevated at 0.5 d following injury. The levels of CRP, IL-6, and IL-10 returned to pre-operative levels at 7 d after injury (Figures 3a-3e, 4a-4f, and 5a-5f). However, the level of TNF-α returned to pre-operative levels at 3 d (Figures 6b-6d). Therefore, the injury and operative procedure affected these differences.

**LGG Inhibits Expression of Pro-Inflammatory Cytokines IL-6, TNF-α and Systemic Marker CRP**

Compared with animals that did not receive LGG (sham group), the LGG-treated (LGG Tre) and LGG-prevention-treated (LGG Pre) animals had significantly suppressed levels of CRP at 0.5, 1, 2 and 3 d after injury and fixation (Figures 3a-3d), and IL-6 at all time points (Figures 4a-4f). The differences were significant ($p<0.05$ for sham vs. LGG Tre and sham vs. LGG Pre). However, the TNF-α upregulation was ameliorated only at 1 and 2 d after injury (Figures 6b-6c) ($p<0.05$ for sham vs. LGG Tre and sham vs. LGG Pre). The differences between the LGG Tre and LGG Pre groups were not statistically significant.

The values of CRP, IL-6, and TNF-α are shown in Table I. These data indicated that LGG intake was related to the suppression of pro-inflammatory cytokine expression.

**LGG Elevates Expression of Anti-inflammatory Cytokine IL-10**

IL-10 is an anti-inflammatory cytokine, which plays a central role in the regulation of wound healing and angiogenesis. A higher level of IL-10 was found in the LGG-treated group compared to the sham group (Figure 5A). The differences at the four time points (0.5, 1, 2, and 3 d after injury) are presented in Figures 5a-5d, and the values are shown in Table I. These differences were significant ($p<0.05$ for sham vs. LGG Tre and sham vs. LGG Pre). Therefore, the level of anti-inflammatory cytokine IL-10 was significantly upregulated in the LGG-treated group, suggesting that LGG diminished the induction of inflammatory cytokines following DFF injury.

**DFF Injury and Fixation Induce Gut Microbial Perturbation**

To characterize the dynamic alterations in gut microbiota during DFF injury and subsequent fixation, we compared the alpha and beta diversities of fecal samples from the sham group at four

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**Figure 3.** Effect of DFF and surgical injury on serum levels of C-reactive protein (CRP). Data for both LGG-treated animals, including LGG prevention (LGG Pre) and LGG treatment (LGG Tre) groups and saline-solution treated controls (Sham group) at 8 time points included 1d and 7d in advance of injury and 12h, 1d, 2d, 3d, 5d, and 7d after injury (A), as well as these groups analyzed at 12h, 1d, 2d, 3d, 5d, and 7d after injury are given as the mean and the standard deviation (a-f). Symbols and colors represent data from individual rat from three independent groups. Data for the Sham group are shown as green spots, data for the LGG Tre group are shown as red triangles and data for the LGG Pre group are shown as blue squares. * indicates that the difference is significant compared LGG Tre group with the sham group. # indicates that the difference is significant compared LGG Pre group with the sham group and + indicates the difference is significant compared LGG Tre group with the LGG Pre group. Unpaired t-test: * or # or $p<0.05$, ** or ## or ++ $p<0.01$ and *** or ### or +++ $p<0.001$. All the $p$-values were according to two-tailed unpaired t-test.
Figure 4. Effect of DFF and surgical injury on serum levels of interleukin-6 (IL-6). Data for both LGG-treated animals, including LGG prevention (LGG Pre) and LGG treatment (LGG Tre) groups and saline-solution treated controls (Sham group) at 8 time points included 1d and 7d in advance of injury and 12h, 1d, 2d, 3d, 5d, and 7d after injury (A), as well as these groups analyzed at 12h, 1d, 2d, 3d, 5d, and 7d after injury are given as the mean and the standard deviation (a-f). Symbols and colors represent data from individual rat from three independent groups. Data for the Sham group are shown as green spots, data for the LGG Tre group are shown as red triangles and data for the LGG Pre group are shown as blue squares. * indicates that the difference is significant compared LGG Tre group with the sham group. # indicates that the difference is significant compared LGG Pre group with the sham group and + indicates the difference is significant compared LGG Tre group with the LGG Pre group. Unpaired t-test: * or # or + p< 0.05, ** or ## or ++ p< 0.01 and *** or ### or +++ p< 0.001. All the p-values were according to two-tailed unpaired t-test.

Table I. Comparison of hematology results of different groups after DFF and surgery.

<table>
<thead>
<tr>
<th>P-values</th>
<th>Sham vs. Tre</th>
<th>Sham vs. Pre</th>
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<tbody>
<tr>
<td>CRP (ug/L)</td>
<td></td>
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<tr>
<td>0.5 d post op.</td>
<td>3535.52 ± 288.94</td>
<td>3995.17 ± 143.98</td>
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<tr>
<td>1 d post op.</td>
<td>5668.49 ± 315.98</td>
<td>5281.93 ± 172.08</td>
</tr>
<tr>
<td>2 d post op.</td>
<td>6811.43 ± 372.77</td>
<td>6266.61 ± 183.15</td>
</tr>
<tr>
<td>3 d post op.</td>
<td>4604.10 ± 318.66</td>
<td>3900.73 ± 210.34</td>
</tr>
<tr>
<td>5 d post op.</td>
<td>3029.19 ± 180.92</td>
<td>2903.93 ± 168.22</td>
</tr>
<tr>
<td>7 d post op.</td>
<td>2665.13 ± 128.24</td>
<td>2777.56 ± 175.59</td>
</tr>
<tr>
<td>IL-6 (pg/mL)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5 d post op.</td>
<td>136.96 ± 8.44</td>
<td>146.00 ± 7.64</td>
</tr>
<tr>
<td>1 d post op.</td>
<td>206.42 ± 10.23</td>
<td>185.53 ± 6.32</td>
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<tr>
<td>2 d post op.</td>
<td>193.05 ± 12.39</td>
<td>174.25 ± 6.77</td>
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<tr>
<td>3 d post op.</td>
<td>173.68 ± 8.50</td>
<td>159.68 ± 8.20</td>
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<tr>
<td>5 d post op.</td>
<td>136.87 ± 5.99</td>
<td>127.93 ± 7.98</td>
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<tr>
<td>7 d post op.</td>
<td>99.29 ± 6.09</td>
<td>85.47 ± 5.97</td>
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<tr>
<td>IL-10 (ng/L)</td>
<td></td>
<td></td>
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<tr>
<td>0.5 d post op.</td>
<td>207.95 ± 9.36</td>
<td>238.47 ± 13.38</td>
</tr>
<tr>
<td>1 d post op.</td>
<td>268.60 ± 12.23</td>
<td>307.29 ± 11.24</td>
</tr>
<tr>
<td>2 d post op.</td>
<td>251.50 ± 8.78</td>
<td>268.41 ± 9.65</td>
</tr>
<tr>
<td>3 d post op.</td>
<td>198.75 ± 9.78</td>
<td>219.78 ± 11.91</td>
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<tr>
<td>5 d post op.</td>
<td>171.08 ± 9.41</td>
<td>178.80 ± 13.18</td>
</tr>
<tr>
<td>7 d post op.</td>
<td>143.61 ± 10.77</td>
<td>147.40 ± 11.74</td>
</tr>
<tr>
<td>TNF-α (ng/L)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5 d post op.</td>
<td>477.14 ± 20.60</td>
<td>516.26 ± 23.45</td>
</tr>
<tr>
<td>1 d post op.</td>
<td>419.10 ± 19.62</td>
<td>385.17 ± 14.37</td>
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<tr>
<td>2 d post op.</td>
<td>358.16 ± 20.39</td>
<td>317.93 ± 21.34</td>
</tr>
<tr>
<td>3 d post op.</td>
<td>312.98 ± 15.15</td>
<td>298.55 ± 12.12</td>
</tr>
<tr>
<td>5 d post op.</td>
<td>296.55 ± 16.06</td>
<td>303.36 ± 16.60</td>
</tr>
<tr>
<td>7 d post op.</td>
<td>295.90 ± 10.77</td>
<td>300.64 ± 11.25</td>
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Values are expressed as mean ± SD (n=8). Abbreviations: CRP, C-reactive protein; IL-6, interleukin-6; IL-10, interleukin-10; TNF-α, tumor necrosis factor-α, LGG Tre, LGG-treated group; LGG Pre, LGG-prevention-treated group.
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time points (TPs), including one pre-injury time point on day 1 (TP1), day 1 (TP2), day 3 (TP3), and day 7 (TP4) after DFF injury plus fixation progression in the sham group. Although we did not find a significant difference in bacterial richness and diversity in the Chao indices (p>0.05,}

Figure 5. Effect of DFF and surgical injury on serum levels of interleukin-10 (IL-10). Data for both LGG-treated animals, including LGG prevention (LGG Pre) and LGG treatment (LGG Tre) groups and saline-solution treated controls (Sham group) at 8 time points included 1d and 7d in advance of injury and 12h, 1d, 2d, 3d, 5d, and 7d after injury (A), as well as these groups analyzed at 12h, 1d, 2d, 3d, 5d, and 7d after injury are given as the mean and the standard deviation (a-f). Symbols and colors represent data from individual rat from three independent groups. Data for the Sham group are shown as green spots, data for the LGG Tre group are shown as red triangles and data for the LGG Pre group are shown as blue squares. * indicates that the difference is significant compared LGG Tre group with the sham group. # indicates that the difference is significant compared LGG Pre group with the sham group and + indicates the difference is significant compared LGG Tre group with the LGG Pre group. Unpaired t-test: * or # or + p< 0.05, ** or ## or ++ p< 0.01 and *** or ### or +++ p< 0.001. All the p-values were according to two-tailed unpaired t-test.

Figure 6. Effect of DFF and surgical injury on serum levels of tumor necrosis factor-α (TNF-α). Data for both LGG-treated animals, including LGG prevention (LGG Pre) and LGG treatment (LGG Tre) groups and saline-solution treated controls (Sham group) at 8 time points included 1d and 7d in advance of injury and 12h, 1d, 2d, 3d, 5d, and 7d after injury (A), as well as these groups analyzed at 12h, 1d, 2d, 3d, 5d, and 7d after injury are given as the mean and the standard deviation (a-f). Symbols and colors represent data from individual rat from three independent groups. Data for the Sham group are shown as green spots, data for the LGG Tre group are shown as red triangles and data for the LGG Pre group are shown as blue squares. * indicates that the difference is significant compared LGG Tre group with the sham group. # indicates that the difference is significant compared LGG Pre group with the sham group and + indicates the difference is significant compared LGG Tre group with the LGG Pre group. Unpaired t-test: * or # or + p< 0.05, ** or ## or ++ p< 0.01 and *** or ### or +++ p< 0.001. All the p-values were according to two-tailed unpaired t-test.
Figure 7A), analysis of the beta diversity based on the weighted UniFrac distances showed that the bacterial composition and distribution at TP2 was distinct from TP1. We further performed an analysis of similarities (ANOSIM). The results indicated that the composition of the gut microbiome was significantly affected by DFF injury plus fixation progression (ANOSIM, $r=0.337$, $p<0.01$, weighted UniFrac, Figure 7B).

To further examine the shift of the community structure in terms of taxonomic and functional composition during the injury, we assessed the hierarchical clustering result based on the relative abundance of different microbial phyla. The total distribution of bacterial taxonomy showed significant variations in the bacterial communities between pre- and pro-operation. A significant increase in the relative abundance of Proteobacteria was observed at TP2 compared with TP1 (Figure 7C). Moreover, the relative abundance of Firmicutes and Bacteroidetes was reduced (Figure 7C). Interestingly, the intestinal bacterial community composition at TP3 was similar to that at TP1. Thus, DFF injury plus fixation induces gut microbial perturbation.

**LGG Prevents and Remedies Gut Dysbiosis Caused by the Induction of DFF Injury and Fixation**

Next, we next examined the effect of LGG administration on the gut microbial composition. A comparison of the alpha and beta diversities of fecal samples among the sham, LGG Tre group, and LGG Pre groups at TP2 revealed distinct gut microbiome profiles. Beta diversity analysis based on the weighted UniFrac distances showed that the rats treated with LGG exhibited significant differences in bacterial composition and distribution at TP2 compared with the sham group ($p=0.01$, weighted UniFrac, Figure 8B), while analysis of alpha diversity showed that there was no significant difference at TP2 ($p>0.05$, Figure 8A). These data indicate that LGG regulates injury-induced gut dysbiosis. However, the alterations of microbial composition in the LGG Tre group were highly similar to those in the LGG Pre group at TP2 (ANOSIM, $r=0.038$, $p>0.05$, weighted UniFrac, Figure 8C), suggesting that preventive probiotic intake did not alleviate gut dysbiosis.

To identify how the gut microbiota profile changed upon LGG feeding during the induction of DFF injury and fixation, we next examined the relative abundance of microbes at the phylum level between the sham and LGG Tre groups at TP2. The stack map (Figure 8D) showed that LGG intake could restore the composition and diversity of gut microbiota. In the sham group (Figure 8E), the relative abundance of Deferribacteres, Fusobacteria, Acidobacteria, Proteobacteria and Epsilonbacteraeota were increased, while those of Actinobacteria, Elusimicrobia, Bacteroidetes, Tenericutes, Gemmatimonadetes, and Firmicutes were reduced (Figure 8C).
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Cyanobacteria, Firmicutes, Verrucomicrobia and Patescibacteria were decreased after injury, indicating that these microbes may be influenced by the induction of experimental injury severely. We further investigated the alterations in the species in the LGG-treated rats. Unlike rats in the sham group, a cluster of microbes, including Epsilonbacteriota, Actinobacteria, Elusimicrobia, Bacteroidetes, Tenericutes, Gemmatimonadetes, Patescibacteria, Firmicutes and Verrucomicrobia were upregulated, and Deferribacteres, Fusobacteria, Acidobacteria, Proteobacteria and Cyanobacteria were downregulated in LGG-treated rats (Figure 8E). Collectively, these conclusions shifted toward the alleviation of upregulated Bacteroidetes, Firmicutes, Patescibacteria, Verrucomicrobia and downregulated Fusobacteria, Acidobacteria, Proteobacteria suggesting that LGG can rebalance the gut microbiota to a healthy status.

Linear discriminant effect size (LEfSe) analysis between the sham and LGG Tre groups revealed signature microbiome profiles and predominant bacterial biomarkers of the LGG Tre group. Significant increases in the relative abundance of Firmicutes, Actinobacteria, and Bacteroidetes (at the phylum level) as well as Lactobacillus, Bifidobacterium, and Paramuribaculum (at the genus level), in addition to a significant reduction in Proteobacteria (at the phylum level), were observed in the LGG Tre group compared with the sham group, as indicated by the linear discriminant analysis (LDA). All potential biomarkers (LDA score >3) are shown in Figure 8F. Therefore, the function of LGG to reshape the microbial community structure and function is closely associated with its ability to restore the relative abundance of Proteobacteria, Firmicutes, Actinobacteria and Bacteroidetes to normal levels.
**Inhibition of Expression of Pro-Inflammatory Cytokine Expression Correlates with the Abundance of Bacteria Affected by LGG**

To better understand the association between the microbial abundances and cytokine expression in the LGG-treated group, we analyzed whether the relative abundance of intestinal bacterial species was correlated with the expression of inflammatory cytokines. Results showed that the abundance of only *Firmicutes* and *Proteobacteria* were correlated with the expression of pro-inflammatory cytokines detected previously. The relative abundance of *Firmicutes* in the LGG-treated group showed significant negative correlations with the expression level of CRP ($r=-0.69$ and $p<0.05$ for LGG Tre, $r=0.70$ and $p<0.05$ for sham Group, Figure 9A). In contrast, the relative abundance of *Proteobacteria* showed significant positive correlations with the expression level of IL-6 ($r=0.85$ and $p<0.05$ for LGG Tre, $r=0.78$ and $p<0.05$ for sham Group, Figure 9B). These results suggest that the effect of LGG on the maintenance of immune homeostasis in rats after DFF injury and fixation is associated with the homeostasis of intestinal microflora.

**Discussion**

Fractures of the femoral shaft are relatively common injuries in children and adolescents\(^1\). The surrounding soft-tissue injury, fracture hematoma, and the method of fracture fixation can result in an abnormal immune response leading to unbalanced systemic inflammation\(^7\). Pediatric patients are particularly susceptible to profound inflammatory responses to trauma that, if exaggerated, can disturb the fracture-healing cascade and jeopardize successful bone repair. Therefore, understanding the inflammatory status of patients suffering from skeletal trauma may be a useful adjunct in clinical decision-making.

Many studies\(^4,6,8,24-26\) have described the impact of a traumatic insult on the cytokine profile in the inflammatory response in aged rats or humans, but little data exist concerning posttraumatic inflammatory changes in pediatric rats or patients. The increase in inflammatory cytokine levels following injury indicates their role in the body’s response to trauma. Gan et al\(^27\) studied the serum levels of TNF-α in elder hip fracture rats, which peaked at 8 and 72 h after treatment and found that they were significantly higher than the sham group. Zhang et al\(^28\) reported that serum levels of TNF-α, which peaked at 2 h and then at 24 h, were slightly lower than its peak value in a model of elderly rats hip fracture. In our experiment, we confirmed the impact of a traumatic insult on the immune system in adolescent rats. First, we found that the level of TNF-α was significantly upregulated only at 12 h, 1 d, and 2 d after injury in adolescent rats, and the concentration at 48 h after injury was close to normal. Regarding TNF-α, the geriatric group showed a relatively profound pro-inflammatory response in the early postoperative stage. Therefore, we concluded that elderly rats or patients were more susceptible to the inflammatory response in the early postoperative stage compared to the adolescent group suffering from similar injuries. Nevertheless, previous studies\(^8,29\) found no significant elevation in

![Figure 9. Associations of plasmatic cytokines with the abundance of bacteria affected by L. rhamnosus. A, The negative correlation between the relative abundance of Firmicutes and the expression level of CRP. ($r=-0.69$ and $p<0.05$ for LGG Tre, $r=0.70$ and $p<0.05$ for Sham Group). B, The positive correlation between Proteobacteria and IL-6. ($r=0.85$ and $p<0.05$ for LGG Tre, $r=0.78$ and $p<0.05$ for Sham Group). The correlations according to the Pearson correlation test.](image-url)
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the pattern of serum TNF-α detected in elderly patients treated for hip fracture. The reason may be that TNF-α levels have been reported to increase very early after trauma, which made their detection difficult.

IL-6, a major pro-inflammatory cytokine, is mainly produced by TNF-α, can stimulate the hepatic release of CRP. While our data showed a significant increase in the IL-6 and CRP levels in adolescent rats after skeletal fractures and subsequent fixation, other studies have reported similar changes in the expression profile of these cytokines in the elderly groups. However, Vester et al. reported a significant increase in IL-6 concentrations after hip fracture and surgery in older patients whereas young patients did not respond to these insults. So far, only little data exist concerning the immune response of pediatric patients after skeletal injury. It is unclear whether the effects occur in adolescents who are different from the elderly. Here, we have investigated the expression of inflammatory cytokines in a randomized controlled trial to understand the systemic inflammatory status in adolescent rats following skeletal injury. Our results showed that the levels of TNF-α, IL-6, and IL-10, along with CRP in adolescent rats with DFF and fixation, were significantly increased. Therefore, the above-mentioned data indicate that the elderly rats or patients are more susceptible to the inflammatory response in the early postoperative stage, and there was no significant difference between the adolescent and elderly groups after activation of the inflammatory response.

According to the current literature, we hypothesize that the differences in the cytokine expression profiles are associated with the following changes. First, the innate immune system is not completely developed in children. Second, there is an imbalance between the pro-inflammatory and anti-inflammatory regulators in elderly rats or patients, which results in the low-grade chronic pro-inflammatory status. The steady-state inflammatory status of elderly rats or patients can be considered as a stimulus in the face of injury, making them more susceptible to the inflammatory response. Lastly, the ability to produce anti-inflammatory cytokines and hormones is reduced in the elderly.

We then compared the data of the sham group with the LGG Tre and LGG Pre groups. The results showed that LGG intake efficiently alleviated the upregulated serum concentrations of pro-inflammatory cytokines (TNF-α, IL-6) and the systemic marker CRP, while it increased those of the regulatory cytokine (IL-10) compared to placebo, thus showing significant anti-inflammatory effects.

Lactobacillus rhamnosus GG (LGG) is one of the most widely studied probiotic strains. LGG has been extensively studied in clinical trials and human intervention studies. Recommendations have been published for its use in the treatment of acute gastroenteritis and the prevention of antibiotic-associated diarrhea. It also provides potential benefits in reducing the incidence of acute otitis media and upper respiratory infections in children. Bajaj et al. studied 30 cirrhosis patients and found that TNF-α levels decreased, and the composition of the microbiome changed in the LGG-randomized group. Moreover, self-limited diarrhea was more frequent in LGG patients than in controls. Khailova et al. showed that LGG administration regulated the TNF-α and IL-6 balance in a mouse model of puncture peritonitis, allowing a more effective modulation of the inflammatory response. Additionally, several previously published studies have confirmed that LGG exerts considerable influence on activating innate immunostimulation by modulating the innate immune signaling pathway and cytokine responses. Overall, LGG mediates the inflammatory cytokine secretion and regulation of the immune system, benefiting the host. However, these studies were limited to the digestive or respiratory systems and rarely involved the motor system. Our research confirmed the dynamic changes in the gut microbiome throughout the progression of skeletal fractures, and the effect of LGG on modulating intestinal microbial homeostasis and diminishing injury-induced inflammatory response in pediatric rats with skeletal fractures.

The mechanisms by which probiotics benefit the gut environment and the health of the host are diverse. Some of them include improvement of the intestinal barrier function through effects on the epithelium and mucus lining, production of anti-microbial substances, competition with pathogenic bacteria, and regulation of luminal acidity. Most importantly, probiotics manipulate the intestinal microbial communities and improve overall health. L. rhamnosus strains have been shown to have an optimal effect in restoring the intestinal balance in several in vitro and in vivo studies. However, the results were conflicting and showed strain-specific differences.
Wang et al. found that high levels of TNF-α, IL-1β, and IL-6 in obese mice were mitigated in response to gut microbiota manipulation by decreasing the abundance of Bacteroides and Desulfovibrio and increasing that of Lactobacillus and Bifidobacterium. Therefore, after supplementation with L. rhamnosus strains, modulation of gut microbiota would lead to the amelioration of the inflammatory response. In the present study, the TNF-α, IL-6, and CRP levels were significantly decreased and the anti-inflammatory cytokine IL-10 level was significantly increased in the LGG-treated groups probably due to the higher abundance of Bacteroidetes, Firmicutes, Patescibacteria, and Verrucomicrobia and the lower abundance of Fusobacteria, Acidobacteria, and Proteobacteria caused by LGG. Consistent with our conclusion, Li et al. observed that the colitis-affected mice had more severe inflammatory effects (TNF-α and IL-6 levels increased, while the IL-10 content decreased) and was accompanied by disrupted gut microbial balance (decreased relative abundance of Firmicutes, and increased relative abundance of Bacteroidetes and Proteobacteria). However, these changes were distinctly ameliorated by the administration of LGG. Hence, intervention with LGG modulated the level of the inflammatory response via its action on the gut microbiota. Consequently, the results indicated that the gut microbiota could act as an important checkpoint for the inflammatory response and further lead to the amelioration of diseases. Moreover, LGG acts as a beneficial bio-modulator of gut microbiota.

Meanwhile, LGG was able to modulate the intestinal microbiota and influence the expression of pro-inflammatory cytokines in our study. The decreased expression of CRP had a significant negative correlation with the relative abundance of Firmicutes, while decrease IL-6 had a positive correlation with the relative abundance of Proteobacteria. It is suggested that the anti-inflammatory effect of L. rhamnosus strains was highlighted by the higher relative abundance of Firmicutes and the lower relative abundance of Proteobacteria. Proteobacteria are a minor constituent within a balanced gut-associated microbial community. A higher relative abundance of Proteobacteria is observed in humans with severe intestinal inflammation, including patients with inflammatory bowel disease, colorectal cancer or necrotizing enterocolitis. Therefore, an increased prevalence of Proteobacteria acts as a pro-inflammatory modulator enhancing the inflammatory state, which can serve as a potential diagnostic signature of dysbiosis. Firmicutes are involved in the maintenance of intestinal homeostasis and prevention of inflammation response, playing a pivotal role in the host’s overall health status.

Interestingly, we did not find a strain-specific biomodulator at the genus or species level. Many factors can negatively influence conclusions. To date, there is little evidence on the ability of probiotics to affect the inflammatory response triggered by skeletal injury in pediatric patients. Moreover, factors such as the types of inflammatory cytokines detected, administration of different probiotic species, duration of feeding, use of monostrain or multistrain products, and presence of numerous variables related to the host lifestyle can lead to different scientific conclusions.

In conclusion, our data highlighted that the TNF-α, IL-6, IL-10, and CRP were elevated and the composition and diversity of gut microbiota were disrupted by femoral shaft fracture injury and surgical treatment. The study also showed that intake prevented the inflammatory response by reducing the relative abundance of potentially harmful bacteria and increasing that of beneficial ones, thereby restoring intestinal dysbacteriosis and conferring health benefits.

Certainly, the trial had some limitations that should be acknowledged. First, we only detected the levels of TNF-α, IL-6, IL-10, and CRP, although the levels of other cytokines such as IL-4, IL-8, IL-12, IL-17, and IL-1B have also been reported. Second, we did not evaluate the gut microbial composition in fecal samples continuously after fracture and surgery. A reduction of potentially harmful bacteria and an increase in beneficial bacteria may be important probiotic features of LGG. However, further clinical and pre-clinical studies are needed to demonstrate the application of these probiotic strains in the clinical field.

**Conclusions**

The present study indicated the impact of a traumatic insult on the immune system in adolescent rats. Femoral shaft fracture injury followed by the surgical procedure can elevate the levels of the systemic inflammatory cytokines, namely, TNF-α, IL-6, IL-10, and CRP and disrupt the normal composition and diversity of gut microbiota. Our study provides data on the patterns of inflammatory cytokines and intestinal flora in non-digestive or respiratory diseases.
Moreover, the present study also indicated that LGG possessed a potent anti-inflammation effect, which can alleviate the intestinal dysbacteriosis in the rat model of femoral diaphyseal fracture. Analysis of the inflammatory cytokines and gut microbiota profile showed that the oral intervention of LGG strains modulated the gut microbiota, which resulted in the attenuation of the inflammatory response. The results of the present study provide new knowledge about the anti-inflammatory effects of LGG on skeletal-injured adolescent rats. Our findings also support the development of the proposed strains as functional food for the prevention or treatment of skeletal injury and its associated complications. Future studies should focus on the administration of LGG and the molecular mechanisms underlying the inflammatory response alleviation and gut microbiota regulation.

**Conflict of Interest**
The Authors declare that they have no conflict of interests.

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