

Probiotic consumption modulates pain and the systemic metabolome in mice after fracture

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INTRODUCTION: There is mounting evidence that the gut microbiota is both a significant regulator of bone fracture healing and a key contributor to inflammatory pain. Importantly, the gut microbiota can be easily targeted for therapeutic benefit using nutrition-based approaches such as probiotics, which are live micro-organisms that when consumed in adequate amounts confer a health benefit. We and others recently reported that dietary supplementation with beneficial probiotic bacteria can influence fracture repair in young and aged mice (1,2,3). However, the analgesic effects of probiotics during fracture healing remains unexplored. Here, we investigated whether consumption of two probiotic supplements would alleviate pain after fracture.

METHODS: Animals and fracture experiments: Female 12-week-old C57BL/6J mice were subjected to experimental closed mid-diaphyseal unilateral femoral fractures. Six hours post-injury, mice were randomly assigned to receive the probiotic species *Bifidobacterium longum* ATCC 15707 (*B. longum*; 1×10^8 CFU) or *Lactobacillus casei* ATCC 393 (*L. casei*; 1×10^8 CFU) or PBS vehicle control via a daily oral gavage. **DXA:** Mice were subjected to longitudinal DXA scans (Kubtec 4-parameter) assess whole body fat and lean tissue, and BMD and BMC of the lumbar spine. **Behavioral Assessments:** Rearing behaviors (defined as simultaneously lifting forelimbs) were recorded over a two-minute window and spatiotemporal gait was recorded to assess the impact of probiotic supplementation on fracture-induced behavioral and functional outcomes. **Untargeted Metabolomics:** Sera was isolated from fasted mice (6 hours) and analyzed using liquid chromatography with high-resolution mass spectrometry (LC-HRMS) techniques (Thermo Scientific Q-Exactive HF). Each sample was analyzed in triplicate. Two technical columns, hydrophilic interaction liquid chromatography (HILIC) and C18 hydrophobic reversed-phase chromatography in negative and positive modes were used to enhance the coverage of metabolic features. **Statistical analyses:** Parametric data were tested using repeated-measures two-way ANOVA followed by Tukey's post-hoc using GraphPad Prism software (v. 9.5.1) and statistical significance was established at $P < 0.05$. All animal procedures were approved by the IACUC and was conducted in accordance with federal and institutional guidelines.

RESULTS: We first sought to determine if probiotic consumption would attenuate the systemic consequences of fracture. Probiotic supplementation did not influence whole-body bone mineral density (BMD), bone mineral content (BMC), fat tissue, lean tissue, or body weight post-fracture. However, *B. longum* supplementation prevented post-traumatic decreases in BMD and BMC within the lumbar spine (L1-L5) at day 7 compared to PBS controls. We next investigated whether probiotic supplementation would impact pain and function during the early post-fracture period when pain and inflammation is most severe. Femoral fracture decreased the number of rearing events in the control and *L. casei* supplemented mice. However, at day 3 post-fracture, *B. longum*-supplemented mice reared significantly more than controls; whereas, at day 7 post-fracture both *B. longum*- and *L. casei*-supplemented mice reared significantly more than controls (Fig. A). We next investigated spatiotemporal measurements of gait using Experimental Dynamic Gait Arena for Rodents (EDGAR) which revealed an expected compromised gait at day 3 and 7 post-fracture in all groups (Fig. B). However, *B. longum* supplementation improved hindlimb temporal symmetry and led to a more balanced gait at day 3 post-fracture compared to control mice, suggesting that *B. longum* may prevent the severity of gait deficits after fracture (Fig. B). Untargeted metabolomics of sera samples was performed to identify potential microbial-derived metabolites that may be driving these effects. PLSDA of metabolic features revealed a clear effect of femoral fracture on the systemic metabolome. KEGG pathway analysis further revealed a significant change in several metabolic pathways in response to fracture, including linoleic acid metabolism and tryptophan metabolism. Due to the stronger analgesic effect of *B. longum*, we next investigated the effects of *B. longum* consumption on the post-fracture systemic metabolome compared to the control mice. PLSDA of serum metabolites at day 3 and 7 post-fracture shows clear separation between PBS control and *B. longum*-supplemented mice (Fig. C). Correlation analyses identified 108 metabolites that were significantly associated with rearing behavior, an indicator of hindlimb pain.

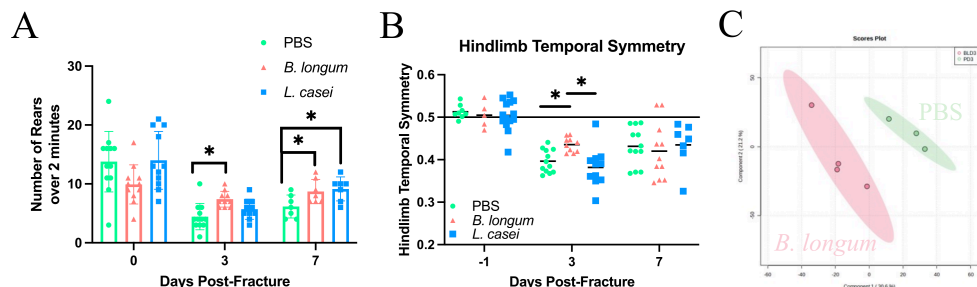


Figure. Probiotic supplementation decreases hindlimb pain and discomfort and alters the systemic metabolome. (A) *B. longum*-supplemented mice reared significantly more at day 3 post-fracture, while at day 7 *B. longum*- and *L. casei*-supplemented mice reared more than the control group. (B) Analysis of gait revealed an improvement in hindlimb temporal symmetry at day 3 post-fracture compared to PBS or *L. casei*-supplemented mice. (C) PLSDA of serum metabolites at day 3 and 7 post-fracture shows clear separation between PBS control and *B. longum*-supplemented mice. * $P < 0.05$.

DISCUSSION: In this study, we demonstrated that targeting the gut microbiota using dietary probiotics can decrease pain, improve function, and alter the systemic metabolome during the early post-fracture period when inflammation and pain are most severe. Moreover, we demonstrate that consumption of *B. longum* after fracture can protect the intact axial skeleton from post-traumatic bone loss. We further revealed that these effects are species-dependent as *B. longum* had more potent effects than *L. casei*.

SIGNIFICANCE/CLINICAL RELEVANCE: This study highlights the potential utility of targeting the gut microbiota using dietary approaches, such as health-promoting probiotics, for improved outcomes during recovery from bone injury.

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