

# Intermittent Fasting Enhances Bone Fracture Healing in Obese Mice Through Shifts in the Gut Microbiome

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**INTRODUCTION:** Obesity is a known risk factor for reduced bone strength, delayed fracture healing, and increased incidence of fracture nonunion. Previous studies of fracture healing in obese mice have delineated characteristics such as reduced size and mineralization of the callus and increased adiposity within its structure. Intermittent fasting (IF) has emerged as a dietary strategy to ameliorate various negative physiological impacts of obesity and improve metabolic profile. To investigate whether IF can enhance fracture healing in obese mice, we conducted a pilot study on a high-fat diet (HFD) fed obese mice with tibial fracture. By employing micro-CT and histological analysis, we identified enhanced callus mineralization and reduced adiposity because of IF (poster presentation at the ORS Annual Meeting 2022). Numerous studies have attributed the benefits of IF to the altered composition of dysbiotic gut microbiota and released IF microbial metabolites. In light of this intriguing connection, we have conducted the present study to delve deeper into the impact of IF on both fracture healing and metabolic profile. To unravel the potential causal relationship between the gut microbiome and the effects of IF, we used fecal microbiota transplantation (FMT) and microbiota ablation interventions to modulate the gut microbiota. This comprehensive investigation aims to shed light on the interplay between dietary interventions, the gut microbiome, and fracture healing.

**METHODS:** To induce obesity, male mice at 4 weeks of age were subjected to a high-fat diet containing 60% of total calories from fat (Open Source D12492i) for a duration of 12 weeks. Following the establishment of obesity, mice were subsequently categorized into distinct regimen groups as follows: 1) **HFD-Ad libitum:** Mice were allowed unrestricted access to food, 2) **HFD-IF:** Every-other-day access to food, 3) **HFD-Ad libitum +AB:** Ad libitum food access and concurrent antibiotics in water, 4) **HFD-IF +AB:** Every-other-day access to food and concurrent antibiotics in water, 5) **HFD-Ad libitum FMT:** Ad libitum food access plus FMT from mice on a similar regimen (HFD-Ad libitum), 6) **HFD-IF FMT:** Ad libitum food access plus FMT from mice on HFD-IF regimen, 7) **Lean Control:** Non-obese mice with ad libitum access to control diet (10% of total calories from fat, Open Source D12450Ji). For ablation, we used a cocktail of 9 antibiotics in water, and for transplantation, 20mg/200µl of feces in sterile anaerobic PBS was gavaged every other day. Four weeks after initiating the abovementioned regimens, all mice underwent open tibial fracture surgery with intramedullary fixation. At the twenty-one-day post-surgery mark, we employed micro-CT assessments of the callus. Additional parameters evaluated included glucose tolerance (GTT test), body weight, food intake, body composition (DEXA Scans), energy expenditure (Indirect Calorimetry), and activity levels. For a comprehensive understanding of alterations in gut microbiome diversity, abundance, predictive function, and the efficacy of ablation and transplantation techniques, 16S rRNA sequencing of fecal samples was conducted (EzBiome, MD). This study was approved by the University of Colorado IACUC.

**RESULTS:** Micro-CT evaluation of callus detected a small callus and low mineralization, as indicated by lower Bone volume/ Total volume ratio (BV/TV) in HFD-Ad libitum cohort that was restored to lean control levels in HFD-IF mice. Notably, the transplantation of the IF microbiome into HFD-IF FMT mice improved callus BV/TV. However, HFD-Ad libitum FMT mice did not show the same BV/TV enhancement. In mice with ad libitum access to food and ablated gut microbiome (HFD-Ad libitum +AB), callus had the same low BV/TV observed in HFD-Ad libitum mice. In contrast, HFD-IF +AB mice showed significant improvement in callus BV/TV (Fig1. A). Despite the tendency of IF mice to consume more on feeding days, aiming to compensate for energy intake (reaching nearly 100% compensation), a reduction in body weight (still higher than control) was observed in HFD-IF mice. This reduction was comparatively milder in HFD-IF FMT mice and both cohorts that received antibiotics. The GTT test (Fig1. B) and DEXA scan detected a significant improvement in glucose tolerance and body fat % of HFD-IF mice and HFD-IF FMT mice, as both cohorts of mice received antibiotics. Of note, neither fracture nor fasting-induced substantial alterations in total physical activity among the mice. Analyzing gut microbiome based on Generalized Unifrac detected significant dissimilarity between HFD-Ad libitum and HFD-IF microbiome (Fig1. C). Analysis of operational taxonomic units (OTUs) revealed a decrease in the abundance of obesity-associated *Peptococcaceae\_f* and an increase in propionate-producing *Muribaculaceae\_g*, as well as acetate, butyrate, and caproate-producing *Caproiciproducens\_s* in both HFD-IF and HFD-IF FMT mice (Fig1. D). Predictive functional profiling revealed higher activation of pathways associated with lipid metabolism fatty acid degradation in HFD-IF and HFD-IF FMT mice compared to HFD-Ad libitum.

**DISCUSSION:** In the current investigation, we detected an improvement in callus mineralization of obese mice as a consequence of the fasting regimen. Observation of improved callus BV/TV in mice with ad libitum access to food, which received FMT sourced from intermittent fasting mice, in contrast to FMT from ad libitum donors, substantiates the notion that IF-induced benefits are attributed to alterations in the gut microbiome composition. Notably, the significant increase in BV/TV observed in the HFD-IF +AB raises the possibility of additional mechanisms at play. This outcome implies that mechanisms beyond gut microbiome shifts, such as modifications in somatic gene expression, contribute to the impact of IF on bone healing. Improvement in GTT in obese mice following gut microbiome ablation was reported in a previous study. Despite that, the lack of BV/TV improvement in HFD-Ad libitum +AB suggests that the sole improvement in metabolic parameters may not be a pivotal factor in enhancing callus mineralization through IF. To fully understand the mechanism of IF's impact on fracture healing, future studies should prioritize investigations into changes in fasting-related microbial metabolites. Exploring their interactions with progenitor cells and their potential to influence shifts in osteogenic differentiation of these progenitors could provide crucial insights. Moreover, a comprehensive understanding of the temporal and spatial expression of somatic genes affected by IF throughout the healing process is imperative to fully unravel the impact of IF on bone fracture healing.

**SIGNIFICANCE:** This study confirms that IF-caused shifts in the gut microbiome have a role in improved callus mineralization in obese mice. This enhancement is possibly mediated by the release of microbial metabolites that exert a substantial influence on fracture healing mechanisms at both the cellular and molecular levels. Understanding the gut-bone axis will unravel how food and dietary interventions can impact bone health and regeneration and will guide us to design innovative non-pharmacological approaches to optimize bone health and healing.

**ACKNOWLEDGEMENTS:** CTSA Grant UL1 TR02535 (H.H), NIH R01 AR078414 (M.J.Z.)

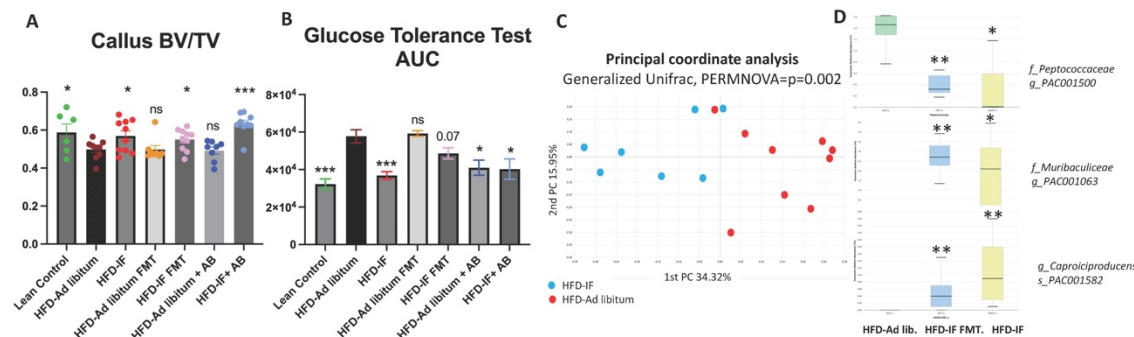


Fig. 1) A) Micro-CT analysis of callus BV/TV. For the t-test, each cohort is compared to the HFD-Ad libitum. B) GTT, t-test, each cohort is compared to the HFD-Ad libitum. C) PCOA plot. D) Taxonomic Relative Abundance %, Wilcoxon rank sum.