AWARD NUMBERS: W81XWH-19-1-0807

TITLE: Targeting the Gut Microbiome to Treat Post-Traumatic Osteoarthritis

PRINCIPAL INVESTIGATOR: Michael Zuscik, PhD

CONTRACTING ORGANIZATION: University of Colorado at Denver

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6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

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University of Colorado at Denver

8. PERFORMING ORGANIZATION REPORT NUMBER

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13. SUPPLEMENTARY NOTES

14. ABSTRACT
While osteoarthritis (OA) is a debilitating condition with no disease modifying treatments, the gut microbiome may play a role in its development and progression. The establishment of a disease modifying treatment of OA has immense ramifications, including improved quality of life, lowered economic burden of treatment, and increased productivity of patients with OA. The purpose of this project is to study the pathogenic role of the microbiome in the development of OA as well as to develop microbiome-targeting treatments of the disease. Fecal microbiota transplants (FMTs) will be used to examine the causal relationship between microbiome dysbiosis that may develop in veterans diagnosed with post-traumatic osteoarthritis (PTOA) and belonging to the Military and Veteran Microbiome: Consortium for Research and Education (MVM-CoRE). Recruitment of the Veterans into the study is completed, setting up analysis of their fecal samples and FMTs which will occur in 2023. Treatment of PTOA with microbiome pre and probiotics has been ongoing, and we find the dietary supplement hydrolyzed hylaline cartilage (hHC) to have protective effects on cartilage degeneration in a mouse model of PTOA. Progress on all these fronts is significant, with 3 manuscripts that are submitted or about to be submitted for peer review. Despite substantial institutional shutdowns caused by the COVID-19 pandemic, we are now moving ahead quickly, anticipating completion of all proposed aims in 2024.

15. SUBJECT TERMS

NONE LISTED

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1. Introduction

This project is focused on 1) studying the pathogenic role of the gut microbiome in the development of osteoarthritis (OA), and 2) developing approaches that target the gut microbiome to treat posttraumatic OA (PTOA), a disease that does not currently have an accepted disease modifying treatment. Our first objective is to study the microbiome dysbiosis that develops in a mouse model of PTOA and military veterans that have been diagnosed with PTOA and have been recruited into the Military and Veteran Microbiome: Consortium for Research and Education (MVM-CoRE). To prove the causal role of this dysbiosis, fecal microbiota transplants (FMTs) are planned (mouse to mouse or human to mouse), and specific potentially pathogenic microbial species will be gavaged to pinpoint taxa that are PTOA-accelerating. Our second objective is to build on or historical work studying dietary supplements as disease modifiers for OA by examining the impact of daily supplementation with hydrolyzed hyaline cartilage (hHC) on PTOA progression and on the gut microbiome. Again, FMTs between supplemented and non-supplemented mice will test the causal role of the gut community in any joint protection that is observed, and specific microbial species will be studies as active participants in the protective effects. Finally, in the third objective, we will focus on developing pre- and probiotic combinations for dietary supplements involving hHC and taxa identified in the second objective that promote joint protection and decelerated PTOA progression. Multiple dosing regimens will be tested, with the most protective combinations studies with deep analysis of the gut microbiome using cutting edge metagenomic, metatranscriptomic and metabolomic profiling to drill down on specific molecular fractions that are contributing to disease modification that we document. We have completed three years of work on this project, and despite a major interruption by the COVID-19 pandemic, we have continued to make progress in the first and second objectives, nearing completion of those studies, with work now moving forward on Aim 3. This progress is described below in the various sections of this report.

Note: This report provides information on the third year of a partnering-PI project, and the specific activity for each aspect of progress is attributed to the contributing PI (CU for Zuscik, UR for Gill). In general, all OA analysis, animal work, and human work occurs at CU. All analysis of gut microbiome and microbiology work to expand and maintain taxa of interest is performed at UR.

2. Key Words

Osteoarthritis (OA)
Posttraumatic osteoarthritis (PTOA)
Gut microbiome
Fecal microbiota transplant (FMT)
Military and Veteran Microbiome: Consortium for Research and Education (MVM-CoRE)
Hydrolyzed hyaline cartilage (hHC)
Destabilization of the medial meniscus (DMM)
Peptococcaceae *rc4-4*
Anaeroplasmataceae
Firmicutes
Tenericutes
Cartilage
Chondrocyte
Synovium
Tumor Necrosis Factor-alpha (TNF)

3. Accomplishments

- What were the major goals of the project?

The major goals of the project are 1) to define the role of the gut microbiome in PTOA, 2) to establish that hHC-induced chondroprotection in PTOA is due to effects on the gut microbiome, and 3) to test the efficacy of combined pre- and pro-biotic strategies to treat PTOA. Our aim is to accomplish these goals using state-of-

the-art approaches and methods, culminating with a dataset supporting the effectiveness of gut microbiome interventions in treating PTOA and setting the stage for the first in-human trial work to test targeting of the gut microbiome as an OA disease modifying approach.

- What was accomplished under these goals?

i) Experiments have been performed to study the gut microbiome dysbiosis that occurs in PTOA (Objective #1). Using a mouse model of PTOA that involves surgical destabilization of the medial meniscus (DMM) to initiate PTOA, we have collected fecal samples weekly for 16s rDNA analysis, for study of the metagenome and for study of the metabolome. DMM surgeries were initially performed on a cohort of mice in January of 2020, but these samples were destroyed when the COVID-19 pandemic caused CU and UR to shut down operations in March of 2020. Mice, which were in mid-protocol, were euthanized because of the shutdown, essentially rendering collected samples unusable since the full experiment could not be performed. Once the Universities re-opened in the summer of 2020, a new cohort of mice was purchased, and DMMs were performed again in September of 2020. Collection protocols played out (CU), and terminal endpoints in December 2020 and January of 2021 provided fecal and cecal samples that we have now fully analyzed. Microbial 16S rDNA and metatranscriptomic analyses were carried out (UR), and interesting functional alterations were observed that correlate with metabolomic changes that were uncovered (CU). These findings are summarized in a poster from the 2022 Military Health System Research Symposium (Appendix 1) and the draft of a manuscript that is currently under development (Appendix 2).

ii) To support humanization FMT experiments (Objective #1), an IRB-approved protocol was developed within the MVM-CoRE to collect fecal material from Veterans with a diagnosis of advanced knee osteoarthritis that are otherwise healthy, along with a cohort of healthy control participants. The goal was to enroll and collect samples from 20 for each group. To date, we have collected 14 samples from the OA cohort, and 20 from the healthy cohort. The stringent inclusion and exclusion criteria, which will enhance power to see gut microbiome differences between these cohorts, have made recruitment slower than expected. As a team, we have decided to proceed with the key experiments, which include deep analysis of the fecal material (metagenome, metatranscriptome, metabolome) and transplant into mice for humanization experiments in Objective 1. This work will take place this spring summer.

iii) We initiated work with hHC supplements in the past period of activity in the award (Objective #2 & #3). This work has led to a dataset that was presented at the American Society for Bone and Mineral Research conference, with an updated version of the dataset presented at the Orthopedic Research Symposium and D'Ambrosia Diversity Lectureship at the University of Colorado (included in the previous report and included here as well, Appendix 2). The findings are currently in preparation as a manuscript (CU and UR), with the early structure denoted in a previously presented poster included in Appendix 3. This study, which involved quantifying the impact of hHC on PTOA (CU), provides the first data delineating the gut microbiome impact of this supplement (UR), setting the stage for our ongoing studies which involve isolation and expansion of several interesting taxa for in vivo work as a probiotic intervention, which in underway (CU and UR). The net results of this study were collected in the broader context of our work to study the role of the gut microbiome in the action of various nutraceutical products that set the stage for a publication in 2020, and a follow-on White Paper published this year in Current Rheumatology Reports at Springer Nature (see publications).

- What opportunities for training and professional development have the project provided?

During year 3, we had two trainees that were involved in this project: predoctoral student David Villani and Instructor Honey Hendesi MD PhD. We had another postdoctoral fellow on the team in year 2 (Andrew Wu MD), but he graduated from the program and is currently in an Orthopaedic Residency at Johns Hopkins. We also have technical-level staff (Jake Guzzetti) and an Instructor (Lacey Favazzo PhD) that help carry forward the work. These individuals are involved in all aspects of the management and execution of the experimental plan, and thus gain knowledge on how to administer a program of research, how to consider budgets in the context of the work, and scientific aspects of the project. The training and professional development plays out in daily work on the project, weekly work in progress meetings and journal clubs associated with a T32 Training Grant (Zuscik, PI), and ultimately in the presentation of the work in broader contexts.

- How were the results disseminated to communities of interest?

Besides the 2 publications so far, posters at the ORS, ASBMR, MHSRS, and our Regional Symposium are our primary modes of dissemination. Some of the work was also presented at the Steadman Philippon Research Institute Science Summit in Vail, Colorado on August 23, 2022, and at Vanderbilt University on August 29, 2022 as well.

- What do you plan to do in the next reporting period to accomplish the goals?

We plan to complete the bulk of our work on all Objectives by the end of 2023. By summer we will see where the final OA experiments stretch to, with the possibility of requesting a 12 month no cost extension pending administrative advice on this possibility.

4. Impact

- What was the impact on development of the principle disciplines of the project?

The central impact of the work so far:

- While the microbial community in the gut is not altered significantly by advanced PTOA in the mouse, the community's functional capacity is significantly altered; the first evidence of this unique phenotype that we will publish in 2023.
- Data generated on this project has provided the first evidence suggesting that nutraceutical supplements may impact joint health and OA degenerative disease via effects on the gut microbiome. This is a novel concept that provides the first explanation for the purported effects of such agents in the context of joint homeostasis and disease.

- What was the impact on other disciplines?

Nothing to report

- What was the impact on technology transfer?

Nothing to report

- What was the impact on society beyond science and technology?

Nothing to report

5. Changes/Problems

- Changes in approach and reasons for change.

Nothing to report

- Actual or anticipated problems or delays and action plans to resolve them

We are in the final stages of the project. By summer we will see where the final OA experiments stretch to in terms of timeline, with the possibility of requesting a 12 month no cost extension pending administrative advice on this possibility.

- Changes that have significant impact on expenditures

Nothing to report

- Significant changes in use and care of human subjects, vertebrate animals, biohazards, and/or select agents.

Nothing to report

6. Products

- Publications, conference papers and presentations

a. Publications:

Mobasheri A, Mahmoudian A, Kalvaityte U, Uzieliene I, Larder CE, Iskandar MM, Kubow S, Hamdan PC, de Almeida CS Jr, Favazzo LJ, van Loon LJC, Emans PJ, Plapler PG, Zuscik MJ. A White Paper on Collagen Hydrolyzates and Ultrahydrolyzates: Potential Supplements to Support Joint Health in Osteoarthritis? Curr Rheumatol Rep. 2021 Oct 30;23(11):78. doi: 10.1007/s11926-021-01042-6. PMID: 34716494; PMCID: PMC8556166.

Currently, manuscripts are in development (see Appendices)

b. Books or other non-periodical, one-time publications

Nothing to report

c. Other publications, conference papers and presentations:

David A Villani, Toru Ishii, Honey Hendesi, Samantha H Landgrave, Ann Gill, Lisa Brenner, Lacey Favazzo, Steven R Gill, Michael J Zuscik. Post Traumatic Osteoarthritis Causes Significant Changes in the Functional Output of the Gut Microbiome in Injured Mice. Poster at 2022 MHSRS Conference

Zuscik MJ. Thinking outside the joint: Can gut interventions support disease modification in OA? 7th Annual Vail Scientific Summit, Steadman Philippon Research Institute, August 23, 2022. (This award was acknowledged)

Zuscik MJ. From Gut to OA. Vanderbilt University, Department of Orthopaedics Grand Rounds, August 29, 2022. (This award was acknowledged)

- Website(s) or other internet site(s)

Nothing to report

- Technologies or techniques

Nothing to report

- Inventions, patent applications, and/or licenses

Nothing to report

- Other products

Nothing to report

7. Participants & Other Collaborating Organizations

- What individuals have worked on the project?

University of Colorado School of Medicine (Zuscik Partnering PI)

Name:	Michael Zuscik PhD
Project role:	Partnering PI
Researcher identifier:	0000-0003-0461-8708 (ORCID)
Nearest person month worked:	3
Contribution to project:	Contribution to design and planning of all aspects of the project
Funding support:	

Name:	Lisa Brenner PhD
Project role:	Co-I
Researcher identifier:	Not available
Nearest person month worked:	0.5
Contribution to project:	IRB development and planning for fecal collection from humans
Funding support:	

Name:	Honey Hendesi MD PhD
Project role:	Instructor-level significant contributor
Researcher identifier:	Not available
Nearest person month worked:	6
Contribution to project:	Contribution to design, planning and execution of all aspects of the project
Funding support:	

Name:	David Villani
Project role:	Graduate student
Researcher identifier:	Not available
Nearest person month worked:	12

Contribution to project:	Contribution to design, planning and execution of all aspects of the project
Funding support:	

Name:	Kelly Stearns-Yoder
Project role:	Clinical Coordinator
Researcher identifier:	Not available
Nearest person month worked:	0.6
Contribution to project:	IRB development
Funding support:	

Name:	Jake Guzzetti
Project role:	Clinical Coordinator
Researcher identifier:	Not available
Nearest person month worked:	1.8
Contribution to project:	IRB development, recruitment, and sample collection
Funding support:	

University of Rochester School of Medicine and Dentistry (Gill Partnering PI)

Name:	Steven Gill PhD
Project role:	Partnering PI
Researcher identifier:	0000-0002-2408-1373 (ORCID)
Nearest person month worked:	3
Contribution to project:	Contribution to design, planning and execution of all aspects of the project, particularly as related to microbiome analysis and microbiology work.
Funding support:	

Name:	Ann Gill MS
Project role:	Senior technical associate
Researcher identifier:	Not available
Nearest person month worked:	6
Contribution to project:	Contribution to microbiome analysis and microbiology work.
Funding support:	

Name:	Cal Palumbo MS
Project role:	Bioinformatician and Data Analyst

Researcher identifier:	Not available
Nearest person month worked:	2.4
Contribution to project:	Contribution to microbiome analysis.
Funding support:	

- Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

A summary of active support for each PI is listed below.

Zuscik

*Title: AIM-for-RA
*Major Goals: The goal of this project is to implement a multidisciplinary team for RA patient recruitment, longitudinal clinical data accrual, and synovial biopsy tissue analysis for the molecular deconstruction and reconstruction of disease pathogenesis.
*Status of Support: Active
Project Number: 1UC2AR081025-01
Name of PD/PI: Moreland, L. (PI); Anolik, J. (Contact PI)
*Source of Support: NIAMS
*Primary Place of Performance: University of Rochester and University of Colorado Denver
Project/Proposal Start and End Date: (MM/YYYY) (if available): 3/2022 – 12/2026
*Total Award Amount (including Indirect Costs): (subcontract)
*Person Months (Calendar/Academic/Summer) per budget period.

Year (YYYY)	Person Months (##.##)
2. 2023	0.24 calendar
3. 2024	0.60 calendar
4. 2025	0.60 calendar
5. 2026	0.60 calendar

*****NO OVERLAP*****

*Title: Interdisciplinary Training in Musculoskeletal Research
*Major Goals: This training program establishes a curriculum and various education elements to support pre- and post-doctoral training in musculoskeletal research, with 4 funded pre-doctoral seats and 2 funded post-doctoral seats.
*Status of Support: Active
Project Number: T32AR080630-01
Name of PD/PI: Zuscik, M. (Contact PI)
*Source of Support: NIAMS
*Primary Place of Performance: University of Colorado Denver
Project/Proposal Start and End Date: (MM/YYYY) (if available): 07/2022 – 06/2027
*Total Award Amount (including Indirect Costs):
*Person Months (Calendar/Academic/Summer) per budget period.

Year (YYYY)	Person Months (##.##)
2. 2023	2.4 calendar
3. 2024	2.4 calendar
4. 2025	2.4 calendar
5. 2026	2.4 calendar

*****NO OVERLAP*****

*Title: Studies on gut microbiome-joint connections in arthritis

*Major Goals: This project aims to 1) definitively establish that a pro-inflammatory dysbiotic gut microbiome is causal in the osteoarthritis of obesity, and 2) demonstrate that correction of this dysbiosis using strategies to expand B. pseudolongum will decelerate the progression of osteoarthritis in the obese context.
*Status of Support: Active
Project Number: 5R01AR078414-02
Name of PD/PI: Zuscik, M. (Contact PI)
*Source of Support: NIAMS
*Primary Place of Performance: University of Colorado Denver
Project/Proposal Start and End Date: (MM/YYYY) (if available): 04/2021 – 02/2026
*Total Award Amount (including Indirect Costs):
*Person Months (Calendar/Academic/Summer) per budget period.

Year (YYYY)	Person Months (##.##)
2. 2023	3.0 calendar
3. 2024	3.0 calendar
4. 2025	3.0 calendar
5. 2026	3.0 calendar

*****NO OVERLAP*****

*Title: Collaborative Research: RECODE: Organoid model of growth plate development
*Major Goals: This research will provide fundamental insight into the mechanisms that govern stem cell differentiation and organization into a mature, functional growth plate organoid.
*Status of Support: Active
Project Number: NSF 2135032
Name of PD/PI: Payne, K.A. (PI), Zuscik, M. (Co-PI)
*Source of Support: National Science Foundation
*Primary Place of Performance: University of Colorado Denver
Project/Proposal Start and End Date: (MM/YYYY) (if available): 12/2021 – 11/2025
*Total Award Amount (including Indirect Costs):
*Person Months (Calendar/Academic/Summer) per budget period.

Year (YYYY)	Person Months (##.##)
2. 2023	0.6 calendar
3. 2024	0.6 calendar
4. 2025	0.6 calendar

*****NO OVERLAP*****

(Reduced effort from 2.40 calendar months to 1.20 calendar months)
*Title: Development of Chondroregenerative Therapy for Human Osteoarthritis
*Major Goals: This phase II, double blind, placebo controlled clinical trial will determine if teriparatide is chondroregenerative in early-mid stage osteoarthritis in humans.
*Status of Support: Active
Project Number: Hansjörg Wyss Award
Name of PD/PI: Zuscik, M.
*Source of Support: Wyss Medical Foundation & Eli Lilly LLC
*Primary Place of Performance: University of Rochester and Duke University
Project/Proposal Start and End Date: (MM/YYYY) (if available): 11/2016 – 12/2023
*Total Award Amount (including Indirect Costs):
*Person Months (Calendar/Academic/Summer) per budget period.

Year (YYYY)	Person Months (##.##)
7. 2023	1.20 calendar

*****NO OVERLAP*****

*Title: Investigation of the joint protective effect of hydrolyzed hyaline cartilage (hHc)
*Major Goals: This phase II, double blind, placebo controlled clinical trial will determine if hHC, via prebiotic effects playing out in the gut microbiome, is chondroprotective in early-mid stage osteoarthritis in humans.

*Status of Support: Active
Project Number: Contract Agreement 200709
Name of PD/PI: Zuscik, M.
*Source of Support: Rousselot BVBA
*Primary Place of Performance: University of Colorado Denver
Project/Proposal Start and End Date: (MM/YYYY) (if available): 01/2021 – 12/2023
*Total Award Amount (including Indirect Costs):
*Person Months (Calendar/Academic/Summer) per budget period.

Year (YYYY)	Person Months (##.##)
3. 2023	1.0 calendar

*****NO OVERLAP*****

THIS AWARD:
*Title: Targeting the gut microbiome to treat posttraumatic osteoarthritis
*Major Goals: The goal of this project is to study the role of the gut microbiome in the chondroprotective effects of oral dietary supplements containing hydrolyzed type I collagen in posttraumatic osteoarthritis.
*Status of Support: Active
Project Number: W81XWH-19-1-0807
Name of PD/PI: Zuscik, M. (PI) and Gill, S. (Partnering PI)
*Source of Support: Department of the Army
*Primary Place of Performance: University of Colorado Denver
Project/Proposal Start and End Date: (MM/YYYY) (if available): 09/2019 – 09/2023
*Total Award Amount (including Indirect Costs):
*Person Months (Calendar/Academic/Summer) per budget period.

Year (YYYY)	Person Months (##.##)
4. 2023	3.0 calendar

*Title: Abaloparatide as the first chondroregenerative therapy for osteoarthritis
*Major Goals: This project focuses on study of the role of the chondrocyte in the joint preserving effects of abaloparatide, along with in vitro experiments to study the effects of abaloparatide in human articular chondrocytes.
*Status of Support: Active
Project Number: Grubstake 03-2021
Name of PD/PI: Zuscik, M.
*Source of Support: Gates Center for Regenerative Medicine
*Primary Place of Performance: University of Colorado Denver
Project/Proposal Start and End Date: (MM/YYYY) (if available): 01/2021 – 12/2023
*Total Award Amount (including Indirect Costs):
*Person Months (Calendar/Academic/Summer) per budget period.

Year (YYYY)	Person Months (##.##)
3. 2023	0.6 calendar

*****NO OVERLAP*****

*Title: Regulation of microRNA homeostasis: Implications in bone fracture healing
*Major Goals: This work will energize the development of novel therapeutic approaches tailored to correct the specific bone-healing defect that manifests in the obese/type 2 diabetic patients.
*Status of Support: Active
Project Number: 5R01DK121327-04
Name of PD/PI: Elbarbary, R. (PI), Zuscik, M. (Consultant)
*Source of Support: NIDDK
*Primary Place of Performance: Pennslyvania State University
Project/Proposal Start and End Date: (MM/YYYY) (if available): 09/2019 – 06/2024
*Total Award Amount (including Indirect Costs):
*Person Months (Calendar/Academic/Summer) per budget period.

Year (YYYY)	Person Months (##.##)
4. 2023	0.12 calendar
5. 2024	0.12 calendar

*****NO OVERLAP*****

*Title: Therapeutic Targeting of GPCR Gbetagamma-GRK2 in Osteoarthritis
*Major Goals: This study is to determine if Gbetagamma/GRK2 signaling inhibition, either genetically or via inhibitors is protective in osteoarthritis.
*Status of Support: Active
Project Number: 5R01AR071968-04
Name of PD/PI: Kamal, F. (PI), Zuscik, M. (Consultant)
*Source of Support: NIAMS
*Primary Place of Performance: Pennslyvania State University
Project/Proposal Start and End Date: (MM/YYYY) (if available): 02/2019 – 12/2023
*Total Award Amount (including Indirect Costs):
*Person Months (Calendar/Academic/Summer) per budget period.

Year (YYYY)	Person Months (##.##)
4. 2023	0.12 calendar

*****NO OVERLAP*****

PENDING:
*Title: Inhibiting angiogenesis in growth plate injuries to prevent bony repair tissue formation
*Major Goals: This project aims to investigate the role that angiogenic processes play in the formation of the bony bar, and to develop biomaterial delivery systems that are capable of blocking them. The delivery of microRNA to target various parts of angiogenic pathways will be investigated to determine their effect on blocking bony bar formation in growth plate injuries.
*Status of Support: Pending
Project Number: 1R01AR079512-01A1
Name of PD/PI: Payne, K.A. (Contact PI), Zuscik, M. (Co-I)
*Source of Support: NIAMS
*Primary Place of Performance: University of Colorado Denver
Project/Proposal Start and End Date: (MM/YYYY) (if available): 07/2023 – 06/2028
*Total Award Amount (including Indirect Costs):
*Person Months (Calendar/Academic/Summer) per budget period.

Year (YYYY)	Person Months (##.##)
1. 2024	0.25 calendar
2. 2025	0.25 calendar
3. 2026	0.25 calendar
4. 2027	0.25 calendar
5. 2028	0.25 calendar

*****NO OVERLAP*****

Gill

*Title: Understand biological factors underlying early childhood caries disparity from the oral microbiome in early infancy
Major Goals: Leveraging an archived underserved birth cohort, our multidisciplinary team proposes using cutting- edge metagenomic sequencing and analysis tool, combined with a high-dimensional statistical machine learning approach, to examine the early-life oral microbiome development and identify its multilevel determinant and association to ECC. ECC risk factors revealed via prediction models could be used as targets for ECC early prediction and prevention specifically suitable for underserved racial minority children.
*Status of Support: ACTIVE
Project Number: R01 DE031025

Name of PD/PI: Gill, S, Xiao, J. and Wu, T. MPIs
*Source of Support: NIDCR
*Primary Place of Performance: University of Rochester, Rochester NY Project/
Proposal Start and End Date: (MM/YYYY) (if available): 5/2022 – 1/2027
*Total Award Amount (including Indirect Costs):
*Person Months (Calendar/Academic/Summer) per budget period.

Year (YYYY)	Person Months (##.##)
1. 2022	1.92 calendar months
2. 2023	1.92 calendar months
3. 2024	1.92 calendar months
4. 2025	1.92 calendar months
5. 2026	1.92 calendar months

*****NO OVERLAP*****

*Title: Povidone Iodine Efficacy Study (PIES)
Major Goals: Severe Early Childhood Caries (S-ECC) is an infectious disease that continues to be a significant global public health problem among young, preschool children. The clinical, social and public health impact of S-ECC is underscored by its association with increased risk of new cavities in the primary dentition, a higher risk of cavities in the permanent dentition, hospitalizations and emergency room visits, high treatment costs, lost school days, diminished ability to learn and a profound impact on a child's quality of life. Results of treatment of S-ECC are poor; approximately 40% of children treated for S-ECC will develop new cavities within 12 months after dental surgery. This proposal will assess the efficacy of topical antibacterial therapy in improving clinical outcomes for S-ECC.
*Status of Support: Awarded
Project Number: 4UH3DE030434
Name of PD/PI: D. Kopycka-Kedzierawski
*Source of Support: NIH
*Primary Place of Performance: University of Rochester
Project/Proposal Start and End Date: (MM/YYYY) (if available): 5/1/22-4/30/27
*Total Award Amount (including Indirect Costs):

Year (YYYY)	Person Months (##.##)
1. 2022	2.04 calendar months
2. 2023	2.04 calendar months
3. 2024	2.04 calendar months
4. 2025	2.04 calendar months
5. 2026	2.04 calendar months

*****NO OVERLAP*****

*Title: Targeting the gut microbiome to treat posttraumatic osteoarthritis
Major Goals: The goal of this project is to study the role of the gut microbiome in the chondroprotective effects of oral dietary supplements containing hydrolyzed type I collagen in posttraumatic osteoarthritis.
*Status of Support: ACTIVE
Project Number: W81XWH-19-1-0808
Name of PD/PI: Gill, S and Zuscik, M. –partnering PIs
*Source of Support: Department of the Army
*Primary Place of Performance: University of Rochester, Rochester NY
Project/Proposal Start and End Date: (MM/YYYY) (if available): 9/2019-9/2023 NCE
*Total Award Amount (including Indirect Costs):
*Person Months (Calendar/Academic/Summer) per budget period.

Year (YYYY)	Person Months (##.##)
1. 2019	3.0 calendar months
2. 2020	3.0 calendar months

Year (YYYY)	Person Months (##.##)
3. 2021	0.96 calendar months
4. 2022	0.36 calendar months

THIS AWARD

*Title: Role of the gut Microbiome in the osteoarthritis of obesity
Major Goals: The major goals of this project are to definitively establish that a pro-inflammatory dysbiotic gut microbiome is causal in the osteoarthritis (OA) of obesity, and demonstrate that correction of this dysbiosis using strategies to expand Bifidobacterium pseudolongum will decelerate the progression of OA in the obese context.
*Status of Support: ACTIVE
Project Number: R01 AR078414
Name of PD/PI: Gill, S. and Zuscik, M. MPIs
*Source of Support: NIH/NIAMS
*Primary Place of Performance: University of Rochester, Rochester NY
Project/Proposal Start and End Date: (MM/YYYY) (if available): 4/2021-2/2026
*Total Award Amount (including Indirect Costs):
*Person Months (Calendar/Academic/Summer) per budget period.

Year (YYYY)	Person Months (##.##)
1. 2021	3.0 calendar months
2. 2022	1.8 calendar months
3. 2023	1.8 calendar months
4. 2024	1.8 calendar months
5. 2025	1.8 calendar months

*Title: Biomarker Identification, Viral Susceptibility and Management in S. aureus Colonized AD Patients Major Goals: To determine the impact of the pathogen, Staphylococcus aureus (which is commonly present on the skin of atopic dermatitis patients), on systemic inflammation (measured in the circulation), response to treatments, and viral skin infections in atopic dermatitis patients.
*Status of Support: Active
Project Number: U01 AI152011
Name of PD/PI: Beck, L.
*Source of Support: NIH
*Primary Place of Performance: University of Rochester, Rochester NY
Project/Proposal Start and End Date: (MM/YYYY) (if available): 4/2020-3/2027
*Total Award Amount (including Indirect Costs):
*Person Months (Calendar/Academic/Summer) per budget period.

Year (YYYY)	Person Months (##.##)
1. 2020	0 calendar months
2. 2021	1.8 calendar months
3. 2022	1.8 calendar months
4. 2023	1.8 calendar months
5. 2024	1.2 calendar months
6. 2025	1.2 calendar months
7. 2026	1.2 calendar months

*****NO OVERLAP*****

*Title: Neurobiological and neurocognitive consequences of diverse microbiome functional trajectories
Major Goals: We are testing the central hypothesis that neurodevelopment is dependent on age-driven biosynthesis of NAMs through the postnatal period of infant gut-microbiome (IGM) development. In Aim 1, we use metagenomic analysis of the prenatal maternal vaginal microbiome (MVM) to identify species and functional biosynthetic pathways for NAMs associated with PNA. We also assess the potential transfer of

maternal anxiety through the initial colonization of the infant gut microbiome by an anxiety “imprinted” MVM. In Aim 2, we use metagenomic and metabolomic analyses to determine the association between key stages of IGM development and differential synthesis of NAMs over the first year, attending to confounds and competing exposures, most notably, maternal diet and infant feeding. In Aim 3, we apply this rich data to predict neurocognitive assessments from age 1 to 4 years to formally test the temporal relationship between microbiome phase and neurodevelopment in the first year of life and durability of the microbiota-neurodevelopment relationship through 4 years of age.
*Status of Support: Active
Project Number: R01 MH125103
Name of PD/PI: Gill, S./O’Connor, T./Scheible, K. MPI
*Source of Support: NIH
*Primary Place of Performance: University of Rochester, Rochester NY Project/
Proposal Start and End Date: (MM/YYYY) (if available): 7/2022-05/2027
*Total Award Amount (including Indirect Costs):
*Person Months (Calendar/Academic/Summer) per budget period.

Year (YYYY)	Person Months (##.##)
1. 2022	2.28 calendar months
2. 2023	2.28 calendar months
3. 2024	2.28 calendar months
4. 2025	2.28 calendar months
5. 2026	2.28 calendar months

*****NO OVERLAP*****

*Title: Center of Research Translation on the Osteoimmunology of Bone Infection (CoRTOBI)
Major Goals: Bone infection caused by S. aureus remains the bane of orthopaedic surgery, as diagnostics, prophylaxis and treatments have significant shortcomings that result in catastrophic outcomes for patients, and crippling healthcare costs. To address this, we propose renewal of the Center of Research Translation on the Osteoimmunology of Bone Infection (CoRTOBI), which will increase our knowledge on pathogenesis and immunity, and develop novel diagnostics and interventions for patients with serious bone infections.
*Status of Support: Active
Project Number: P50 AR072000
Name of PD/PI: Schwarz, E.
*Source of Support: NIAMS
*Primary Place of Performance: University of Rochester, Rochester NY
Project/Proposal Start and End Date: (MM/YYYY) (if available): 09/2022 – 08/2027
*Total Award Amount (including Indirect Costs):
*Person Months (Calendar/Academic/Summer) per budget period.

Year (YYYY)	Person Months (##.##)
1. 2022	0 calendar months
2. 2023	0 calendar months
3. 2024	1.2 calendar months
4. 2025	1.8 calendar months
5. 2026	1.2 calendar months

*****NO OVERLAP*****

*Title: Complement C1q and sepsis associated fatalities
Major Goals: This study will determine the underlying mechanisms that lead to the accumulation of neutrophils in the organs that causes fatal hyper-inflammatory responses in septic patients
Aim 1. Determine the relationship between C1q expression in peripheral blood neutrophils and organ failure and death in critically ill septic patients.
Aim 2. Explore the mechanisms by which neutrophil C1q hastens sepsis resolution.
Aim 3. Investigate the mechanisms underlying the heterogeneous C1q expression associated with sepsis mortality

*Status of Support: Active
Project Number: R01 HL160723
Name of PD/PI: Kim, M./Pietropaoli A. MPI
*Source of Support: NIH
*Primary Place of Performance: University of Rochester, Rochester NY Project/
Proposal Start and End Date: (MM/YYYY) (if available): 7/2022-06/2027
*Total Award Amount (including Indirect Costs):
*Person Months (Calendar/Academic/Summer) per budget period.

Year (YYYY)	Person Months (##.##)
1. 2022	0.48 calendar months
2. 2023	0.48 calendar months
3. 2024	0.48 calendar months
4. 2025	0.48 calendar months
5. 2026	0.48 calendar months

*****NO OVERLAP*****

PENDING
*Title: Interactions of polyamines and macrophages on obesity-related type 2 diabetes during implant associated osteomyeltis
Major Goals: In this study, we will examine polyamine-mediated immunomodulatory mechanisms during *S. aureus* implant-associated osteomyelitis in obesity/T2D. We will assess if dysfunctional macrophages that are recruited to the site of S. aureus bone infection accentuate disease severity in obese/T2D mice (AIM 1) and if oral polyamine treatments correct macrophage dysfunction to decrease the disease burden in obese/T2D mice (AIM 2).
*Status of Support: PENDING
Project Number: R21 AI178267
Name of PD/PI: Gill, S. - MPI, Muthukrishnan, G. - MPI
*Source of Support: NIAID
*Primary Place of Performance: University of Rochester, Rochester NY
Project/Proposal Start and End Date: (MM/YYYY) (if available): 7/1/2023-06/30/2025
*Total Award Amount (including Indirect Costs):
*Person Months (Calendar/Academic/Summer) per budget period.

Year (YYYY)	Person Months (##.##)
1. 2024	0.6 calendar months
2. 2025	0.6 calendar months

*****NO OVERLAP*****

PENDING
*Title: Microbiome-Host-RSV Interactions and Illness Severity
Major Goals: This proposal will study the mechanisms by which non-typeable *Haemophilus influenzae* (NTHi) delays interferon responses (IFN) and enhances infant Respiratory Syncytial Virus (RSV) disease severity and the role of defective viral genomes (DVGs) in regulating the NTHi-induced responses. We will use in vitro and ex vivo models to characterize the host molecular and cellular mechanisms, the role of RSV DVGs in mediating the IFN response, and investigate NTHi functions that potentiate severe host responses and effect respiratory host-microbiota community interactions.
*Status of Support: PENDING
Project Number: R01 AI179777
Name of PD/PI: Gill, S./Mariani, T. - MPI
*Source of Support: NIH
*Primary Place of Performance: University of Rochester, Rochester NY
Project/Proposal Start and End Date: (MM/YYYY) (if available): 9/1/2023-08/31/2028
*Total Award Amount (including Indirect Costs):
*Person Months (Calendar/Academic/Summer) per budget period.

Year (YYYY)	Person Months (##.##)
1. 2023	1.8 calendar months
2. 2025	1.8 calendar months
3. 2026	1.8 calendar months
4. 2027	1.8 calendar months
5. 2028	1.8 calendar months

*****NO OVERLAP*****

PENDING

*Title: Metabolic Effects of Differing Carbohydrate Sources in Infant Formula – A Randomized Control Trial
Major Goals: This Randomized Controlled Trial (RCT) will study the impact of different carbohydrate sources in infant formula on infant physiology. Formula-fed infants will be randomized to consume a formula that has a carbohydrate source of either lactose, glucose, or glucose/sucrose. Impact on infant glycemic control, intestinal gene expression, and metabolomic profile will be studied. A group of exclusively breastfed infants will be included as a control group.
*Status of Support: PENDING
Project Number: R01 HD113587
Name of PD/PI: Young, B. (Role: Co-Investigator)
*Source of Support: NIH
*Primary Place of Performance: University of Rochester, Rochester NY
Project/Proposal Start and End Date: (MM/YYYY) (if available): 12/01/2023 – 11/30/2028
*Total Award Amount (including Indirect Costs):
*Person Months (Calendar/Academic/Summer) per budget period.

Year (YYYY)	Person Months (##.##)
1. 2023	0.0 calendar months
2. 2024	0.0 calendar months
3. 2025	0.0 calendar months
4. 2026	0.6 calendar months
5. 2027	0.6 calendar months

*****NO OVERLAP*****

PENDING

*Title: Statistical methods to facilitate the translation of microbiome research into clinical practice
Major Goals: This project aims to develop statistical and computational methods for human microbiome data analysis. The proposed methods are expected to 1) reduce the irreproducibility issue of microbiome studies; and 2) gain more insights into the mediating roles (i.e., being affected by external factors and modulating phenotypes) of the microbiome. These are minimum requirements to determine microbes truly contributing to a specific disease, thus facilitating the translation of microbiome research into therapeutic and preventative applications.
*Status of Support: PENDING
Project Number: R01 DE033388
Name of PD/PI: Sohn, M. (Role: Co-Investigator)
*Source of Support: NIH
*Primary Place of Performance: University of Rochester, Rochester NY
Project/Proposal Start and End Date: (MM/YYYY) (if available): 01/01/2024 – 12/31/2028
*Total Award Amount (including Indirect Costs):
*Person Months (Calendar/Academic/Summer) per budget period.

Year (YYYY)	Person Months (##.##)
1. 2024	1.2 calendar months
2. 2025	1.2 calendar months
3. 2026	1.2 calendar months
4. 2027	1.2 calendar months

Year (YYYY)	Person Months (##.##)
5. 2028	1.2 calendar months

*****NO OVERLAP*****

- What other organizations were involved as partners?

a. Organization Name:
Rousselot BVBA

b. Location of Organization:
Gent, Belgium

c. Partner's Contribution to the Project:
Other: Study material, hydrolyzed hyaline cartilage

8. Special Reporting Requirements

- Collaborative Award

This is a collaborative award. Partnering PIs Michael Zuscik PhD and Steven Gill PhD are located at the University of Colorado and University of Rochester, respectively. This report has denoted which aspects of the project have been completed at each site, so both partnering PIs will be submitting this same document.

- Quad Charts

Not applicable

University of Colorado Anschutz Medical Campus

Osteoarthritis Causes Significant Changes in the Functional Output of the Gut Microbiome in Injured Mice

David A Villani[1,2], Toru Ishii[1], Honey Hendesi[1], Samantha H Landgrave [1,2],Ann Gill[3] , Lisa Brenner[4,5], Lacey Favazzo[1,6], Steven R Gill[3], Michael Zuscik[1,2,6]

[1]Colorado Program for Musculoskeletal Research, Department of Orthopedics, University of Colorado Anschutz Medical Campus, Aurora, CO, [2]Cell Biology, Stems Cells and Development PhD Program, University of Colorado Anschutz Medical Campus, Aurora, CO, [3] Department of Microbiology and Immunology, University of Rochester, Rochester , NY [4] Department of Psychiatry and Neurology, University of Colorado Anschutz Medical Campus, Aurora, CO 80045, USA [5] Veterans Health Administration, Rocky Mountain Mental Illness Research Education and Clinical Center (MIRECC), Rocky Mountain Regional Veterans Affairs Medical Center (RMRVAMC), Aurora, CO 80045, USA [6] University of Colorado Interdisciplinary Joint Biology Program, University of Colorado Anschutz Medical Campus, Aurora, CO

Introduction

Osteoarthritis (OA) and Injury:

- OA is a degenerative disease that involves the loss and destruction of cartilage.
- OA is the leading cause of disability in the United States
- OA affects more than 1 in 3 people who have served in the military, and military members older than 40 are twice as likely to develop OA.
- Post traumatic OA (PTOA) is a type of OA induced by knee injury.
- 50% of OA patients will exhibit radiographic knee OA symptoms 10 to 20 years after suffering a knee injury

The microbiome and OA development:

- The gut microbiome is an ecosystem of bacteria, fungus, viruses, phages, parasites, and archaea that inhabit the digestive tract
- A gut microbiome dysbiosis leads to an increase in both systemic and localized joint inflammation
- Germ free mice are protected from PTOA development
- Treatment of a gut dysbiosis led to a decrease in inflammation and protection in the OA of obesity

Hypothesis

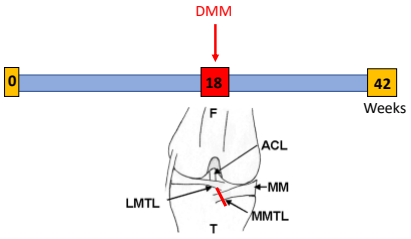
Injury and PTOA development leads to a dysbiosis in the gut that will lead to whole body ramifications.

Methods

- PTOA was induced with destabilization of the medial meniscus (DMM) in C57BL/6 mice at 18 weeks of age
- Blood and fecal samples were collected weekly
- Knee joints were harvested to measure PTOA progression
- 16S sequencing was performed on fecal and cecal samples to characterize the composition of the gut microbiome
- Metabolomics was performed to assess the functional output of the microbiome
- RNA-Seq was performed on the colon tissue to determine the effects of PTOA on the intestine

Experimental Cohorts

10 injured mice received DMM
10 uninjured mice serve as controls



Post-traumatic osteoarthritis model: PTOA development results from the transection of the medial meniscotibial ligament, which destabilizes the knee joint (DMM). Mice develop a reproducible OA phenotype.

Results

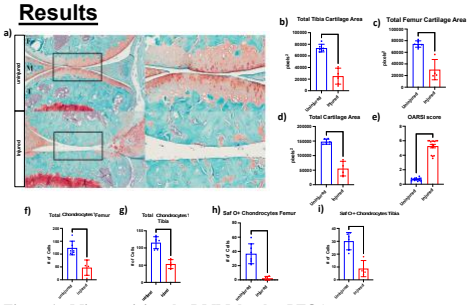


Figure 1 : Mice receiving the DMM develop PTOA
a)Representative histology image of injured and uninjured joints. Injured animals have a decrease in **b)** tibia, **c) f**emur and **d)** total cartilage area. **e)** OARSI scoring confirms PTOA progression in the injured cohort. Animals with PTOA have a decrease in the total amount of chondrocytes on the **f)** femur and **g) t**ibia. A reduction in matrix producing safranin O+ chondrocytes is seen within the **h) f**emur and **i)** tibia of injured animals.

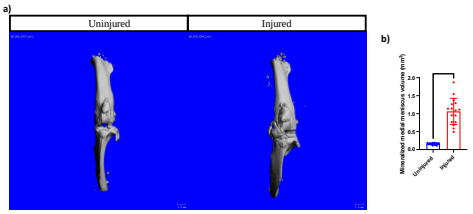


Figure 2 : Injured animals have an increase in mineralized medial meniscus volume
a) Representative 3D reconstructions of uninjured and arthritic joints. Animals that have PTOA have an increase in the amount of **b)** mineralized meniscus volume, a key indicator of PTOA.

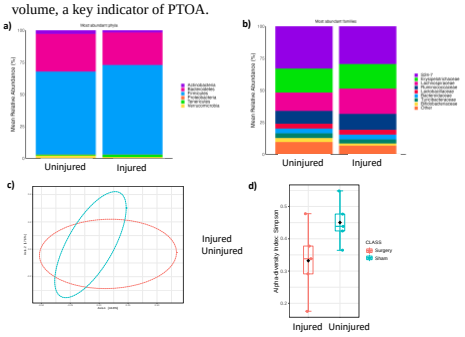


Figure 3: OA has minor effects on the gut microbiome composition
PTOA leads to minimal changes in the gut microbiome at both the **a)** phylum and **b)** family level. **c)** The PCA plot demonstrates that the gut microbiome does not significantly differ between PTOA and control group. **d)**Animals that have developed OA have a decrease in alpha diversity compared to uninjured controls, an indicator of disease and inflammation.

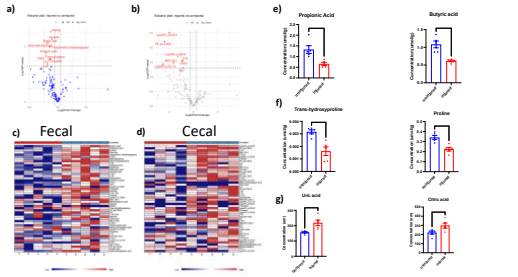


Figure 4: Metabolomics reveals key functional differences between the cecal, fecal, and serum of animals with PTOA.
Volcano plots of **a)** fecal and **b)** cecal samples (red samples indicate significantly different metabolites). The heat maps display all the metabolites found in **c)** fecal and **d)** cecal samples of uninjured and mice with OA. **e)** Small chain fatty acids (SCFAs) propionic acid and butyric acid and **f)** cartilage breakdown products trans-hydroxyproline and proline are decreased in fecal samples of animals with PTOA. **g)** Uric acid and citric acid are elevated in the serum of injured animals.

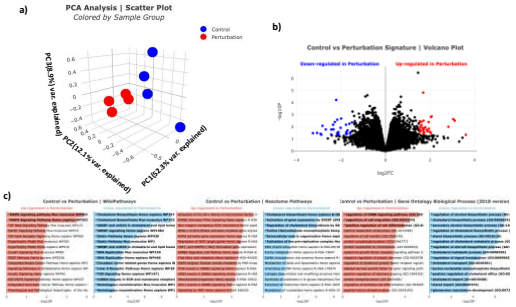


Figure 5: RNA-Seq indicates alterations in the colon transcriptome, with a reduction in pathways associated with cholesterol.
a) PCA shows colon transcriptome differs between PTOA and uninjured animals. **b)** Volcano plot indicates that 75 genes differ between the OA and healthy animals. **c)** Gene ontology and Pathway enrichment analysis demonstrate pathways that are associated with cholesterol are downregulated in the colon of animals with PTOA.

Conclusions

DMM results in OA progression:

- Animals receiving the DMM display a loss of cartilage on both the femur and tibia
- Injured animals had an increase in medial meniscus volume, a sign of PTOA development

OA leads to minor shifts in the bacteria present within the gut microbiome

- The gut microbiome of animals with PTOA does not significantly differ from uninjured animals
- Injured animals display a reduction in diversity of microbes within the gut

Metabolomics reveals functional differences between OA and uninjured animals

- Metabolomics reveals 29 metabolites differently produced in the fecal and cecal contents of injured animals
- SCFAs butyric acid and propionic acid are decreased in the fecal samples of injured animals
- Cartilage breakdown products are decreased within the fecal of animals with PTOA.

Colon RNA-Seq indicates mice with OA have a decrease in cholesterol signaling pathways

- RNA-Seq reveals differences in the transcriptome and function of the colon of animals with PTOA
- Animals with PTOA display alterations in cholesterol signaling

Orthopedics
UNIVERSITY OF COLORADO

10. Appendix 2: Villani et al, Manuscript Draft

INTRODUCTION

Osteoarthritis(OA), the leading cause of disability in the United States (US), is a degenerative disease that involves the loss and destruction of cartilage within diarthrodial joints [1]. OA afflicts more than 25% of the population over 18, with the incidence of OA increasing 102% between 1990 and 2017 [1, 2]. There are a variety of risk factors for OA, including obesity, genetics, age, sex, and injury[3]. Injury is a key risk factor for OA; it has been reported that 35% of people will develop symptomatic knee OA 10 years after sustaining an injury [4]. Understanding the pathogenesis of OA is critical; despite the severe physical and economic impact of OA, all existing therapeutic options are palliative.

The gut microbiome is one area of research that may be important in comprehending the onset of OA. The ecosystem of bacteria, fungi, viruses, phages, parasites, and archaea that inhabit the digestive tract are known as the gut microbiome[5]. It is recognized that the gut microbiome plays a role in host health and illness. Alterations in both the composition and function of the gut microbiome have the potential to affect not only the colon but also distal organ systems [6]. We and other researchers have demonstrated in the past that altering the gut microbiota with either a prebiotic or a probiotic can prevent the development of OA [7-9]. Furthermore, when compared to specific pathogen-free mice, both germ-free and antibiotic treated mice showed protection against the development of OA [10, 11]. Despite these investigations in preclinical models, very little is known about how the microbiome is involved in human OA development.

Numerous findings in human studies suggest that gut flora may contribute to the development of OA. *Streptococcus* abundance is one taxa that has been correlated with the severity of knee pain in OA [12]. Lipopolysaccharide (LPS), a component of bacterial cell walls, is elevated within both the synovial fluid and serum of humans with OA[13]. Metabolomics on fecal samples from obese patients with and without OA indicated that propionic acid, indoles, and other tryptophan metabolites were altered in OA patients[14]. However, obesity is known to cause drastic changes within the microbiome, so metabolites involved in the OA of obesity may differ from those involved in PTOA [15, 16]. Changes in specific bacteria have been harder to elicit in human studies due to the heterogenicity of the human gut microbiome. Thus, a controlled animal study is required to provide insights into candidate bacteria that may be involved in the human OA process.

Despite this body of work, little research has been conducted to fully comprehend how the gut microbiome is involved in OA. Here we report a multi-omics approach to identify the functional changes of the microbiome during the OA process. In addition to 16S rDNA gene sequencing, we performed both metatranscriptomics and metagenomics to further determine what genes and species of bacteria are involved in OA. Both cecal and fecal contents were subjected to metabolomics to provide a functional readout of the microbiome. RNA-Seq a was performed on the colon to determine how these functional alterations affected the tissue that directly communicates with the microbiome. Results from this study provide firm evidence that despite minor compositional changes within the gut of mice with PTOA, the functional capacity of the gut microbiome is altered during PTOA.

RESULTS

Bilateral DMM surgery results in a reproducible OA phenotype. Destabilization of the medial meniscus was utilized to model the pathological OA process. A bilateral surgery was performed to induce OA in both knees (Fig 1A). Control animals remained unmanipulated and had significantly more cartilage present on both the femur and tibia (Fig 1B and C). Injured animals had a significant decrease in total cartilage on both the femur and tibia compared to healthy controls (Fig 1D). The total chondrocytes and Safranin O+ matrix-producing chondrocytes were reduced in mice with PTOA (Fig 1E-H). Osteoarthritis Research Society International (OARSI) scores were significantly different between injured and uninjured animals, confirming the presence of OA within injured joints (Fig 1I).

Micro CT indicates an increase in medial meniscus volume in animals with PTOA. Our previous studies have shown an increase in the mineralization of the medial meniscus in animals developing OA [7]. As expected, microCT analysis revealed a significant increase in medial meniscus volume within injured animals compared to uninjured controls (Sup Fig). Representative images of the joint show increased ectopic mineralization around the meniscus of injured animals. These results, combined with histomorphometric data, indicate that injured mice have a severe and reproducible OA phenotype.

16S sequencing reveals PTOA does not drastically alter the fecal microbiome. We and others have demonstrated that targeting the microbiome is protective against OA development [7, 17]. However, characterization of the gut microbiome in a controlled study has not been performed in the context of PTOA. In order to determine if PTOA induces shifts in the gut microbiome, we collected fecal pellets biweekly from injured animals and uninjured controls and performed 16S rRNA sequencing. Principle coordinate analysis (PCoA) of microbial community dissimilarity utilizing the Bray-Curtis index of all fecal samples collected throughout the study reveals that the microbiome of animals with PTOA remains similar to control animals (Fig 2A). Nonmetric multidimensional scaling (NMDS) analysis over the course of the study indicates that only one distinct microbial profile is present between both groups, signifying joint injury and PTOA progression does not alter the gut microbiome composition (Fig 2B). At the phylum level microbial communities did not differ dramatically over the course of 24 weeks, as evidenced by nearly identical bar charts (Fig 2C). This was also true at the family level, as bar plots again did not show any discernible differences (Fig 2D).

To further characterize the fecal gut microbiome, a PCoA plot of fecal samples collected at the time of harvest was generated (Sup Fig 2A). At both the phylum and genus levels the fecal microbiome at the time of harvest did not differ between the animals with PTOA and control animals (sup Fig 2 B and C)). This was further evidenced by the lack of statistical differences in any of the members of the microbiome in the 24-week fecal samples (Sup Fig 2D). These results indicate that the composition of the fecal microbiome shows little change due to the presence of OA.

16S sequencing reveals PTOA does not drastically alter the cecal microbiome. Numerous studies have demonstrated that cecal and fecal contents differ in taxonomic structure; thus to further understand the effects of PTOA on the gut microbiome, we performed 16S rRNA characterization of the cecal contents of animals with end-stage PTOA. The microbial composition of the cecal contents changed slightly, but the general organization of the microbiome remained largely similar between injured and uninjured animals, as demonstrated by the overlap in the PCoA plot (Fig 3A). The Firmicutes to Bacteroides ratio (F/B), a ratio that has been shown to increase in inflammatory states, was increased in the injured cohort, mirroring published data [18]. Firmicutes accounted for 79% of the cecal microbiome composition in injured animals, with that number decreasing to 69% in uninjured animals. Bacteroides made up 15% of the , compared to 26% in uninjured animals (Fig 3 B and C). No significant alterations in bacterial species were present within the ceca (Fig 3D). *Bifidobacterium pseudolongum* and family Bacillaceae were reduced in the cecal microbiome of injured animals, whereas the family Lachnospiraceae and genera Anaeroplasma increased in animals with PTOA (Fig 3C and D). *Bifidobacterium psuedolongum* has long been linked to positive health outcomes, including protection in the OA of obesity [7, 19]. These results indicate that like the fecal microbiome, the cecal microbiome structure is not significantly altered due to PTOA onset and progression.

Metagenomics identify novel species of bacteria that are altered in the cecum of animals with PTOA. Following initial characterization with 16S rRNA sequencing, we performed shotgun metagenomic sequencing to determine if species level alterations occurred in the cecal contents of animals with PTOA. Shotgun sequencing revealed four species of bacteria that were significantly altered between injured and matched controls, three of which were not present in our 16S rRNA sequencing. *Asaccharobacter celatus, Adlercreutzia equolifaciens*,*Clostridium sp. ASF502*, and *Bifidobacterium psuedolongum* were altered in the ceca of animals with PTOA (Fig 4 A and B). Interestingly, *Adlercreutzia equolifaciens* has been implicated in other

musculoskeletal diseases but has not been studied in the context of OA[20]. Validating our 16S rRNA findings, the F/B ratio was again increased in injured animals, a phenotype that was largely driven by the increase of *Clostridium sp. ASF502 (*Fig 4 A and B). The PCoA plot reiterated that although these novel species are altered in PTOA, the general structure of the community did not change dramatically (Fig 4C). Mirroring previously established human findings, these data indicate that the microbiome of animals with and without PTOA does not display significant compositional changes.

The functional capacity of the cecum is altered in the colon of animals with PTOA. Although metagenomic data revealed modest changes in the composition of the gut microbiome, it did reveal extensive pathway changes. To this end, we performed in silico bacterial pathway profiling utilizing HUMAnN 3 [21]. DNA metagenomic analysis revealed 110 pathways that were differentially abundant between uninjured and PTOA animals. Of these pathways, 71 were associated with biosynthesis, 21 with degradation, utilization, or assimilation, 14 with the generation of precursor metabolites and energy, 2 with super pathways, and 1 with macromolecule modification (Fig 5 A)(supplemental table). The Bifidobacterium shunt pathway, a key indicator of Bifidobacterium activity was overly abundant in uninjured animals (Fig 5 B). The result of this pathway is the production of ATP, acetate, and lactate. This reaction is symbiotic in nature, as Bifidobacterium gains ATP while the host gains acetate, which can be used for host ATP production, and lactate, an antimicrobial metabolite that can hamper the growth of pathogenic microbes.

Of note, the pathway for 2-oxobutanoate degradation II was overrepresented in the microbiome of uninjured mice compared to animals with PTOA (Fig 5 C). This pathway is associated with the production of propionate, a SCFA that has known anti-inflammatory effects on the colon and other distal tissues [22, 23]. Six methionine synthesis pathways were more abundant in uninjured animals, L-methionine can be broken down into 2-oxobutanoate (Fig 5 D and E)(table). To further understand our metagenomic dataset, we utilized SqueezeMeta to determine molecular functions via KeggOntology [24]. In total, 111 orthologs were identified, with 42 of these mapping to metabolic pathways and 17 mapping to the biosynthesis of secondary metabolites (supplemental table). These data indicate that although the composition of the gut microbiome of animals with PTOA does not show large-scale compositional shifts, the functional capacity of the gut microbiome has shifted.

Meta transcriptomic analysis reveals altered metabolism pathways in the gut microbiome of animals with PTOA. Meta transcriptomics on the cecal contents provided insight into active pathways within the cecal microbiota. SqueezeMeta uncovered a total of 8 KEGG orthology groups that were differentially expressed between uninjured controls and injured animals, 5 of which were downregulated in the injured animals and 3 of which were activated in the injured animals. Of the 5 orthology groups downregulated during OA, 2 were involved in environmental information and processing (Fig 6 A and C). Interestingly, all 3 of the ortholog groups upregulated in the cecal microbiome during PTOA were associated with metabolism (Fig 6 B and C) . In addition to Kegg orthology groups, 125 significant Clusters of Ortholog Groups (COGs) and 30 Protein Families (PFAMs) were identified, with the top 25 of each indicated in (supplement table x). One of the most upregulated PFAMs was the Membrane Attack Complex/Perforin domain (MACPF) (coefficient=4.792) (Fig 6 D). The MACPF has been implicated as an important component of microbial virulence. In fact, certain microbial species can also utilize this domain as a competitive advantage by releasing MACPF in vesicles, which can act on competing commensals of the gut [25]. The GLUG motif was elevated in the ceca of animals with PTOA (coefficient =2.81) (Fig 6 E). The GLUG motif is a bacterial immunoglobulin protease that can cleave IgA proteins produced by the host immune system. This bacterial defense system is another example of how pathogenic bacteria in the microbiome of animals with PTOA could avoid host surveillance. These findings show that the cecal microbiome of PTOA animals is functionally altered, with increases in ortholog groups associated with bacterial metabolism and bacterial competition altered in the cecum of animals with PTOA.

PTOA alters metabolites produced by the gut microbiome To further understand the functional alterations in the gut microbiome, we performed targeted metabolomics on fecal and cecal samples. Although the composition of the microbiome only showed modest changes, metabolite analysis of the ceca and feces revealed large-scale changes (Fig 7 A)(Sup Fig). In total, 8 metabolites were altered in the cecum and 18 were differentially produced in the feces of animals with PTOA (Sup Fig). PcoA plot demonstrates the shift in the metabolites in the fecal (Fig 7A) and cecal (Sup Fig). Volcano plots display significantly altered metabolites in the fecal (Fig 7B) and cecal (Sup Fig) samples. Butyric acid, a SCFA that has previously demonstrated protective properties in pre-clinical models of PTOA, was reduced in the fecal pellets of animals with PTOA (Fig 7C) [26, 27]. Butyrate supplementation in PTOA is protective through direct action on both chondrocytes and cells of the synovium [28, 29]. To date, no studies have determined how the loss of butyrate effects PTOA

progression; however, given its decrease in our injured cohort, the loss of butyrate could play a role in the OA disease process.

The SCFA propionic acid levels were also lower in the feces of animals with PTOA (Fig 7D). Propionic acid has not been studied in OA but has been shown to possess anti-inflammatory capabilities in other models [30, 31]. The pathway for 2-oxobutanoate degradation II, responsible for propionate production, was also decreased in the metagenomic data.

The cartilage breakdown products trans-hydroxyproline and proline were reduced in fecal samples from PTOA animals(Fig 7 E and F). Trans-hydroxy proline has been demonstrated to have anti-inflammatory effects on the colon but has mainly been studied as a biomarker in OA [32, 33]. Trans-hydroxyproline may also play a role in the shaping of the bacteria present in the gut, favoring microbes with the capability to utilize trans-hydroxyproline [34].

Polyamines spermidine and spermine were found decreased in the fecal pellets of injured animals (Fig 7 G and H). Polyamines have recently been implicated as playing a role in other musculoskeletal diseases, with evidence indicating that these metabolites can interact with both cells of the joint and immune system cells in the colon.

Many fatty acids were also altered in the fecal and cecal contents of animals with PTOA. Steric acid, a metabolite that has been shown to have anti-inflammatory effects on the colon was decreased in the feces of animals with PTOA (Sup Fig). These data definitely demonstrate that the functional output of the gut microbiome differs between animals with PTOA compared to healthy controls. Understanding the functional consequences of these metabolites may provide novel insight into the PTOA disease process.

Citric acid and uric acid are altered in the serum of animals with PTOA. Serum was harvested from the mice at time of euthanasia for targeted metabolomics. Only two metabolites differed in the serum of animals with PTOA. Citric acid and uric acid were both elevated in the serum of injured animals (Sup Fig).

The colon transcriptome is altered in animals with PTOA. To our surprise, SCFAs, polyamines, cartilage breakdown products, and fatty acids identified in the cecum and feces by metabolomics were not elevated in the serum. Thus, in order to determine if these metabolites have a biological effect on the tissue they most closely interact with, we performed RNA-Seq on the colon of animals with and without PTOA. Transcriptome analysis of the colon tissue isolated from mice with and without PTOA demonstrated significant changes in the colon gene profile in injured animals (Fig 8A). Principle component analysis (PCA) based on quantification of gene signature dissimilarity indicated that the colons of animals with PTOA differed from healthy control animals (Fig 8B). In order to further understand the differences in gene expression, we performed cell type enrichment utilizing cTen, which utilizes an enrichment algorithm that identifies cell type demographics based on differentially expressed genes (DEG) [35]. Cell type enrichment of the upregulated DEG from injured animals revealed an enrichment of genes associated with the macrophage lineage, suggesting increased macrophage activity in the colon of animals with PTOA (Fig 8C). Macrophages in the colon are known to produce inflammatory cytokines that can disrupt intestinal integrity; however, they have not been studied in the context of PTOA [36].

We utilized Enrichr, a tool that contains a variety of gene libraries capable of identifying gene ontology, to perform enrichment analysis. In the top 25 enriched ontologies, 11 of these terms were associated with lipid and fat signaling (Fig 8D). The effects of decreased lipid signaling in the colon have scarcely been studied, with very little known of how this decreased signaling may impact colon biology.

Two distinct gene ontologies associated with phagocytosis were reduced in the top 25 ontologies, indicating that macrophage function may have been altered in PTOA mice (supplementary table). When taken together with cTen cell type enrichment, these data indicate that although macrophage activity is increased in the colon, the phagocytic function of these macrophages maybe altered.

To identify pathways that are involved in the onset of PTOA in the colon, we performed KEGG analysis utilizing Enrichr with the DEGs. Two pathways were identified as significantly altered: terpenoid backbone biosynthesis and fat digestion and absorption (Sup Fig). Similar to signatures identified in gene ontology, fat digestion and absorption pathways are altered in animals with PTOA, indicating the alteration of metabolic pathways in the colon may be involved in the onset of PTOA.

DISCUSSION

The microbiome is now recognized as an important factor in the OA process. Despite this finding, no controlled studies have been carried out to determine not only the compositional changes in the gut microbiome but also the effect of PTOA on the its function . Evidence from human studies has demonstrated that little to no change occurs in the gut microbiome composition in patients with PTOA [12]. Understanding the alterations in the gut microbiome could lead to a better understanding of the itsmes function in PTOA progression and the identification of potential therapeutic candidates generated from microbes. As a result, the purpose of this study is to better comprehend the functional alterations that occur in the microbiome during PTOA. In this study we identify that PTOA induces minimal changes in the composition of the gut microbiome, but results in large functional changes evidenced by meta transcriptomic and metabolomic shifts. Pathways associated with SCFA production and bacterial competition emerge as important factors in PTOA progression which lead to alterations in the colon transcriptome.

Human studies of OA using 16S rRNA sequencing have revealed minimal alterations in the composition of the microbiome in patients with OA. Smaller compositional changes have been difficult to characterize due to the heterogeneity of the microbiome, with research in human PTOA studies not designed to control for this heterogeneity. Boer et al. demonstrated that Streptococcus was associated with knee joint pain in the Rotterdam and Lifeline-DEEP cohorts, but, similar to our findings in mice, they found no significant variation in microbial composition when evaluating OA development [12]. Loeser et al. also performed 16S rRNA compositional analyses on patients with and without OA who had a BMI greater than 30, finding no significant compositional differences between the two groups [37]. Metabolomics on the serum and fecal contents of obese OA patients revealed changes in LPS, osteopontin, propanoic acid, indoles, and other tryptophan metabolites [14, 37]. Obesity, by contrast, is known to have substantial effects on the microbiota and the metabolites it produces, making the effect of OA harder to examine due to the obesity overlay [38]. Preclinical studies have provided some insight into the gut microbiome; Collins et al previously demonstrated a shift in the B/F ratio during OA progressionand identified 9 taxa correlated with cartilage loss, irrespective of diet status or fat composition [18]. To this end, a controlled study of the microbiome and its functional output is required to further our understanding of PTOA and to provide validation for future human studies.

To determine the effects of PTOA on the gut microbiome composition, we performed 16S rRNA sequencing longitudinally over the course of the study to uncover compositional shifts in the microbiome that develop during PTOA progression. Surprisingly, the microbiomes of PTOA-affected animals remained comparable to those of control animals, with very minor species differences found over the length of the study (Fig 2). To examine the composition of the microbiome in animals with end-stage PTOA, cecal contents were extracted and 16S rRNA analysis was performed. Sequencing indicated little change in the bacteria found in the gut microbiome. (Fig 3) The Firmicutes/Bacteroides ratio increased in animals with PTOA (Fig 3). An increase in the F/B ratio has been linked to an increase in inflammation and a decline in overall health [8, 39]. This conclusion is consistent with clinical studies of OA, which have shown that the F/B ratio increases throughout OA development and progression [40].

Bifidobacterium psuedolongum, a microorganism altered in the cecum (Fig 3 and 4) , has previously been linked to general health [41]. *B. psuedolongum* has also been linked to the treatment of obesity-related OA [7]. Although utilized as a probiotic, its mechanism of action remains unknown. Previous research has shown favorable effects in the colon, with *B. pseudolongum* outcompeting mucin-degrading bacteria and, as a result, boosting mucus thickness [42]. Furthermore, Bifidobacterium reduces inflammation in the colon while enhancing barrier function by upregulating tight junctions and inhibiting the NF-kB pathway [43]. The expansion of *Bifidobacterium psuedolongum* in the gut microbiome may also explain the increases in both SCFAs and polyamines that were observed via metabolomics, since previous data indicate that *Bifidobacterium psuedolongum* can increase butyric Acid, propanoic Acid, spermine and spermidine [43-45]. Using *Bifidobacterium psuedolongum* as a PTOA therapy remains an attractive proposition.

To further understand the gut microbiome's role in PTOA, we performed shotgun metagenomic and meta transcriptomic analyses. Shotgun metagenomic analysis revealed altered abundance of pathways and orthologs in the cecal contents of animals with PTOA compared to controls (Fig 5) . Bifidobacterium shunt was a pathway that decreased in abundance in the injured cohort. This pathway is centered around the enzyme fructose-6-phosphate phosphoketolase. It involves the breakdown of hexose sugars such as glucose and fructose, with the metabolites from this pathway being used to produce SCFAs.

SCFAs are metabolites produced in the microbiome via bacterial fermentation of dietary fibers and starches. Dietary fibers are typically broken down in the distal colon, where SCFAs can directly function as an energy source for colonocytes while also influencing immune cells [46]. SCFAs are known to play several roles in the colon, including intestinal barrier integrity, mucus production, and inflammation reduction [47, 48]. The anti-inflammatory nature of SCFAs makes them an appealing candidate for studies in PTOA.

In metagenomic pathway analysis of the ceca, SCFA pathways were discovered differentially abundant, with the oxobutanoate degradation II pathway decreased animals with PTOA (Fig 5). Oxobutanoate degradation II results in the formation of propionate, a SCFA with numerous anti-inflammatory properties [22, 49]. Propionic acid was likewise one of the most drastically reduced SCFAs in the feces of mice with PTOA (Fig 7). Its decrease in abundance at the metagenome and metabolite levels could imply that targeting propionate-producing bacteria may have anti-PTOA therapeutic potential. The effects of propionate in PTOA have not been investigated; however, its study in rheumatoid arthritis may provide insight into its role in PTOA. Propionate has been shown to have protective effects in collagen-induced rheumatoid arthritis models via a reduction in inflammation. Further, supplementation of propionate in drinking water or by injection, reduced antigen-induced arthritis, targeting synoviocytes, a key cell type involved in the OA process [50, 51]. In addition to cells of the joint, propionate effects macrophage populations and cells of the colon by reducing the expression of pro-inflammatory cytokines and the limiting the activation of the NF-kB pathway, a key pathway that has been implicated in OA development [52, 53]. With no studies performed in the context of OA, propionic acid emerges as an enticing target for future microbially derived DMOADs.

Butyric acid was another metabolite found elevated in the fecal contents of mice with PTOA (Fig 7). Butyric acid has been studied in a variety of disease contexts and has been shown to have anti-inflammatory effects in a variety of tissues [54]. Butyrate, in addition to its anti-inflammatory properties, serves as one of the primary energy sources for colonocytes and has been demonstrated to influence immune cell development [48]. Butyric acid has been shown to decrease autophagy and mediate inflammatory chondrocyte death in murine models of OA, making it a target for therapeutic development [27]. Furthermore, cell culture experiments utilizing human chondrocytes revealed butyrate has both chondrogenic and anti-inflammatory effects on chondrocytes, demonstrated by a decrease in pro-inflammatory cytokines and type 2 collagen degradation [26]. These findings are consistent with our discovery that butyrate levels are lowered in injured animals and may explain its success in pre-clinical models of OA.

Metabolomics also revealed a decrease in hydroxyproline in the fecal contents of injured animals (Fig 7). Hydroxyproline is one of the most abundant amino acids found in collagen, derived via posttranslational hydroxylation of proline. In OA, hydroxyproline has been implicated as a potential biomarker, with arthritic patients demonstrating an increase in serum hydroxyproline [32]. Studies of hydroxyproline derived from the microbiome have demonstrated that it may have protective effects on the colon, with protection seen in colitis studies via the targeting of the NF-kB pathway [33]. These anti-inflammatory capabilities of hydroxyproline were also evident in co-culture experiments with macrophages [33]. As we show in this paper and others have previously demonstrated, pathways in the colon and macrophages are altered in PTOA, making them an attractive target for further investigation.

Two polyamines, spermine and spermidine, were found to be lower in the feces of animals with PTOA (Fig 7). Polyamines have previously been shown to provide protection in PTOA models. Spermidine can decrease pro-inflammatory cytokines and act directly on both chondrocytes and synoviocytes in the joint [55, 56]. Further, in collagen-induced models of RA, spermidine inhibits macrophage activation and ultimately reduces disease progression [57]. Both spermine and spermidine are also important in the activation of T and B cells in the colon [58]. The significant decrease in proline and glutamic acid in the feces of uninjured animals may explain the decrease in spermine and spermidine in the feces of PTOA animals. Of note, acetyl-ornithine is also decreased in the feces of animals with PTOA, although this decrease was not significant. Since acetyl-ornithine is a microbially derived product involved in the formation of polyamines, this decrease could provide an explanation of the loss of spermine and spermidine in the microbiome of mice with PTOA.

Our meta-transcriptomic study of the microbiome revealed three KEGG pathways that were enriched in PTOA mice. All three processes are involved in metabolism. K18687 is a HIP-CoA ligase is involved in steroid degeneration and a cholesterol degeneration pathway active in the microbiome. The elevation of this pathway in the microbiome of injured mice could be one plausible explanation for the decrease in cholesterol signaling seen in the colon of the PTOA cohort, as the degeneration of cholesterol may be limiting the availability of cholesterol in the colon. Meta-transcriptomic analysis of the microbiome provides definitive evidence that the

gut microbiome is functionally altered during PTOA and could potentially explain some of the biological differences seen in other tissues and systems.

Serum metabolomics did not reveal an increase in microbially derived metabolites, leading us to believe that the biological effects of these metabolites are occurring in the colon of these animals. To this end, we performed RNA-Seq on the colon of animals with PTOA to gain a deeper understanding of how PTOA alone impacts the tissue that most closely interacts with the gut microbiome. Cell type enrichment analysis uncovered a macrophage signature in the colon (Sup Fig). We have previously demonstrated that a macrophage enrichment in the colon is a driver of inflammation in the OA of obesity [7]. Recently, Cho et al. demonstrated that animals with OA have an increase in MCP-1 production within the intestinal epithelium, one of the key recruiters of monocytes and macrophages to sites of inflammation [27]. In OA, both prebiotic and probiotic treatment restored inflammatory gene networks and MCP1 expression in the colon [27](Schott et al unpublished data). Depletion of infiltrating macrophages into the colon has been shown in different disease systems to reduce inflammation and improve overall health [36, 59]. Of the top 25 enriched ontologies identified by Enrichr, two are associated with phagocytosis. These ontologies were reduced in PTOA animals. With an enrichment of macrophages but a decrease in genes associated with phagocytosis, further investigations into both the presence and function of infiltrating macrophages in the colon could not only provide valuable information on the PTOA disease process but also potential therapeutic options.

Surprisingly, seven of the top ten gene ontologies found in the colons of PTOA mice involved a decrease in metabolic processes involving lipid and fat signaling. The most significant GO term in the colon was lipid biosynthetic process, which was decreased in PTOA (Fig 8). This, combined with the other enriched ontologies in the top ten, such as fatty acid metabolism and steroid metabolism, imply that the colon of animals with PTOA undergoes fundamental metabolic changes. These changes in colon pathways have been linked to obesity, but with no change in weight in these animals (data not shown), understanding the metabolic state of these colon cells could provide vital insight into the role of the colon in PTOA. Numerous studies have indicated increases in lipid signaling within colon cells during different disease states; however, little research has been performed in the context of decreased lipid signaling [60]. With fatty acids and lipid molecules decreasing in the ceca and feces of PTOA-affected animals, these metabolites may explain the decrease in lipid signaling within the colon. Steric acid has been shown to have anti-inflammatory effects on the colon [61]; decreases in steric acid and other lipid molecules in the ceca could offer explanations for the altered fatty acid and lipid signaling within the colon of animals with PTOA.

Cholesterol biosynthetic pathway was the third most significant Gene Ontology, with three cholesterol-related ontologies decreasing among the top 25 enriched ontologies in the colons of mice with PTOA (Fig 8). Previous work in OA, particularly the OA of obesity, has targeted elevated cholesterol as a treatment; however, this has resulted in conflicting results [62-64]. The liver is considered the central location for cholesterol maintenance in the body, but recent work has implicated the colon as a potential source of cholesterol [65].This was further validated by the increase of meta transcriptomic pathways associated with cholesterol degeneration within the microbiome of animals with PTOA. Further investigations need to be conducted to determine if pathways associated with cholesterol signaling in the colon play a role in PTOA development.

Conclusion: This study provides the first meta-transcriptomic analysis of the gut microbiome in the context of PTOA. We validated results found in human clinical studies, which showed that PTOA alone does not cause significant changes in the composition of the gut microbiome. We demonstrate via meta transcriptomics, metagenomics, and metabolomics of the cecal and fecal compartments that, despite the small shift in the composition of bacteria in the gut, the functional capacity and output are altered due to PTOA. The identification of multiple SCFAs provides insights into future therapies that can be tested in human populations with limited safety concerns. Finally, we performed RNA-Seq on the colon of animals with PTOA to determine metabolic pathways that are altered in PTOA development and may provide key insight into the understanding of the microbiomes effect on PTOA development.

METHODS

Mouse model of PTOA: All mouse work was approved by the University of Colorado Anschutz under protocol #904. C57BL/6J mice were purchased from the Jackson Laboratory and housed in groups of 3–5 mice per cage. Mice were housed in microisolator cages with a 12-hour light/dark schedule. Mice were allowed to age, and at 19 weeks of age, they were administered bilateral destabilization of the medical meniscus surgery (DMM). Briefly, mice were anesthetized with inhaled isoflurane, and their knees were shaved. Prior to surgery, mice were given buprenorphine SR at a dose of 1 mg/kg. Under a dissecting microscope, a #10 blade was used to make an initial cut of 5 mm on the medial side to expose the knee joint. Next, using micro-scissors and a #25 syringe, a cut was made on the medial side of the patella tendon to expose the medial meniscus. With a number 11 blade, the medial meniscotibial ligament was transected. Following surgery, incisions were closed with 4-0 silk sutures. Sutures were removed after 10 days.

Fecal collection: Fecal pellets were freshly collected from scruffed animals weekly and flash frozen in cryovials. Vials were then transferred for long-term storage at 80 °C.

*** sequencing info**

***DNA and RNA extractions of fecal/cecal ***

** RNA seq methods***

Serum collection: Submandibular bleeds were used to collect blood samples every two weeks. Blood was collected on ice; samples were then centrifuged at 6800 rcf to separate RBC from serum and frozen at 80 °C for future analysis.

Knee joint tissue harvest: Mice were sacrificed 24 weeks after DMM surgery to ensure advanced OA progression. Mice were harvested using American Veterinary Medical Association-approved methods. Both knee joints were removed, and excess muscle tissue was cut away. Joints were placed in cassettes, secured to plastic brackets at 30-degree joint flexion, and fixed in 10% neutral buffered formalin for 3-5 days. Knee joints were then washed in PBS and decalcified in Webb-Jee EDTA for 14 days. The joints were then processed utilizing a microwave processor and embedded in paraffin as previously described. Tissue blocks were sectioned in the midsagittal plane, with serial sections taken from the joint. Knee tissues were stained with Safranin O/Fast Green, and cartilage was quantified by a blinded observer using Zeiss ****.

Histomorphometric analysis of cartilage: Cartilage area, hypertrophic chondrocytes, and safranin O+ chondrocytes were quantified using Zeiss *** software by a blinded observer. A touch screen and stylus pen were used to outline the cartilage area in six sections of each animal's joint. Two sections at each level (50 uM apart) were averaged. To count chondrocytes, the stylus pen was used to mark and count over 3 levels and 6 sections.

Colon tissue harvest: At the time of harvest, colon tissue was removed from the animal. Fecal pellets and mucous were removed from the colon with 5 mL of PBS and a blunt needle. The tissue was then transected with ball scissors, cut in half longitudinally with a razor blade, and flash frozen in liquid nitrogen. The tissue was then placed at -80 °C for long-term storage.

MicroCT analysis: Prior to decalcification, knee joints were taken from NBF and scanned in PBS using X-ray microcomputed tomography (μCT50, Scanco Medical AG, Bassersdorf, Switzerland). Joints were imaged at 70 kV (200 μA) within a 35 mm diameter field of view, employing 2000 cone beam projections per revolution, and an integration time of 500 msec. Reconstruction of three-dimensional images occurred at 17.2 μm resolution using standard convolution back projection algorithms with Shepp and Logan filtering. Images were rendered at a discrete voxel density of 196,524 voxels/mm3 (isometric 17.2 μm voxels). Images were transposed to longitudinal views, and by manual contouring, menisci were separated from the joint. A mineral density threshold of 550 mg HA/cm^3 was utilized as a density threshold. The mineral was calibrated to a discrete-step hydroxyapatite phantom and segmented from unmineralized meniscal cartilage in conjunction with a constrained Gaussian filter to reduce noise.

RNA isolation from colon tissue: Flash-frozen tissue was submerged in Trizol before being placed in a lysing matrix A tube (MP Biomedicals) and homogenized using Bead Ruptor 12 (Omni International). The tissue was homogenized at a speed of 5 m/s for 15 seconds and placed on ice for 5 minutes; this step was then repeated. Phenol chloroform was added to the homogenized tissue. Tubes were mechanically shaken and then centrifuged at 12000 rcf for 15 minutes at 4 C. The aqueous phase was then extracted and combined with pure ethanol at a ratio of 1:1. RNA was purified from the ethanol: phenol chloroform solution via the Genejet RNA purification kit following the manufacturer's instructions.

OARSI scoring: In order to quantify OA progression and cartilage degeneration, a semiquantitative histopathologic grading system established by OARSI was employed. Safranin O/fast green-stained sections were graded according to the scale previously described [7]. Scoring was performed by four blinded observers (DAV, MJZ, SLP and MD)

Statistical analyses: Non-omics data were analyzed and plotted with GraphPad Prism (Version 9.4.1). Standard t tests were used to test significance, with specifics provided in the figure legends. For all non-omic data, p values < 0.05 are considered significant.

FIGURE LEGENDS

Figure 1: Mice receiving the DMM develop PTOA. a) Representative histological images of injured and uninjured joints. Injured animals have a decrease in (b) the femur, (c) the tibia, and (d) the total cartilage area. Animals with PTOA have a decrease in (e) tibia, (f) matrix-producing SafO+ tibia, (g) femur, and (h) matrix-producing SafO+ femur chondrocytes. **i)** OARSI scoring confirms PTOA progression in the injured cohort. A student t-test was performed to determine statistical differences, with p values represented by *<0.05, ** <0.01, and ***<0.001. All bars represent the group means (± SEM) with each dot representing the average measurement of three levels of histology. Each dot represents an injured or uninjured joint.

Figure 2: PTOA does not alter the composition of the fecal microbiome. (a) Principal coordinate analysis (PCoA) was performed on all samples from initiation to end-stage disease. Each point represents a biweekly fecal sample, with the distance representing the dissimilarity between the samples. (b) Nonmetric multidimensional scaling was performed using averages of the Bray-Curtis distances at each timepoint for injured and uninjured animals. The average relative abundance of (c) phylum and (d) family over the course of the disease process. At each timepoint, an n= 5 was used to generate the relative abundance plots.

Figure 3: End-stage PTOA does not alter the composition of the cecal microbiome by 16S rRNA sequencing. PCoA analysis performed on the cecal microbiome, detailing the composition of the gut, is not dissimilar between injured and uninjured animals. Each point represents a separate animal cecal microbiome. The average (b) phylum and (c) family abundance were determined in cecal samples. Cecal samples (n = 5) were averaged and depicted in the bar charts. The firmicutes-to-Bacteroides ratio was established by averaging the abundance of firmicutes and Bacteroides present within the cecum. (d) A heat map was generated to visualize the abundances of bacteria present within the cecum. Graphs were then generated, displaying the bacteria at the genera, families, and species levels that differed between injured and uninjured animals. A student t-test was performed to determine statistical differences, with p values represented by *<0.05.

Figure 4: Metagenomic sequencing confirms that few compositional changes occur within the cecal microbiome. (a) The average species abundance in the ceca was determined by metagenomic sequencing. The cecal samples (n = 5) were averaged to display the bar charts. The firmicutes-to-Bacteroides ratio was determined by averaging the abundance of firmicutes and Bacteroides in the cecum. (b) graphs showing the mean relative abundance of all significant bacterial species in the cecum (n = 5). (c) PcOA analysis performed at the genus level from metagenomic data, with each dot representing one mouse. The statistical differences between bacteria were determined using a student t-test, with p values represented by *<0.05 and **<0.01.

Figure 5: PTOA alters the functional capacity of the gut microbiome. (a) DNA metagenomic analysis reveals 110 pathways that are altered in abundance due to PTOA. Pathways associated with Bifidocbacterium (b) Bifidobacterium shunt and SCFA production (c) 2-oxobutanoate degradation II were decreased in abundance due to the presence of PTOA at the DNA level. Methionine synthesis pathways (d) the superpathway of L-lysine, L-threonine, and L-methionine biosynthesis I, and (e) methionine biosynthesis I, were significantly increased in abundance within uninjured animals.

Figure 6: Meta transcriptomics of the cecal microbiota indicates that pathways associated with metabolism, bacterial competition, and immune system avoidance are upregulated within the microbiome of animals with PTOA. (a) A total of 8 KEGG ortholog groups were differentially expressed between the gut microbiota of injured and uninjured animals. Blue bars represent ortholog groups that increased in expression in injured animals, with red bars indicating ortholog groups that decreased in expression in the injured cohort. (b) Graphs depicting the expression of all 8 KEGG orthologs are displayed (n = 5). Animals with PTOA have a significant increase in PFAMS (c) the Membrane Attack Complex/Perforin domain (MACPF); and (d) the GLUG motif. All graphs were generated with n = 5 from RNA meta-transcriptomic sequencing.

Figure 7: PTOA alters SCFAs, cartilage breakdown products, and polyamines within the cecal microbiome. PCA analysis was performed on all of the metabolites assayed in the cecum at end-stage PTOA. Each dot represents one animal, and the distance between each dot indicates the dissimilarity between

animals. (b) A volcano plot displaying the metabolic differences between injured and uninjured animals. The concentrations of SCFAs (c) butyric acid and (d) propionic acid. The concentrations of cartilage breakdown products (e) proline and (f) trans-hydroxyproline. The concentrations of polyamines (g) spermidine and (h) spermine. All points represent one animal with an n=4 or 5. A student t test was utilized to determine significance on all graphs, with p values represented by *<0.05 and **<0.01. All graph bars represent the group mean (± SEM).

Figure 8: RNA-Seq indicates alterations in the colon transcriptome, with a reduction in pathways associated with fat signaling. Transcriptome of colon tissue from animals with and without end-stage PTOA. (a) A heatmap displaying the clustering of differentially expressed genes in the colon. (b) A three-dimensional PCoA analysis was performed on the expression profiles of all animals, with each dot representing one animal. (c) Cell type enrichment was performed with cTen using the DEG list. PTOA was associated with a macrophage cell type. (d) Of the top 25 enriched ontologies, 11 of them are associated with lipid and fat signaling. These 11 ontologies are depicted with a log (p-adjusted) value displayed to visualize the differences.

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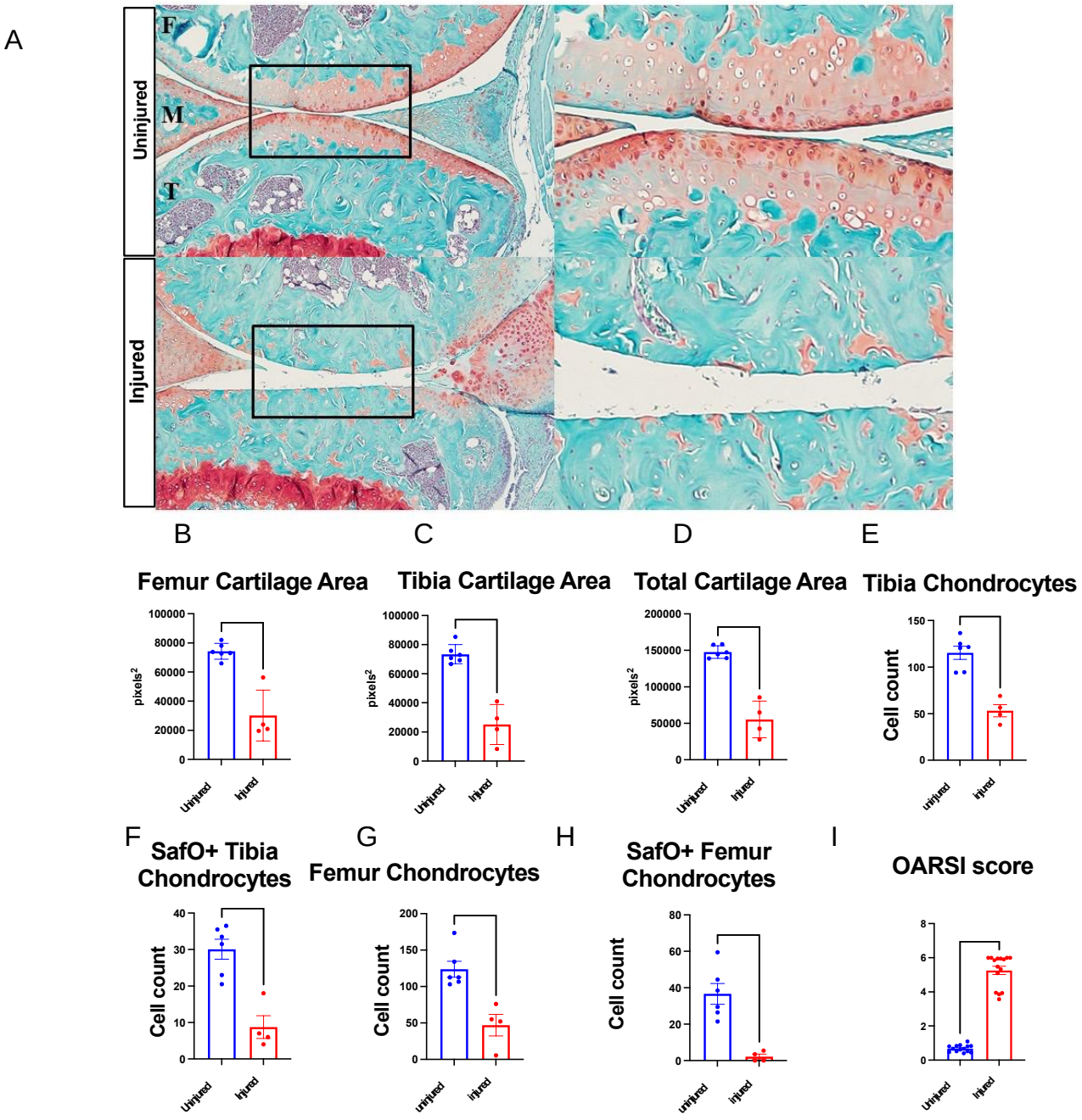

A
Uninjured
Injured
F
M
T
B
Femur Cartilage Area
pixels²
100000
80000
60000
40000
20000
0
Uninjured
Injured
C
Tibia Cartilage Area
pixels²
100000
80000
60000
40000
20000
0
Uninjured
Injured
D
Total Cartilage Area
pixels²
200000
150000
100000
50000
0
Uninjured
Injured
E
Tibia Chondrocytes
Cell count
150
100
50
0
uninjured
injured
F
SafO+ Tibia Chondrocytes
Cell count
40
30
20
10
0
Uninjured
Injured
G
Femur Chondrocytes
Cell count
200
150
100
50
0
uninjured
injured
H
SafO+ Femur Chondrocytes
Cell count
80
60
40
20
0
uninjured
injured
I
OARSI score
8
6
4
2
0
Uninjured
Injured

Figure 1

Supplement

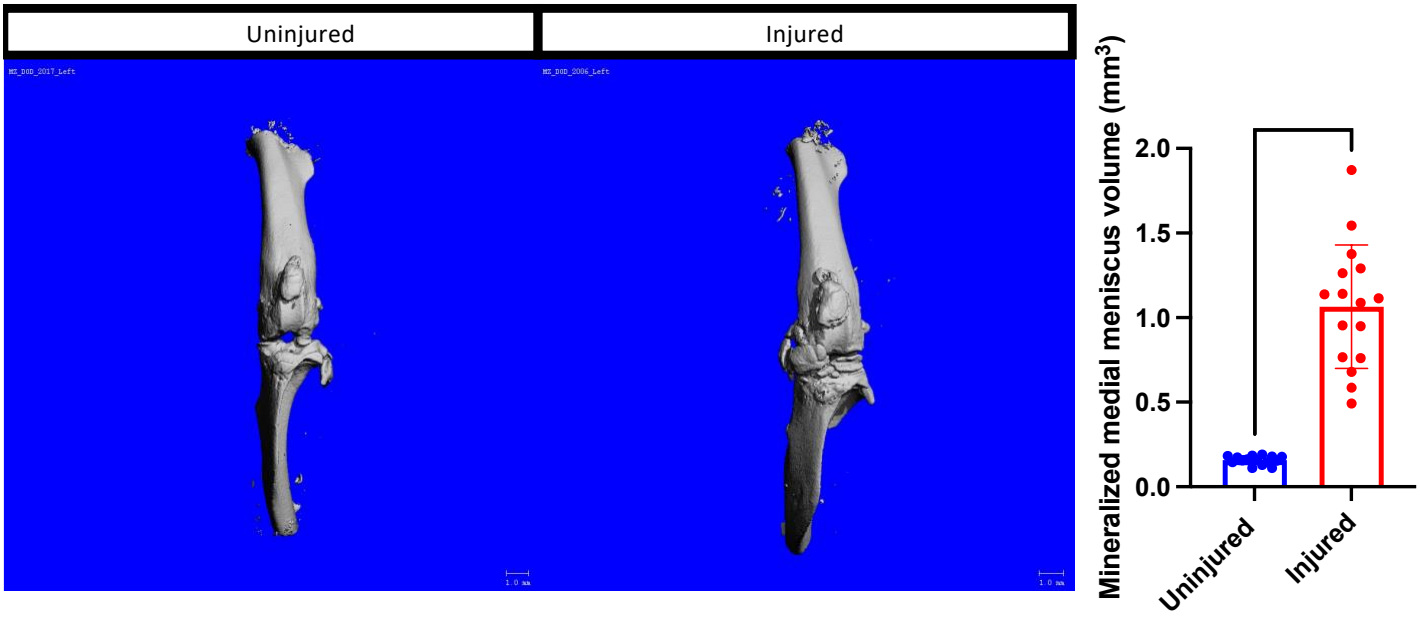
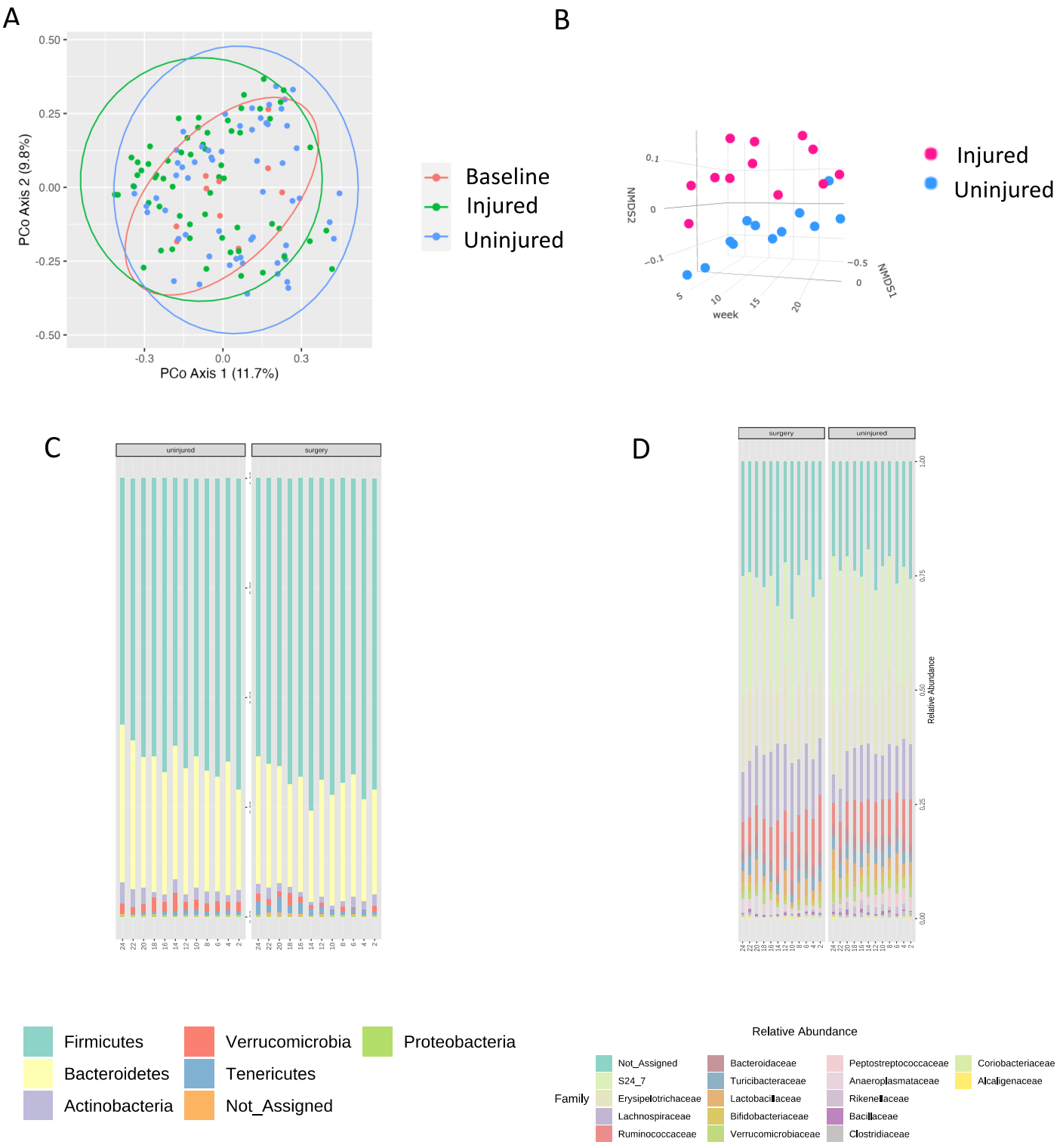
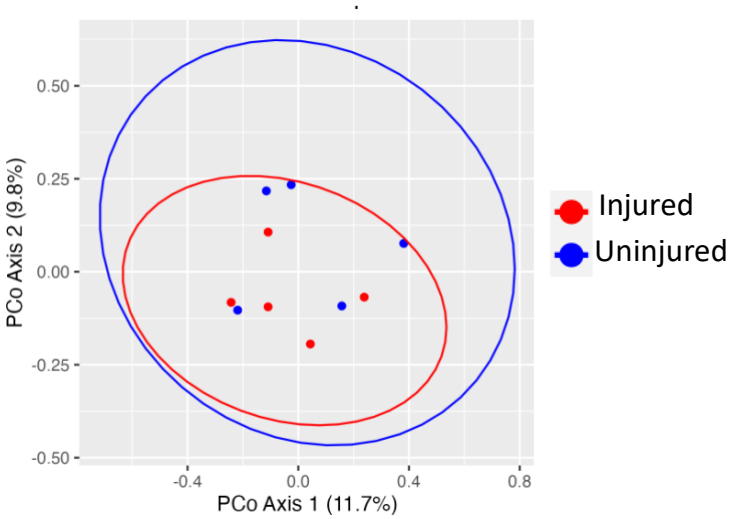

Uninjured
Injured
Mineralized medial meniscus volume (mm3)
2.0
1.5
1.0
0.5
0.0
Uninjured
Injured

Figure 2

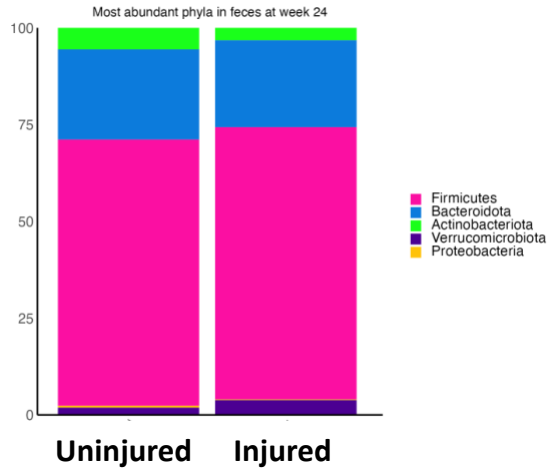

A
0.50
0.25
0.00
-0.25
-0.50
PCo Axis 2 (9.8%)
-0.3
0.0
0.3
PCo Axis 1 (11.7%)
Baseline
Injured
Uninjured
B
NMDS2
NMDS1
week
Injured
Uninjured
C
uninjured
surgery
Firmicutes
Bacteroidetes
Actinobacteria
Verrucomicrobia
Tenericutes
Not_Assigned
Proteobacteria
D
surgery
uninjured
Relative Abundance
Relative Abundance
Family
Not_Assigned
S24_7
Erysipelotrichaceae
Lachnospiraceae
Ruminococcaceae
Bacteroidaceae
Turicibacteraceae
Lactobacillaceae
Bifidobacteriaceae
Verrucomicrobiaceae
Peptostreptococcaceae
Anaeroplasmataceae
Rikenellaceae
Bacillaceae
Clostridiaceae
Coriobacteriaceae
Alcaligenaceae

Supplement

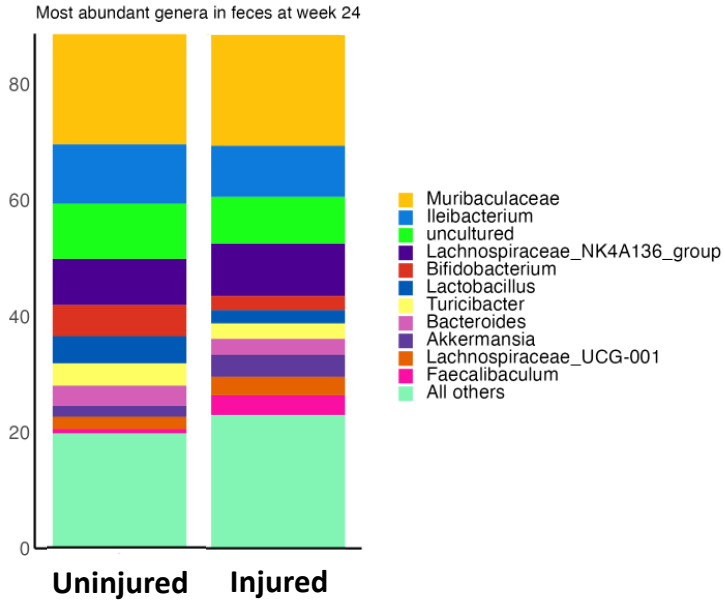
A



B



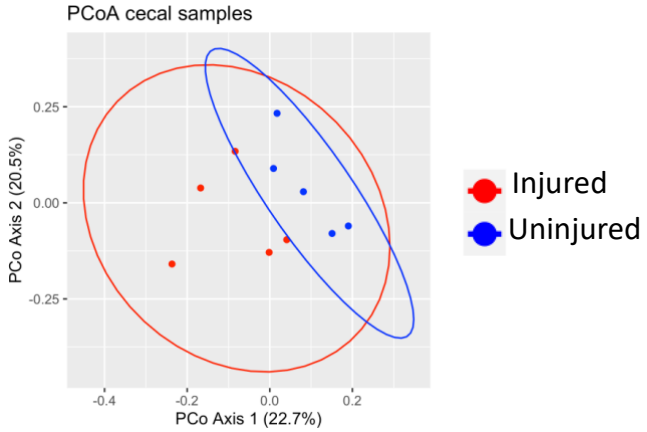
C



D

Figure 3

A



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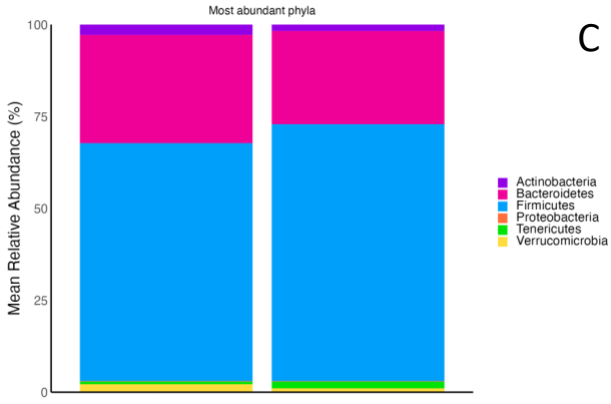
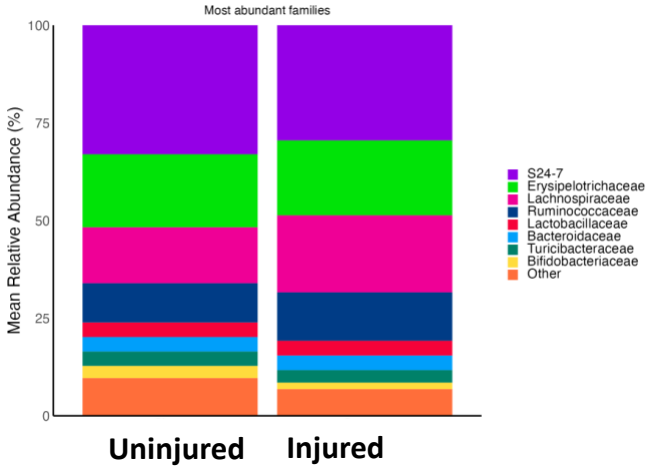
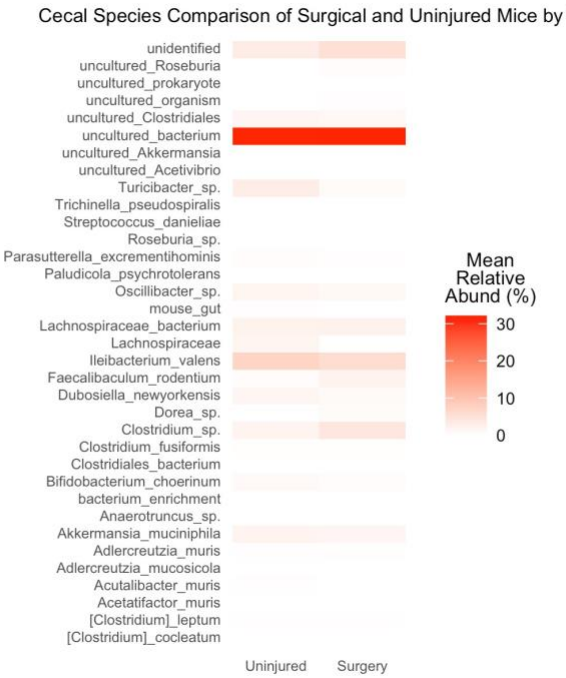


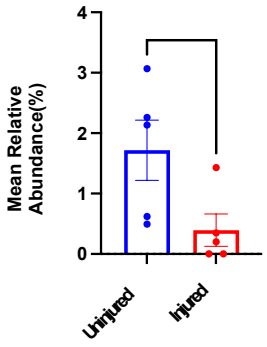
Firmicutes/ Bacteroidetes Ratio	Uninjured	Injured
	2.67	5.14

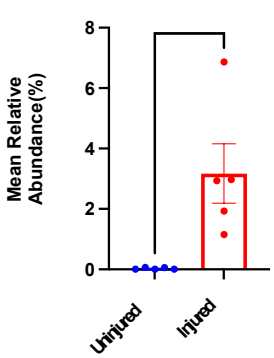
C

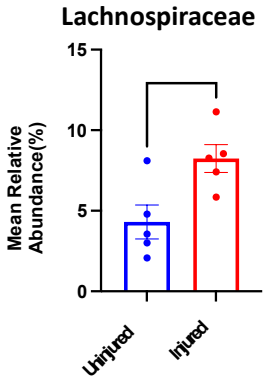


D









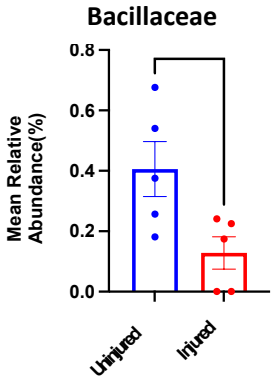
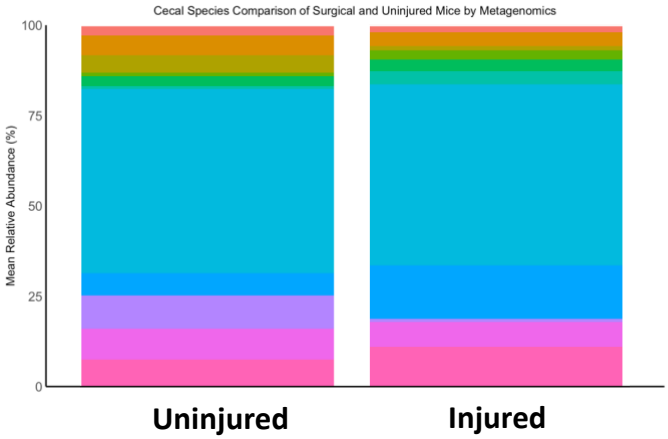


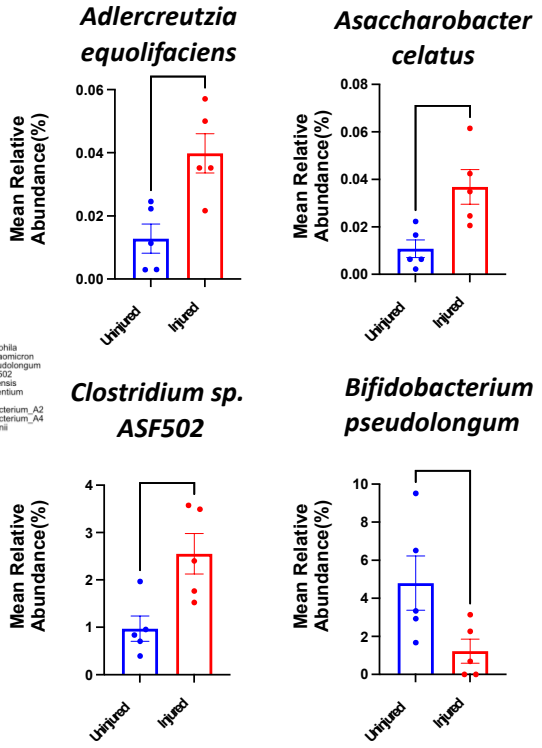
Figure 4

A



	Uninjured	Injured
Firmicutes/ Bacteroidetes Ratio	15.54	24.0

B



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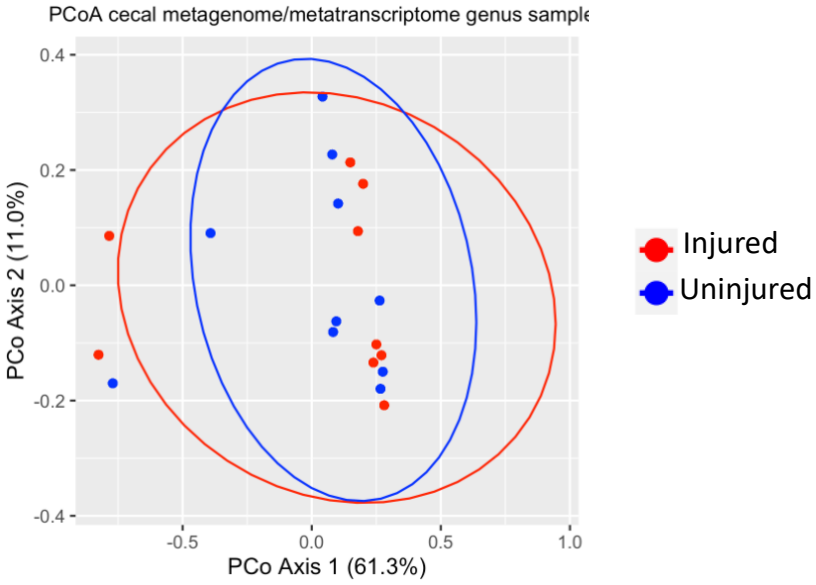
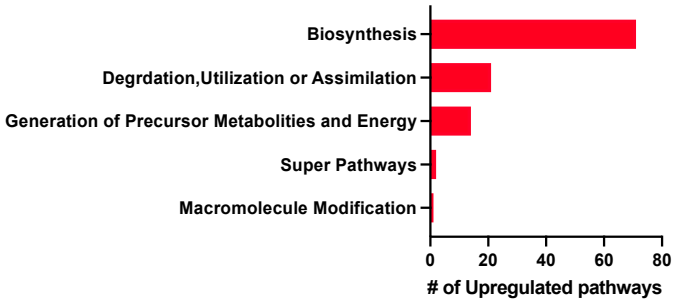


Figure 5

A



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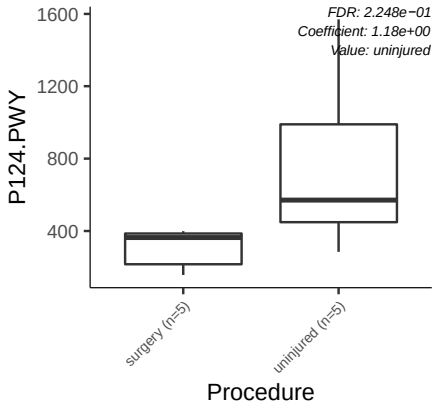

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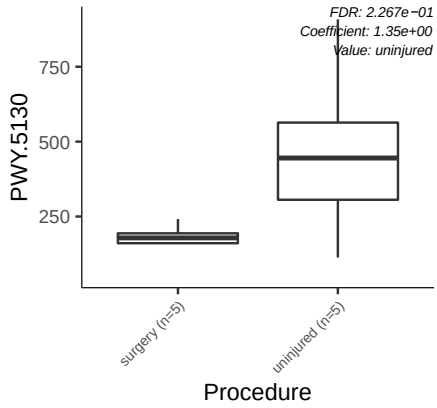

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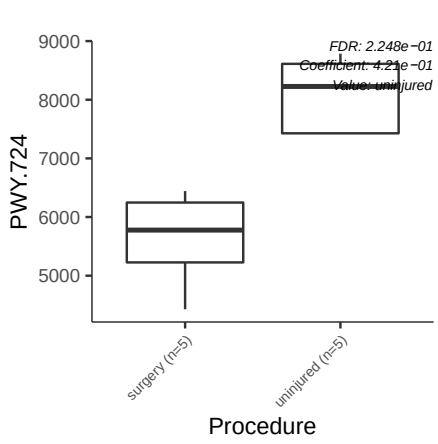
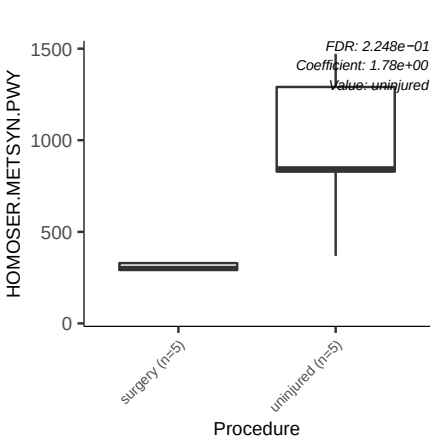


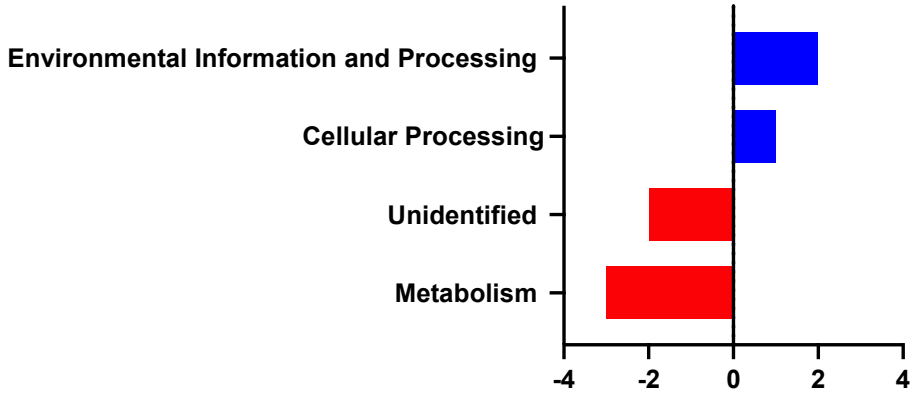
E



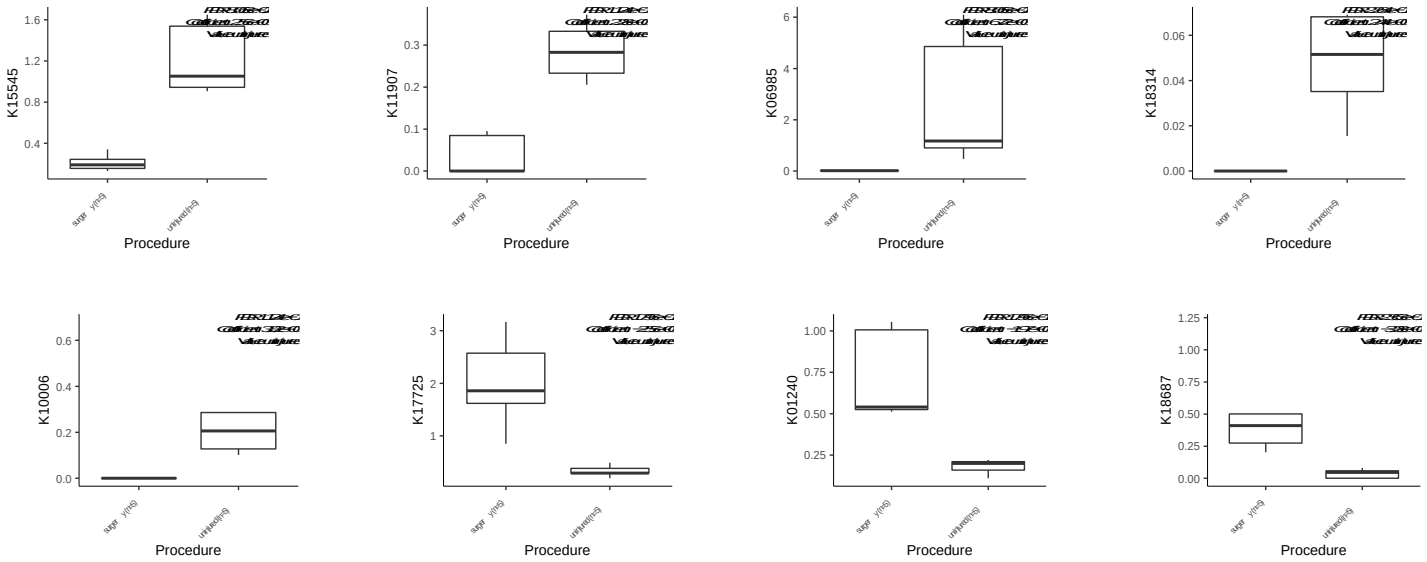
Keggg orthologs DNA table

A

Figure 6


Environmental Information and Processing
Cellular Processing
Unidentified
Metabolism
-4
-2
0
2
4

C


K15545
K11907
K06985
K18314
K10006
K17725
K01240
K18687
Procedure

D

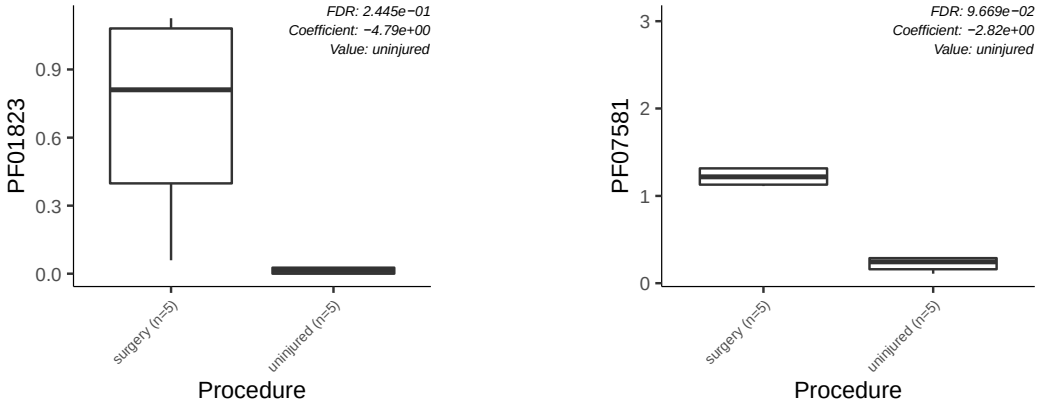
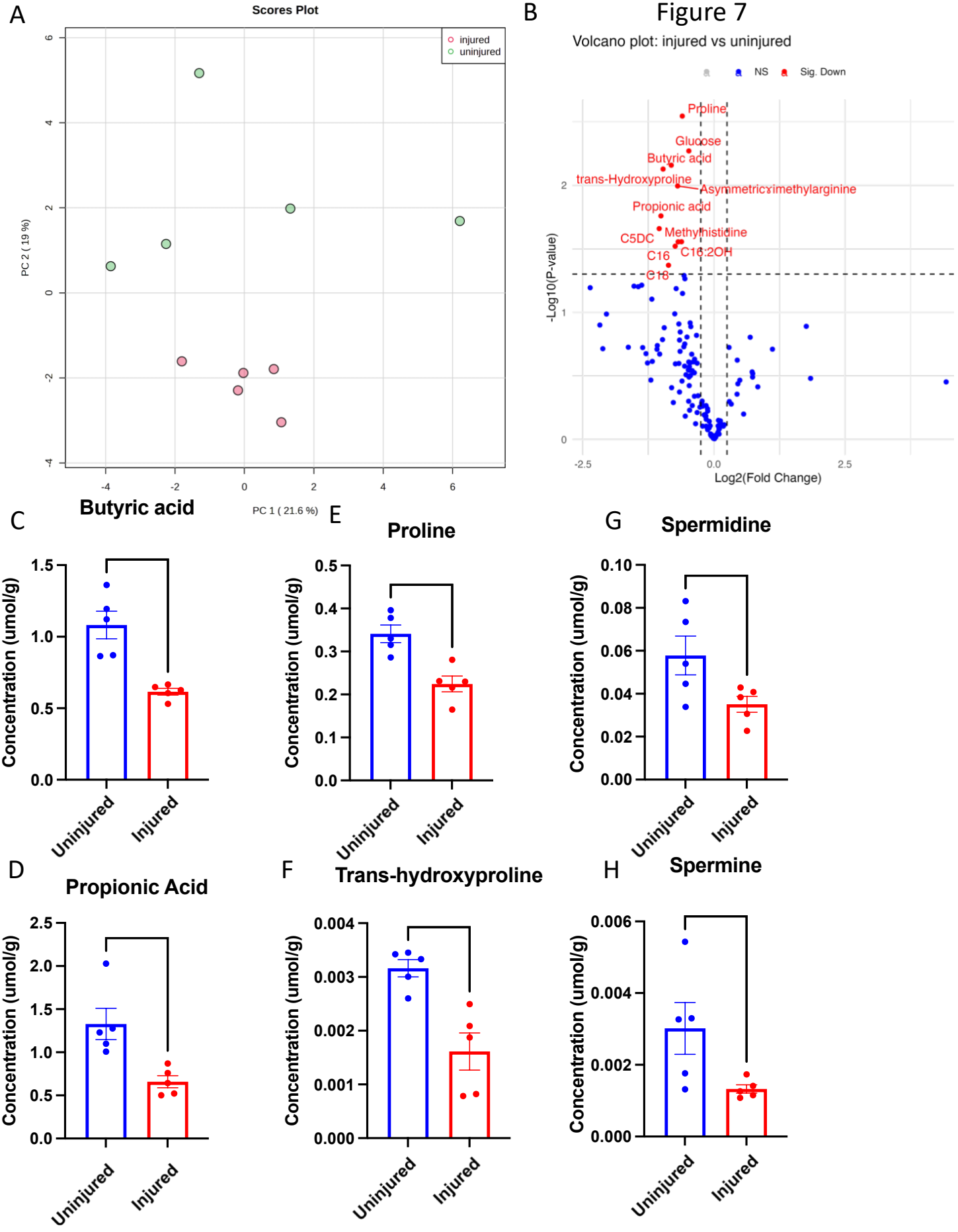
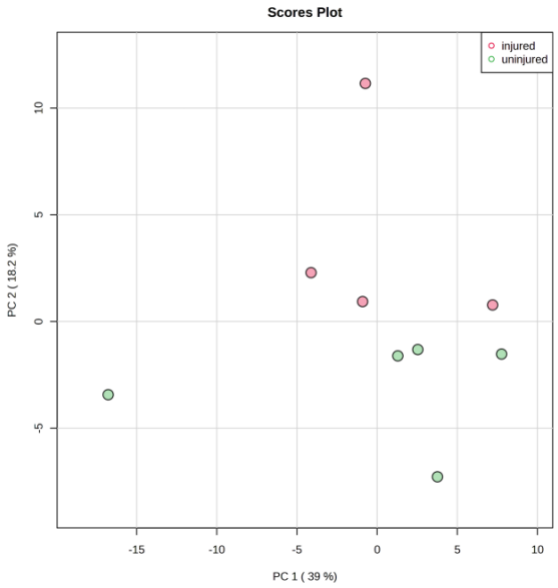
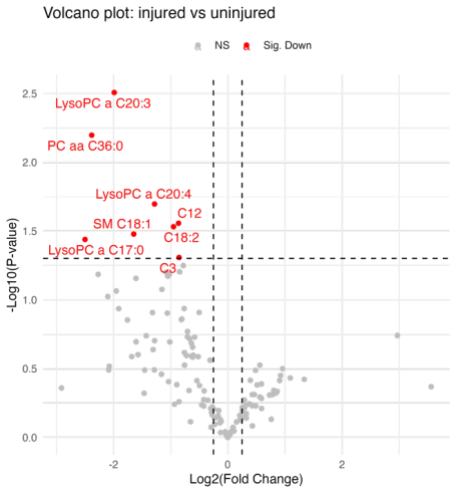

PF01823
FDR: 2.445e−01
Coefficient: −4.79e+00
Value: uninjured
PF07581
FDR: 9.669e−02
Coefficient: −2.82e+00
Value: uninjured
surgery (n=5)
uninjured (n=5)
Procedure

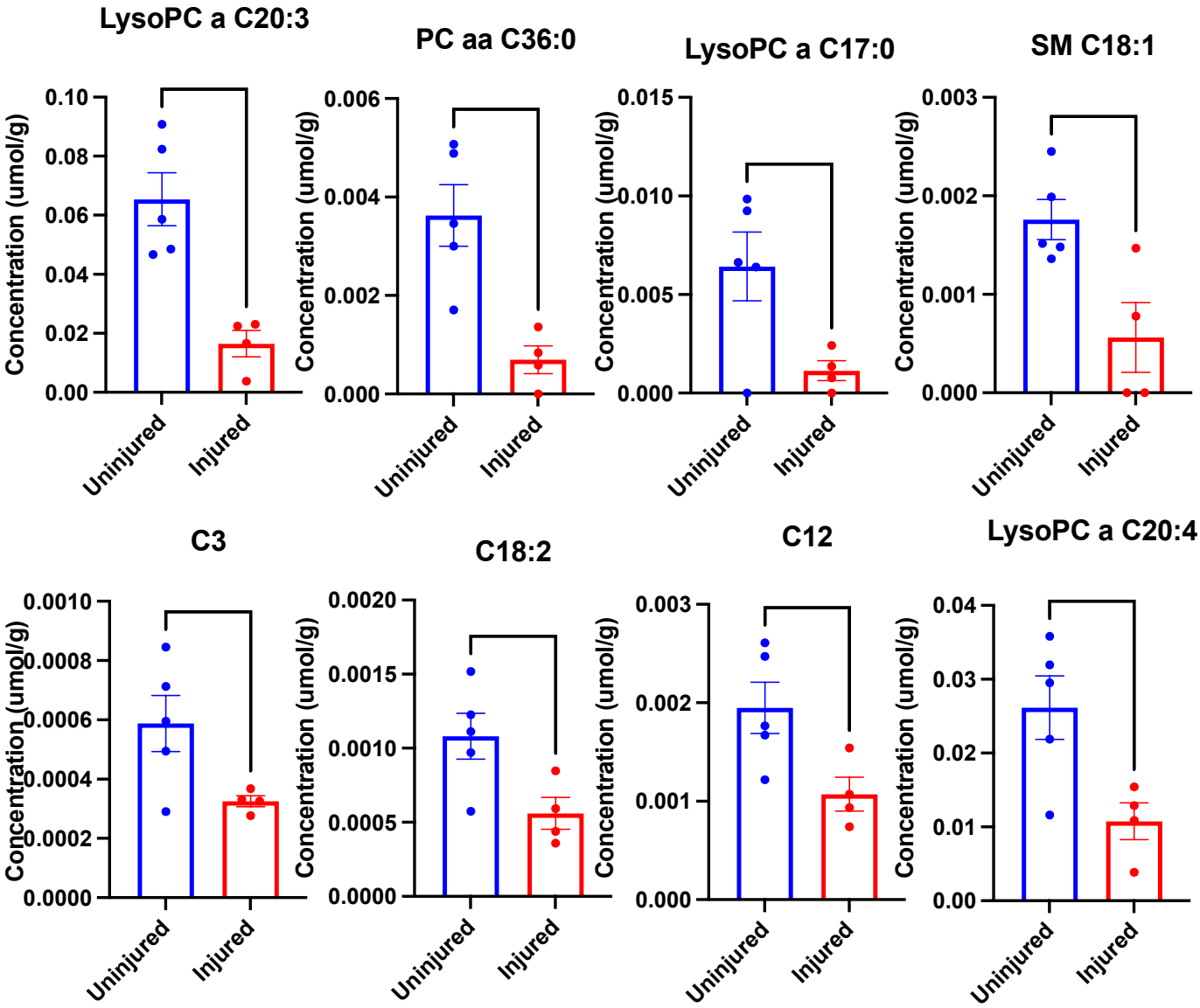
Figure 7

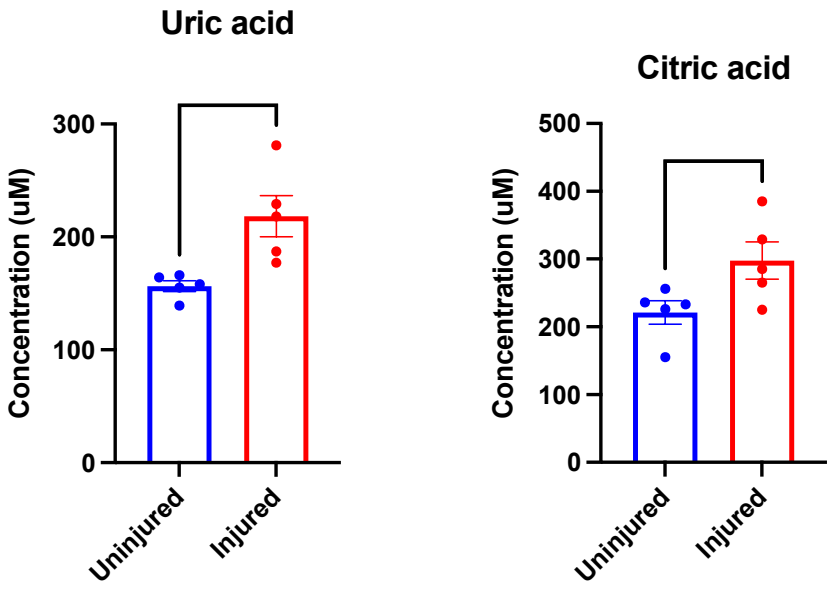

A
Scores Plot
injured
uninjured
PC 2 (19 %)
PC 1 (21.6 %)
B
Volcano plot: injured vs uninjured
NS
Sig. Down
Proline
Glucose
Butyric acid
trans-Hydroxyproline
Asymmetric dimethylarginine
Propionic acid
C5DC
Methylhistidine
C16
C16:2OH
C18
-Log10(P-value)
Log2(Fold Change)
C
Butyric acid
Concentration (umol/g)
Uninjured
Injured
E
Proline
G
Spermidine
D
Propionic Acid
F
Trans-hydroxyproline
H
Spermine

Supplement


Scores Plot
injured
uninjured
PC 2 (18.2 %)
PC 1 (39 %)


Volcano plot: injured vs uninjured
NS
Sig. Down
LysoPC a C20:3
PC aa C36:0
LysoPC a C20:4
C12
SM C18:1
C18:2
LysoPC a C17:0
C3
-Log10(P-value)
Log2(Fold Change)


LysoPC a C20:3
PC aa C36:0
LysoPC a C17:0
SM C18:1
C3
C18:2
C12
LysoPC a C20:4
Concentration (umol/g)
Uninjured
Injured


Uric acid
Concentration (uM)
300
200
100
0
Uninjured
Injured
Citric acid
Concentration (uM)
500
400
300
200
100
0
Uninjured
Injured

Supplement

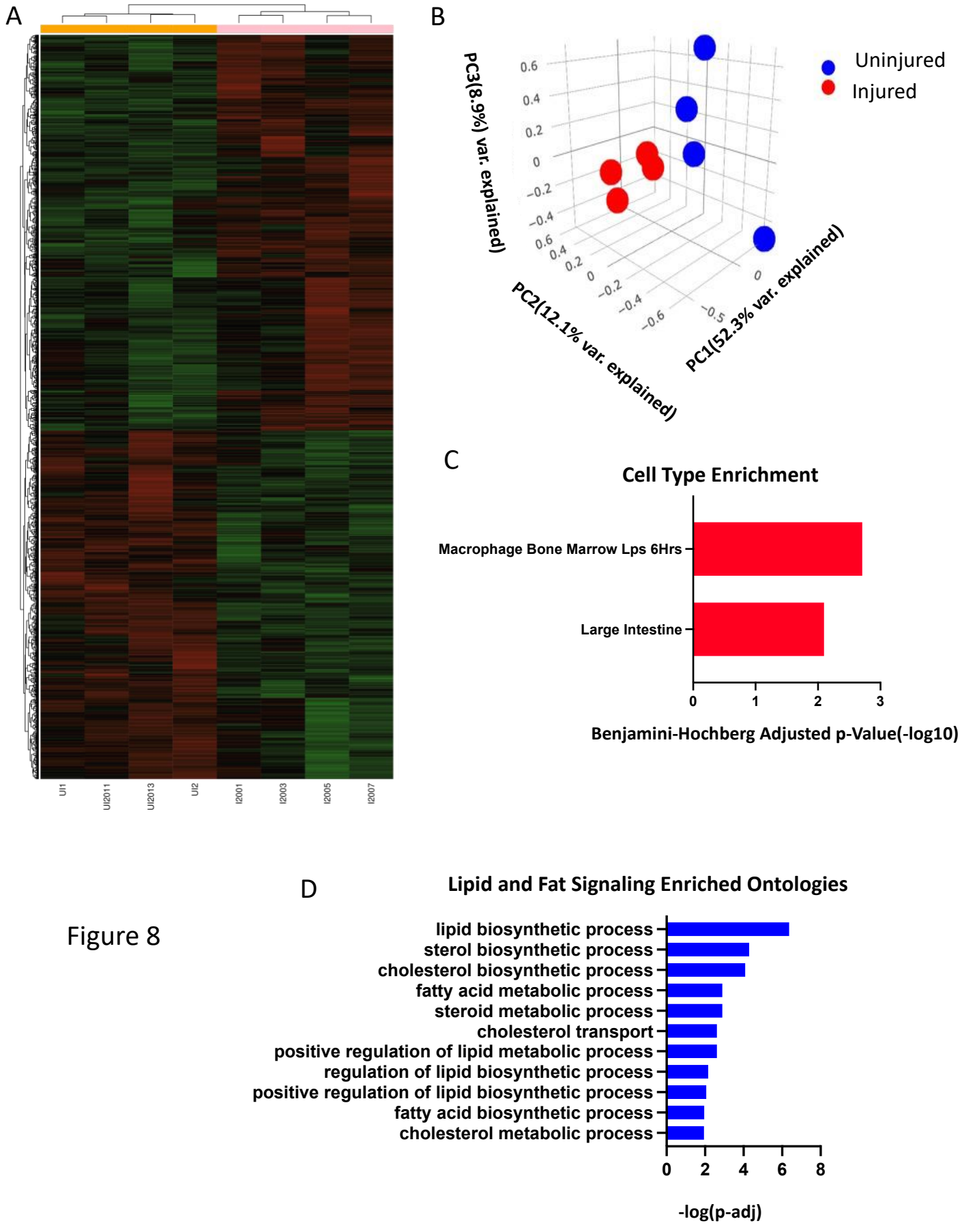

A
UI1
UI2011
UI2013
UI2
I2001
I2003
I2005
I2007
B
Uninjured
Injured
PC3(8.9%) var. explained)
PC2(12.1% var. explained)
PC1(52.3% var. explained)
C
Cell Type Enrichment
Macrophage Bone Marrow Lps 6Hrs
Large Intestine
Benjamini-Hochberg Adjusted p-Value(-log10)
D
Lipid and Fat Signaling Enriched Ontologies
lipid biosynthetic process
sterol biosynthetic process
cholesterol biosynthetic process
fatty acid metabolic process
steroid metabolic process
cholesterol transport
positive regulation of lipid metabolic process
regulation of lipid biosynthetic process
positive regulation of lipid biosynthetic process
fatty acid biosynthetic process
cholesterol metabolic process
-log(p-adj)

Figure 8

Dietary Supplementation with Hydrolyzed Hyaline Cartilage Mitigates Posttraumatic Osteoarthritis: Potential Role of Shifts in Gut Microbiome

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INTRODUCTION

Osteoarthritis (OA) is a leading cause of disability globally. Symptom palliation is the only option for treatment as there is no available disease-modifying therapy. It has recently been suggested that changes in the gut microbiome dysbiosis can influence OA progression ; in fact, the chondroprotective effects of various nutraceuticals, including dietary supplements comprised of cartilage components, may be due to their ability to shift the gut microbiome composition[1,2].

AIM

One supplement in particular, known as hydrolyzed hyaline cartilage (hHC), has anecdotally been identified as joint protective. However, the exact mechanism of this protection and the potential involvement of the gut microbiome is yet to be explored. This study aimed to investigate the basis for joint protection conferred by hHC in OA.

METHOD

- Posttraumatic OA (PTOA) was surgically induced via destabilization of the medial meniscus in male C57BL/6J mice consuming a defined diet from OpenSource (D1245H)
- Injured mice were provided a daily oral supplement of hHC (0.62mg/gm body weight) or vehicle, beginning 2 weeks prior to injury
- Three- and 12-weeks post-injury, knee joints were harvested, fixed, embedded in paraffin and cut sections were stained with Safranin O
- Histomorphometry analyses were performed to measure the area of femur and tibial cartilages
- TNF immunohistochemistry was performed to study joint inflammation and TUNEL staining was performed to assess chondrocyte apoptosis
- Fecal material was also collected to support analysis of the gut microbiome via 16S rDNA sequencing

RESULTS

Histomorphometry revealed that hHC-supplemented mice had more tibial and femoral uncalcified cartilage at both 3- and 12-week post-injury (Figure 1a and b). These results may be related to suppression of inflammation in the hHC cohort, which displayed a trend toward reduced synovial TNF (Figure 2). This paralleled a reduction in chondrocyte apoptosis in hHC-supplemented mice (Figure 3). Suggesting a potential mechanistic association, hHC supplemented mice displayed significant shifts in the gut microbiome that included loss of proinflammatory Peptococcaceae family members, particularly the species rc4-4 (Figure 4).

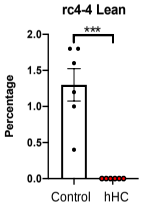

Figure 4: hHC supplemented mice displayed significant shifts in the gut microbiome that included loss of the proinflammatory species rc4-4

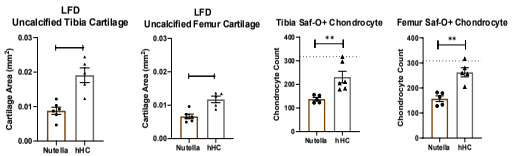

Figure 1a: Cartilage area was evaluated using Osteomeasure System to determine the tibial /femoral uncalcified cartilage area and the total Safranin O-positive chondroctyes in the tibial and femoral cartilage at 12 weeks post-DMM

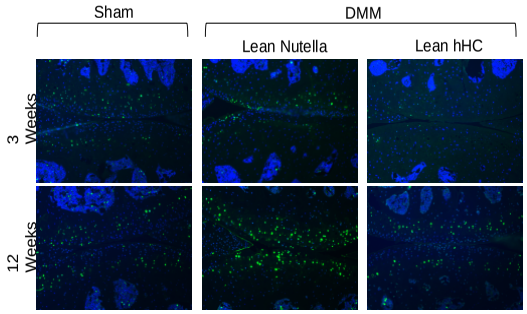

Figure 3: 200x Sagittal sections from the medial compartment of sham and DMM joints 3 weeks and 12 weeks post-DMM were prepared and apoptotic nuclei (green) were identified via TUNE: staining.

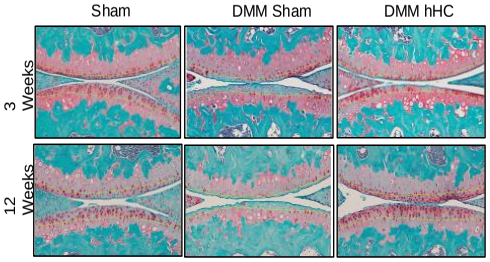

Figure 1b: Array of representative 200x Safranin O/Fast Green stained sagittal sections from the medial compartment of sham and DMM joints 3 weeks and 12 weeks post-DMM

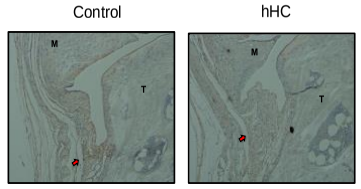

Figure 2: Array of representative 200x TNF immunohistochemistry study, focusing at the synovial located at the junction between tibial plateau (T) and the meniscus (M). Red arrows represent TNF expression positivity

CONCLUSIONS

Findings suggest that oral dietary supplementation with hHC confers joint protective effects in PTOA as well as parallel alterations in the gut microbiome. This sets the stage for follow-on study of the potential mechanistic link between the microbial shifts induced by hHC and its ability to support chondroprotection.

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2. Favazzo LJ, Hendesi H, Villani DA, Soniwala S, Dar QA, Schott EM, et al. The gut microbiome-joint connection: implications in osteoarthritis. Curr Opin Rheumatol. 2020;32(1):92-101.

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