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TITLE: Ghrelin Signaling Regulates Microbiome-Gut-Brain Axis in Inflammatory Bowel Disease and Post-Traumatic Stress Disorder

PRINCIPAL INVESTIGATOR: Chia Shan Wu

CONTRACTING ORGANIZATION: Texas A&M AgriLife Research

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14. ABSTRACT
Gut microbiota is a critical regulator of host's metabolism, immune system and cognitive function. However, the microbiome-gut-brain axis has not been systematically studied in Inflammatory Bowel Disease (**IBD**), much less in Post-traumatic stress disorder (**PTSD**). A novel experimental "2-hit" model (**IBD-PTSD**) is established, where mice are subjected to dextran sulfate sodium (**DSS**)-induced ulcerative colitis and then conditioned fear (**CF**) memory test, to study the role of microbiome-gut-brain axis in these inflammatory pathologies. In previous research period, we found that DSS-induced colitis led to contextual memory deficit in female but not in male mice, even when colitis-associated disease symptoms such as diarrhea and rectal bleeding have subsided. To confirm this sexual dimorphism in inflammatory response, we repeated the experiments and found that the previous negative data in male mice were likely due to technical issues (cage change disturbances during memory consolidation phase). Molecular characterization showed prolonged neuroinflammation and astrogliosis in the mice that had been exposed to DSS. New preliminary data showed that ghrelin attenuated DSS-induced colitis, potentially through PPARγ pathway to regulate intestinal homeostasis.

15. SUBJECT TERMS
Inflammatory Bowel Disease (IBD); Posttraumatic stress disorder (PTSD); Ulcerative colitis; Gut microbiome, Ghrelin

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1. **INTRODUCTION:** *Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.*

Clinical and pre-clinical data pin-point inflammation as the main disease condition underlying inflammatory bowel disease (IBD) and Post-traumatic stress disorder (PTSD), which predisposes the subject to further inflammatory pathologies. The central hypothesis of the current research is that gut microbiota dysbiosis is the unifying factor contributing to pro-inflammatory pathologies underlying IBD and PTSD. Hence, agents that promote rebalancing of the microbiome could ameliorate disease symptoms. A novel experimental model of IBD (dextran sulfate sodium-induced ulcerative colitis) followed by Pavlovian fear conditioning and fear recall testing (referred to as IBD-PTSD paradigm) will be established and used test the hypothesis as well as the therapeutic potential of the gut hormone ghrelin.

2. **KEYWORDS:** *Provide a brief list of keywords (limit to 20 words).*

Inflammatory Bowel Disease (IBD); Posttraumatic stress disorder (PTSD); Gut microbiome, Ulcerative colitis; Ghrelin.

3. **ACCOMPLISHMENTS:** *The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.*

What were the major goals of the project?

List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

Major goals:

- **Aim 1.** Define the dynamic and temporal changes in microbiota composition, metabolomics, inflammation and fear memory in experimental IBD-PTSD. (**Year 1**)
- **Aim 2.** Evaluate the ability of ghrelin to ameliorate experimental IBD-PTSD. (**Year 2**)

Updated Statement of Work for Year 1 (updates in the "Progress" column).

Specific Aim 1: *Define the dynamic and temporal changes in microbiota composition, metabolomics, inflammation and fear memory in experimental IBD-PTSD*	**Proposed Timeline**	**Progress**	**Site 1**
Major Task 1: *in vivo* experiment 1	Months	Calendar	
Local IRB/IACUC Approval	1-2	Approved on 01/17/2020	
ACURO Approval	2-3	Approved on 03/18/2020	
Subtask 1: Breeding of C57BL/6 mice for experimental group	3-4	Jun-Jul	Dr. Wu (n=40)
Subtask 2: Switch to open source diets and monitor body weight and body composition until mice reach 12 weeks of age.	4-6	Jul-Sep	Dr. Wu
Subtask 3: Perform animal experiment DSS-fear conditioning, sample collection	7-8	Oct-Dec	Dr. Wu
Milestone(s) Achieved: completion of sample and data collection from *in vivo* experiment 1.	8	Dec	

Major Task 2: molecular and biochemical characterization			
Subtask 1: fecal microbiome characterization	9-12	planned	Dr. Wu and genomics core facility
Subtask 2: fecal metabolome characterization	9-12	planned	Dr. Wu and metabolomics core facility
Subtask 3: multiplex analysis of serum cytokines and gut hormones, plasma acyl-ghrelin levels	9-12	50%	Dr. Wu
Subtask 4: Molecular characterization of colons: histology, qPCR	9-12	50%	Dr. Wu
Subtask 5: Molecular characterization of brain: histology, qPCR	9-12	80%	Dr. Wu
Milestone(s) Achieved: completion of sample processing and molecular characterization work. Completion of data analysis.	12	delayed	

Updated Statement of Work for **Year 2** (updates in the "Progress" column).

Specific Aim 2: *Evaluate the ability of ghrelin to ameliorate experimental IBD-PTSD*	**Proposed Timeline**	**Progress**	**Site 1**
Major Task 3: *in vivo* experiment 2			
Subtask 1: Breeding of C57BL/6 mice for experimental group	1-2	Jan-Feb	Dr. Wu (n=50)
Subtask 2: Switch to open source diets and monitor body weight and body composition until mice reach 12 weeks of age.	2-4	Feb-Apr	undergrads
Subtask 3: Perform animal experiment DSS-fear conditioning, ghrelin injection, sample collection	5-6	May-Jun	Dr. Wu and undergrads
Milestone(s) Achieved: completion of sample and data collection from *in vivo* experiment 2.	7	(50% complete;) Will generate more cohorts in extended year)	
Major Task 4: molecular and biochemical characterization			
Subtask 1: fecal microbiome characterization	9-12	planned for extended year	Dr. Wu and genomics core facility
Subtask 2: fecal metabolome characterization	8-9	Planned for extended year	Dr. Wu and metabolomics core facility

Subtask 3: multiplex analysis of serum cytokines and gut hormones, plasma acyl-ghrelin levels	9-12	50% complete	Dr. Wu and undergrads
Subtask 4: Molecular characterization of colons: histology, qPCR	9-12	50% complete	Dr. Wu and undergrads
Subtask 5: Molecular characterization of brain: histology, qPCR	9-12	underway	Dr. Wu and undergrads
Milestone(s) Achieved: completion of sample processing and molecular characterization work. Completion of data analysis.	12	Delayed (No-cost extension to year 3)	

What was accomplished under these goals?

For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

The COVID-19 pandemic has significantly impacted the progress of research work; as a result, a no-cost extension has been requested and approved. The PI continued to perform most of the research activities, with assistance from undergraduate students under the PI's direction.

In this reporting period (03/01/2021-02/28/2022), the following research activities had been carried out, while adhering to social distancing and safety guidelines for COVID:

- Completed an additional cohort of male mice in the IBD-PTSD model.
  - In previous reporting period, preliminary data showed that in the remission period after an active episode of colitis, hippocampus-dependent contextual fear memory was significantly decreased in the colitis-exposed female mice but not in male mice. However, further review of the experimental records indicated that there might have been technical issues with the experiment in male mice, as caretakers of the mice colonies had disturbed the mice during the memory consolidation period. Female mice were done in separate time and did not experience this interruption. Therefore, we repeated the experiment in an additional cohort of male mice. New data showed that hippocampus-dependent contextual fear memory was significantly decreased in the colitis-exposed male mice, consistent with female data.
- Molecular analysis of colitis-induced neuroinflammation in the IBD-PTSD model:
  - Downstream analyses were carried out to assess neuroinflammation in the hippocampal regions. New data showed that DSS-exposed mice, at Day 17, had marked signs of astrogliosis (increased GFAP-labeled cells) in the hippocampal regions compared to control (**Figure 1A**). Consistently, there were time-dependent increases in the expression of inflammatory genes *Nfkb*, *Trem2* (microglial marker), *IL-1b*, and *GFAP* (astrocyte marker). Interestingly, expression of *Nfkb* and *Trem2* subsided at Day 42, while *IL-1b* and *GFAP* expression levels remained significantly elevated (**Figure 1B**).
  - Further molecular analysis is underway to characterize reactive astrocytes in the hippocampal regions in response to peripheral inflammation.

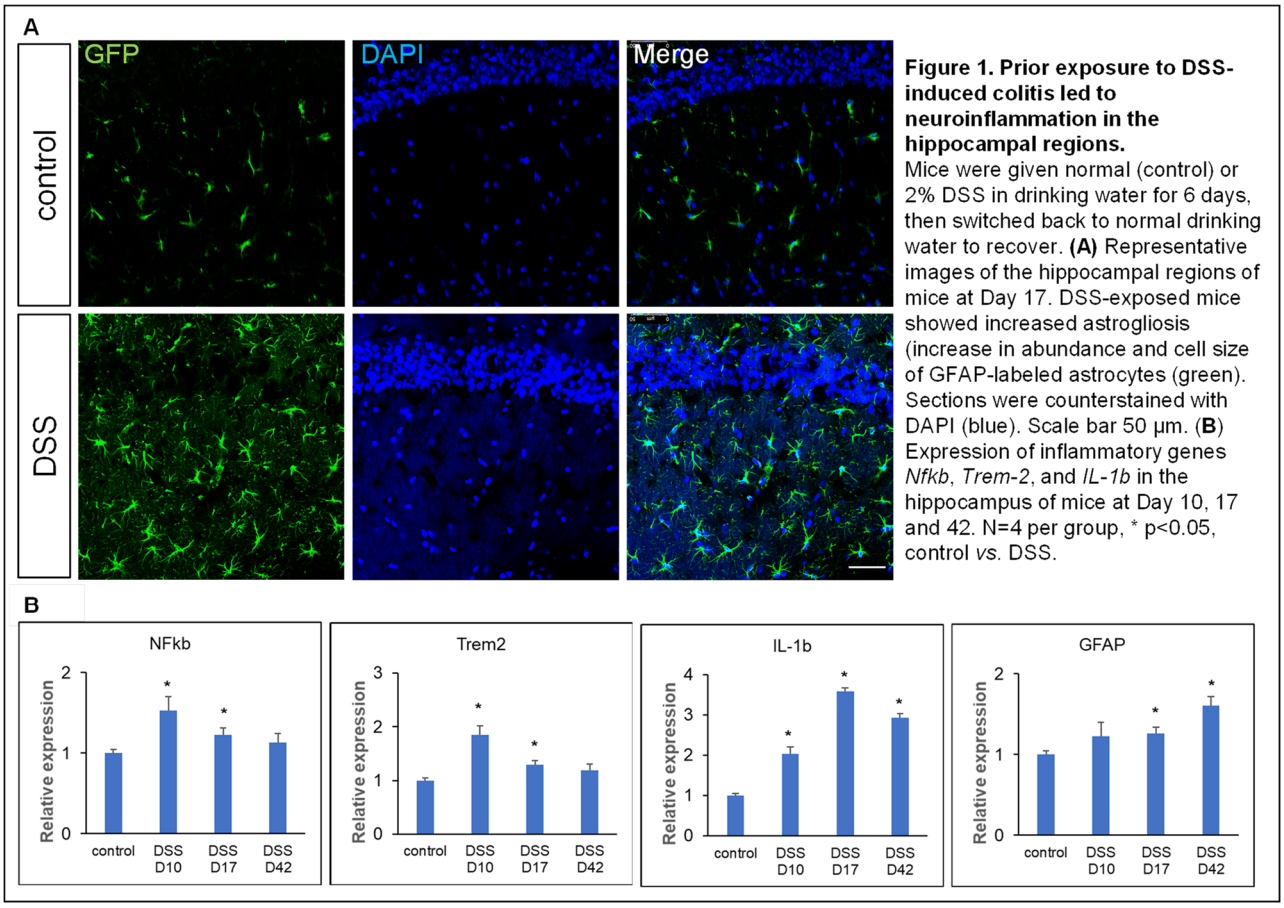


Figure 1. Prior exposure to DSS-induced colitis led to neuroinflammation in the hippocampal regions. Mice were given normal (control) or 2% DSS in drinking water for 6 days, then switched back to normal drinking water to recover. **(A)** Representative images of the hippocampal regions of mice at Day 17. DSS-exposed mice showed increased astrogliosis (increase in abundance and cell size of GFAP-labeled astrocytes (green). Sections were counterstained with DAPI (blue). Scale bar 50 µm. (**B**) Expression of inflammatory genes *Nfkb*, *Trem-2*, and *IL-1b* in the hippocampus of mice at Day 10, 17 and 42. N=4 per group, * p<0.05, control *vs.* DSS.

- Completed a new cohort of *in vivo* experiment, testing the effect of ghrelin in the DSS-induced colitis model:
  - Preliminary data showed that treatment of female mice with acyl-ghrelin, at a dose that we had previously shown to protect against muscle atrophy, attenuated DSS-induced colitis (**Fig. 2**).
  - Extending this finding, we also showed that ghrelin knockout (Ghrl KO) mice exhibited exacerbated disease activities including loss of body weight, fecal consistency and rectal bleeding score (**Fig. 3**).

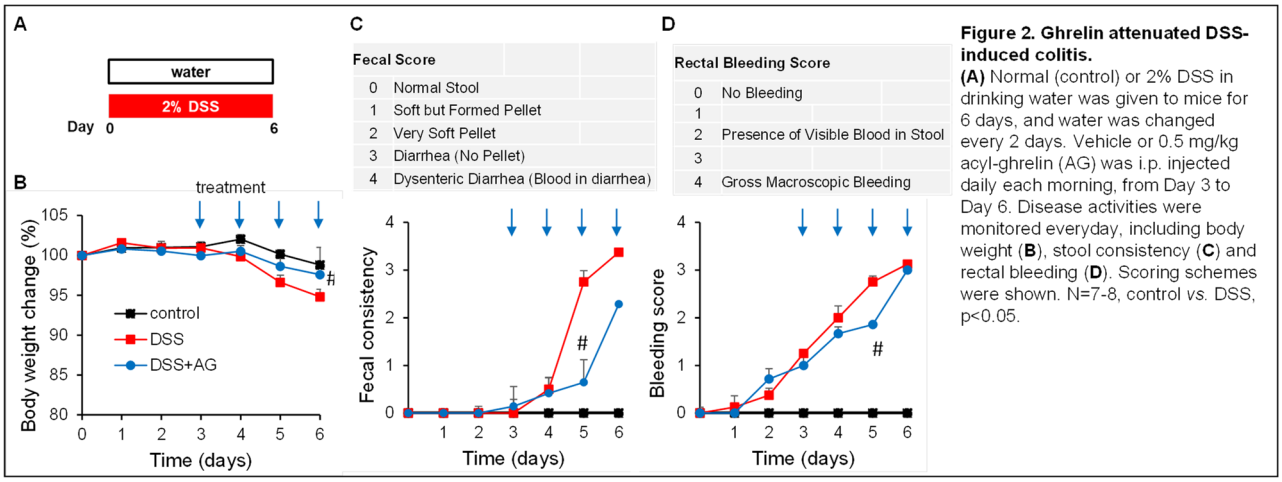


Figure 2. Ghrelin attenuated DSS-induced colitis. **(A)** Normal (control) or 2% DSS in drinking water was given to mice for 6 days, and water was changed every 2 days. Vehicle or 0.5 mg/kg acyl-ghrelin (AG) was i.p. injected daily each morning, from Day 3 to Day 6. Disease activities were monitored everyday, including body weight (**B**), stool consistency (**C**) and rectal bleeding (**D**). Scoring schemes were shown. N=7-8, control *vs.* DSS, p<0.05.

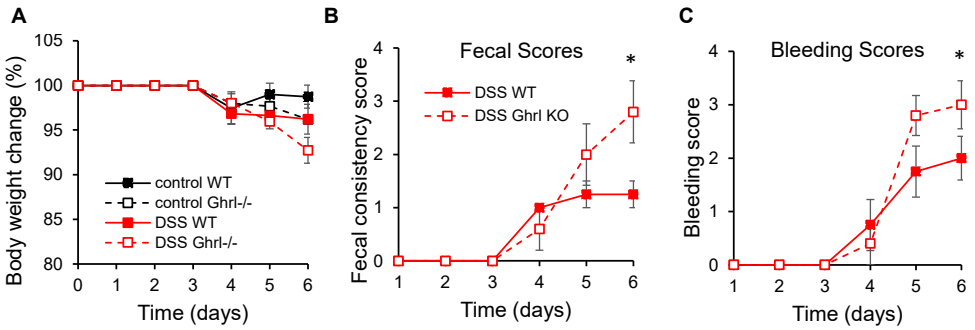


Figure 3. DSS-induced colitis in WT and Ghrl KO mice.
Normal (control) or 2% DSS in drinking water was given to mice for 6 days, and water was changed every 2 days. Disease activities were monitored everyday, including body weight (**A**), stool consistency (**B**) and rectal bleeding (**C**). N=5-6, control *vs.* DSS, $p<0.05$.

- Together, these data suggested that 1) endogenous ghrelin plays a role in intestinal homeostasis, and 2) ghrelin, at pharmacological doses that are above endogenous levels, partially protected against DSS-induced colitis.
- The protective effect of ghrelin on DSS-induced colitis seemed relatively mild. Whether this is due to down-regulation of ghrelin receptor (namely the Growth Hormone Secretagogue Receptor, GHS-R) after repeated dosages of ghrelin is unknown. Interestingly, previous collaborative work with Dr. Yuxiang Sun on global ablation of GHS-R (GHS-R KO) mice showed exacerbated DSS-induced colitis as well as gut dysbiosis in the GHS-R KO mice, in line with the Ghrl KO mice data. This work was recently published in *Int. J. Mol. Sc. 2022*.
- Fecal, serum and tissue samples have been collected. Microbiome and metabolome work are planned in the extension year. The analytical methods have been established with the respective cores in a recently published paper (*J. Nutr. Biochem*, 2022).

- In pursuing the mechanism of how ghrelin signaling functions in maintaining intestinal homeostasis, we hypothesized that the peroxisome proliferator-activated receptor gamma (**PPARγ**) in colonic epithelial cells could be one of the target pathways mediating ghrelin's effects. PPARγ has been shown to regulate epithelial oxygenation and colonization resistance to pathogenic bacteria, and to protect against experimental IBD. We set up new *in vitro* assays in the lab, and preliminary data showed that ghrelin increased oxygen consumption rate in the human colon adenocarcinoma CaCo-2 cells (**Figure 4**) and activated transcriptional activity of PPARγ (**Figure 5**).
- While it is known that epithelial oxygen levels are increased in colitis, precisely how the colonocyte metabolism is altered in disease remission and re-colonization of obligate (healthy) microbes remain largely undetermined. Ghrelin could activate intestinal epithelial PPARγ to maintain healthy colonocyte bioenergetics and low oxygen tension, thereby facilitates intestinal homeostasis for inhabitation and adaptation of the obligate microbes. We also envision that the new *in vitro* assays could be used in screening for compounds that target colonocyte metabolism, to improve host-microbe interactions in IBD.

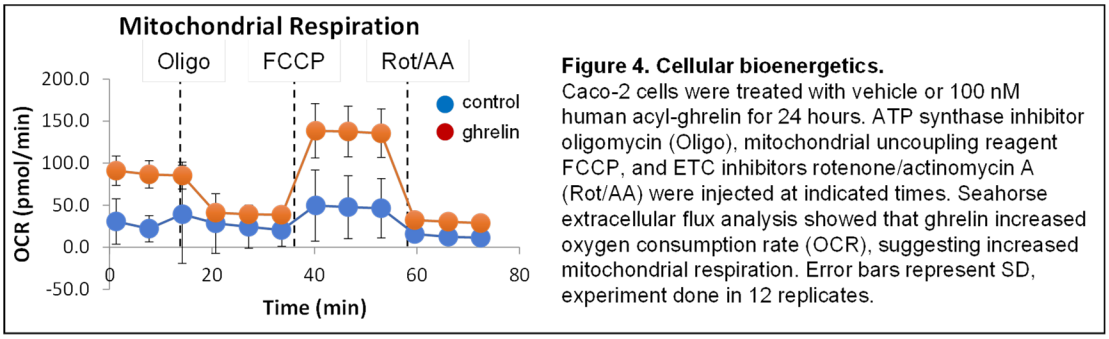


Figure 4. Cellular bioenergetics.
Caco-2 cells were treated with vehicle or 100 nM human acyl-ghrelin for 24 hours. ATP synthase inhibitor oligomycin (Oligo), mitochondrial uncoupling reagent FCCP, and ETC inhibitors rotenone/actinomycin A (Rot/AA) were injected at indicated times. Seahorse extracellular flux analysis showed that ghrelin increased oxygen consumption rate (OCR), suggesting increased mitochondrial respiration. Error bars represent SD, experiment done in 12 replicates.

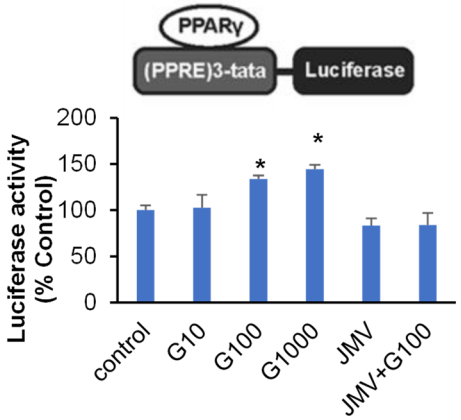


Figure 5. Ghrelin dose-dependently activated PPARγ transcription. PPARγ-reporter assay showing activation of PPARγ by ghrelin in human colon adenocarcinoma Caco-2 cells. PPRE-luciferase plasmids were transiently transfected into Caco-2 cells for 48 hours, cells were then treated for 2 hours with 10, 100 or 1000 nM of ghrelin. JMV: 3 uM of GHS-R antagonist JMV 2959. Error bars represent SEM, experiment done in triplicates, repeated 2 times. * p<0.05 vs. control group.

What opportunities for training and professional development has the project provided?

If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. "Training" activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. "Professional development" activities result in increased knowledge or skill in one's area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

The project had provided opportunities for undergraduate students to be trained in research activities. The PI provided one-on-one trainings on experimental work as well as manuscript preparation, developing their skills in literature search, critical thinking, and problem-solving. One of the studenst, a Cadet at Texas A&M University, will be entering medical school in Fall 2022, intending to pursue a career as military physician.

How were the results disseminated to communities of interest?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

Nothing to Report.

What do you plan to do during the next reporting period to accomplish the goals?
If this is the final report, state "Nothing to Report."

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

The current data suggest that cognitive function was negatively impacted by an active episode of colitis, with prolonged neuroinflammation in the hippocampal regions. Further molecular, biochemical and microbiome analyses are currently underway or planned to corroborate these data. Working relationships with the core facilities at Texas A&M University, including the Molecular Genomics Core and the Integrated Metabolomics Analysis Core have been established, to ensure successful completion of the project. Together with the scientists from the cores, we have recently published a paper on the effects of soluble fibers on microbiomes and adiposity in aging mice in *J. Nutr. Biochem* (see section 6 and appendices), demonstrating successful implementation of the methodologies.

4. **IMPACT:** D*escribe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:*

What was the impact on the development of the principal discipline(s) of the project?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

The preclinical data generated from this project demonstrated that peripheral inflammation, in the form of DSS-induced ulcerative colitis, could induce prolonged neuroinflammation, negatively impacting hippocampal function. These data add to the existing knowledge on the gut-brain axis, and suggest that in addition to clinical management of the symptoms of IBD, other strategies to monitor and reduce neuroinflammation may need to be considered to prevent potential progression to chronic disease conditions such as dementia or neurodegenerative diseases.

What was the impact on other disciplines?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

Nothing to Report.

What was the impact on technology transfer?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

Nothing to Report.

What was the impact on society beyond science and technology?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

Nothing to Report.

5. CHANGES/PROBLEMS: *The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:*

Experimental timeline is delayed, research plan as laid out in the original statement of work will extend 12 months to Feb-2023, due to the COVID-19 pandemic.

Actual or anticipated problems or delays and actions or plans to resolve them
Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

Due to the COVID-19 pandemic, research work has been disrupted and delayed. These delays have significantly impacted on the timeline of the animal experiments and subsequent downstream analyses.

Changes that had a significant impact on expenditures
Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

Due to the COVID-19 pandemic, research work has been disrupted and delayed. These delays have significantly impacted on the timeline of the animal experiments and subsequent downstream analyses, as well as hiring staff. Consequently, a no-cost extension has been requested and approved.

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

Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Significant changes in use or care of human subjects

Nothing to Report.

Significant changes in use or care of vertebrate animals

Nothing to Report.

Significant changes in use of biohazards and/or select agents

Nothing to Report.

6. **PRODUCTS:** *List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."*

- **Publications, conference papers, and presentations**
  Report only the major publication(s) resulting from the work under this award.

  Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

  1. Muthyala, S. D.V., Shankar, S., Klemashevich, C., Blazier, J. C., Hillhouse, A., **Wu, C.-S.** Differential Effects of the Soluble Fiber Inulin in Reducing Adiposity and Altering Gut Microbiome in Aging Mice. Journal of Nutritional Biochemistry, 2022, online preprint published (https://doi.org/10.1016/j.jnutbio.2022.108999); federal support acknowledged.

  2. Tuchaai, E., Endres, V., Jones, B., Shankar, S., Klemashevich, C., Sun, Y., **Wu, C.-S.** Deletion of ghrelin alters tryptophan metabolism and exacerbates experimental ulcerative colitis in aged mice. Experimental Biology and Medicine, 2022, under review; federal support acknowledged.

Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Nothing to Report.

Other publications, conference papers and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

Nothing to Report.

- **Website(s) or other Internet site(s)**
  List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

Nothing to Report.

- **Technologies or techniques**
  Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

Nothing to Report.

- **Inventions, patent applications, and/or licenses**
  Identify inventions, patent applications with date, and/or licenses that have resulted from the research. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

Nothing to Report.

- **Other Products**

  Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:

  - *data or databases;*
  - *physical collections;*
  - *audio or video products;*
  - *software;*
  - *models;*
  - *educational aids or curricula;*
  - *instruments or equipment;*
  - *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
  - *clinical interventions;*
  - *new business creation; and*
  - *other.*

Nothing to Report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change".

Example:

Name:	*Mary Smith*
Project Role:	*Graduate Student*
Researcher Identifier (e.g. ORCID ID):	*1234567*
Nearest person month worked:	*5*
Contribution to Project:	*Ms. Smith has performed work in the area of combined error-control and constrained coding.*
Funding Support:	*The Ford Foundation (Complete only if the funding support is provided from other than this award.)*

Name:	*Chia Shan Wu*
Project Role:	*PI*
Researcher Identifier (e.g. ORCID ID):	*ORCID ID: 0000-0002-6034-939X*
Nearest person month worked:	*5*
Contribution to Project:	*Dr. Wu has performed all work described in this reporting period.*
Funding Support:	*Dr. Wu is also supported by AG061726A (R21), National Institute on Aging.*
Name:	*Brock Jones*
Project Role:	*Undergraduate student*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*1*
Contribution to Project:	*Brock has performed some of the research activities described in this reporting period.*
Funding Support:	*Not applicable.*
Name:	*Valerie Endres*
Project Role:	*Undergraduate student*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*1*
Contribution to Project:	*Valerie has performed some of the research activities described in this reporting period.*
Funding Support:	*Not applicable.*

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

Nothing to Report.

What other organizations were involved as partners?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial

or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.

Provide the following information for each partnership:
Organization Name:
Location of Organization: (if foreign location list country)
Partner's contribution to the project (identify one or more)

- *Financial support;*
- *In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);*
- *Facilities (e.g., project staff use the partner's facilities for project activities);*
- *Collaboration (e.g., partner's staff work with project staff on the project);*
- *Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and*
- *Other.*

Nothing to Report.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: *For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to* ***https://ebrap.org/eBRAP/public/index.htm*** *for each unique award.*

QUAD CHARTS: *If applicable, the Quad Chart (available on https://www.usamraa.army.mil/Pages/Resources.aspx) should be updated and submitted with attachments.*

9. APPENDICES: *Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.*

Award chart, Quad chart and pre-proof of journal article are attached to the report.

W81XWH-20-1-0127: Ghrelin Signaling Regulates Microbiome-Gut-Brain Axis in Inflammatory Bowel Disease and Post-Traumatic Stress Disorder

PI: Chia Shan Wu, Texas A&M AgriLife Research, Texas

Budget: $301,528.00

Topic Area: Inflammatory Bowel Diseases

Mechanism: W81XWH-19-PRMRP-DA

Research Area(s): SCS Coding

Award Status: Period of Performance
01 March 2020 – 28 February 2023

Study Goals:

Accumulating evidence indicate that gut microbiota dysbiosis promote systemic inflammation, which may be the unifying factor contributing to the inflammatory pathologies in inflammatory bowel disease (IBD), which in turn induces neuroinflammation and cognitive dysfunction. In addition, meta-analysis of clinical data suggests that patients with posttraumatic stress disorder (PTSD) exhibit an increased state of inflammation and are at greater risk of developing IBD; however, whether IBD patients are at greater risk of developing PTSD, and whether gut microbiota dysbiosis associated with IBD contribute to the inflammatory pathology in PTSD remain undetermined. Ghrelin, a 28 a.a. peptide hormone produced mainly by the stomach and gut, has been suggested to exert anti-inflammatory effects. Consistently, ghrelin deficiency leads to increased susceptibility to diet-induced adipose tissue inflammation, exacerbated fasting-induced muscle atrophy, and a pro-inflammatory shift in gut microbiota composition. The central hypothesis is that ghrelin contributes to gut barrier function and promotes microbiota symbiosis, thereby protecting the animal to inflammatory pathologies associated with IBD and subsequent PTSD. A novel experimental model of IBD (dextran sulfate sodium-induced ulcerative colitis) followed by Pavlovian fear conditioning and fear recall testing (referred to as IBD-PTSD paradigm) will be established and used test the hypothesis.

Specific Aims:

- Aim 1. Define the dynamic and temporal changes in microbiota composition, metabolomics, inflammation and fear memory in experimental IBD-PTSD.
- Aim 2. Evaluate the ability of ghrelin to ameliorate experimental IBD-PTSD.

Key Accomplishments and Outcomes:

Publications: 1

Patents: none to date

Funding Obtained: none to date

Ghrelin Signaling Regulates Microbiome-Gut-Brain Axis in Inflammatory Bowel Disease and Post-Traumatic Stress Disorder
PR192467
W81XWH2010127

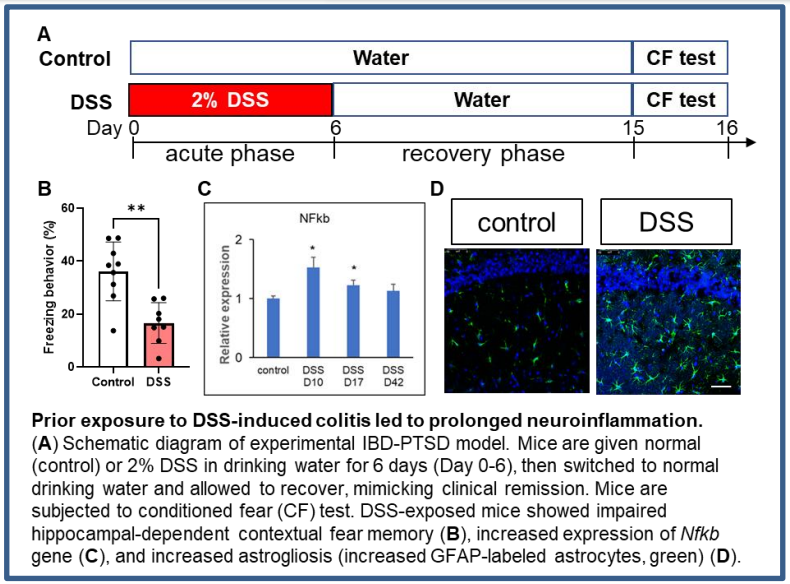
PI: Chia Shan Wu **Org:** Texas A&M AgriLife Research **Award Amount:** $301,528.00

Study/Product Aim(s)

- Aim 1. Define the dynamic and temporal changes in microbiota composition, metabolomics, inflammation and fear memory in experimental IBD-PTSD.
- Aim 2. Evaluate the ability of ghrelin to ameliorate experimental IBD-PTSD.

Approach

Gut microbiota is a critical regulator of host's metabolism, immune system and cognitive function. However, the microbiome-gut-brain axis has not been systematically studied in Inflammatory Bowel Disease (**IBD**), much less in Post-traumatic stress disorder (**PTSD**). A novel experimental "2-hit" model (**IBD-PTSD**) will be established, where mice are subjected to dextran sulfate sodium (**DSS**)-induced ulcerative colitis and then conditioned fear (**CF**) memory test, to study the role of microbiome-gut-brain axis in these inflammatory pathologies.



Prior exposure to DSS-induced colitis led to prolonged neuroinflammation. (**A**) Schematic diagram of experimental IBD-PTSD model. Mice are given normal (control) or 2% DSS in drinking water for 6 days (Day 0-6), then switched to normal drinking water and allowed to recover, mimicking clinical remission. Mice are subjected to conditioned fear (CF) test. DSS-exposed mice showed impaired hippocampal-dependent contextual fear memory (**B**), increased expression of *Nfkb* gene (**C**), and increased astrogliosis (increased GFAP-labeled astrocytes, green) (**D**).

Timeline and Cost

Activities CY	20	21	22	23
In vivo IBD-PTSD model (Aim 1)				
Molecular analyses (Aim 1)				
Ghrelin treatment (Aim 2)				
Molecular analyses (Aim 2)				
Estimated Budget ($K)	**$147K**	**$154K**	**$000**	**$000**

Updated: 03/29/2022

Goals/Milestones

CY20 Goal – IBD-PTSD model
☑ In vivo DSS-induced colitis followed by conditioned fear tests.
☐ Molecular analyses: microbiome, metabolome, inflammation.
Delayed due to COVID-19, 50% complete.

CY21 Goals – Test therapeutic potential of ghrelin
☑ In vivo evaluation of ghrelin's effects in IBD-PTSD.
☐ Molecular analyses: microbiome, metabolome, inflammation.

CY22 Goal – Completion of molecular analyses
☐ Molecular analyses: microbiome, metabolome, inflammation.

Comments/Challenges/Issues/Concerns

- Timeline is delayed, experimental plan will extend 12 months to Feb-2023.

Budget Expenditure to Date
Projected Expenditure: $301,528.
Actual Expenditure: $193,711.

Differential Effects of the Soluble Fiber Inulin in Reducing Adiposity and Altering Gut Microbiome in Aging Mice

Sai Deepak Venkata Muthyala , Smriti Shankar , Cory Klemashevich , John C. Blazier , Andrew Hillhouse , Chia-Shan Wu

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Highlights

- Mice at middle age was most responsive to fat-reducing effects of inulin.
- Inulin intake significantly increased energy expenditure and voluntary wheel running activities in middle-aged mice, but not in old mice.
- The efficacy of inulin in SCFA production, and the subsequent metabolic response was diminished in older mice.

Differential Effects of the Soluble Fiber Inulin in Reducing Adiposity and Altering Gut Microbiome in Aging Mice

Sai Deepak Venkata Muthyala[1], Smriti Shankar[2], Cory Klemashevich[2], John C. Blazier[3], Andrew Hillhouse[3], Chia-Shan Wu[1]

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Key words: *short-chain fatty acids, gut microbiome, aging, metabolism, dietary fiber, inulin*

Running title: *Age-dependent differential response to inulin*

Abstract

Inulin, a soluble dietary fiber, is thought to exert multiple beneficiary effects through promoting growth of bacteria that metabolize the fiber to short-chain fatty acids (SCFAs); however, the effect and efficacy of inulin in aging subjects is unknown. This study aims to systematically evaluate the capacity of SCFAs production and host response in mice of different ages. Male C57BL/6J mice across young (5 months), middle (11 months) and old (26 months) age were subjected to a control diet for two weeks, followed by 6 weeks of inulin-containing diet. Inulin-induced increase in fecal butyric acid levels was most prominent in middle-age group compared to other age groups. In addition, inulin-induced increase in fecal propionic acids showed age-dependent decline. Interestingly, the SCFA-producing *Roseburia* was most abundantly and persistently increased in the middle-age group. Furthermore, inulin intake significantly reduced Firmicutes to Bacteroidetes ratio, and several dysbiotic bacteria associated with pro-inflammatory state. Concomitantly, circulating levels of CXCL1, a chemoattractant for neutrophils, was reduced by inulin intake. Inulin decreased fat mass in all age groups, with middle-aged mice being most responsive to fat-reducing effects of inulin. Moreover, inulin significantly increased energy expenditure and voluntary wheel running in middle-aged mice, but not in old mice. Overall, our data suggest that the efficacy of inulin in altering the microbiome and SCFA production, and the subsequent metabolic response was diminished in old mice, and highlight the importance of including age as a variable in studies determining host-microbe response to diets.

1. Introduction

The gut microbiome has coevolved as a vital determinant of host health, and imbalance in the gut microbiome, the so called microbiota dysbiosis, has been linked to multiple diseases [1-4]. Microbiota enhance and develop the host immune response, confer resistance to infection, and are emerging as key drivers of host metabolism. Gut microbiota synthesize nutrients and metabolites that exert diverse and significant effects on host health. [5]. Hence, gut microbiota functions as an endocrine organ and microbially produced metabolites are crucial executors of diet-based microbial influence on the host [6-8]. On the other hand, the composition, health, and functionality of the gut microbiota can also be influenced by the host [9]. The bi-directional microbiota-host relationship is of great interest for its significance to human health and disease.

Accumulating evidence suggest that leaky gut and microbiota/metabolites are factors contributing to age-associated inflammation [3, 10-13]. In addition to pro-inflammatory shifts in gut microbiota with aging, lack of dietary fiber has been suggested to increase the risk of developing chronic inflammatory diseases [14-16], while supplementation of diets with fiber might offer an array of health promoting benefits [17]. Inulin, a soluble, fermentable dietary fiber, is thought to exert anti-inflammatory effects through promoting growth of bacteria that metabolize the fiber to short-chain fatty acids (SCFA), especially butyrate [18, 19]. Clinical studies have shown that high dietary fiber intake is associated with a lower body weight, as a result of reduced appetite; however, systemic review indicated that dietary fiber intake enhanced satiety in only 39% of all published studies [20]. The production of SCFAs, which are formed from dietary soluble

fiber due to intestinal fermentation, is suggested to contribute to the weight-loss effect. Indeed, numerous studies in animal models have shown protective effects of inulin against diet-induced obesity and the associated metabolic syndrome [21-25]. Whether inulin supplement is equally effective in providing these beneficial effects across different ages is currently unknown.

The goal of the current study was to ~~systematically evaluate the capacity of SCFA production in mice of different ages when given dietary inulin supplement, and its impact on metabolism and inflammation.~~ assess the effects and efficacy of dietary soluble fiber supplementation to aging mice, given the well-known age-dependent structural changes in gut microbiome and the likely reduced capacity to produce SCFAs in older mice. Three representative ages across the lifespan of the inbred mouse strain C57BL/6J were chosen: 5-, 11- and 26 months of age, corresponding to ~25, 40, and 75 years of age in human [26]. We investigated the effects of inulin supplementation in these mice on multiple parameters including gut microbiome composition and SCFA production, food intake, body composition, metabolic parameters, and aging-associated pro-inflammatory cytokines,.

2. Materials and Methods

2.1 Animals

Male C57BL/6J mice were used in the study. Animals were housed in groups of 3-5 under controlled temperature and lighting (72 ± 2° F; 12-hour light–dark cycle, lights on at 6:00 AM), at the Jackson Laboratory. Mice were maintained on 5K0Q (with 22% of

calories from protein, 16% from fat and 62% from carbohydrates, LabDiet), and never exposed to other diet interventions prior to the initiation of the current study. This diet contains 4.3% crude fiber; dietary fiber in the chow diet is derived from a variety of plants including ground wheat, ground corn, wheat middlings, ground oats, dehulled soybean meal, corn gluten meal and dehydrated alfalfa meal. The composition of this diet is now provided in Supplementary Table 1. All *in vivo* experiments were performed at the Jackson Laboratory, and approved by the Animal Care Research Committee of the Jackson Laboratory.

2.2 Experimental design

Male C57BL/6J mice at 3 different ages were used in the study: 5, 11 and 26 months (M) of age (n = 7, 8 and 5, respectively). Mice were switched to a semi-purified, irradiated regular diet (RD; D12450j, based on the AIN-93M formula, Research Diets Inc.) for 2 weeks. After the diet washout period, all mice were switched to inulin-containing "high fiber" diet (HF, D13081108, irradiated), denoted as week 0 (Fig. 1). The diet composition of the two purified diets is provided in Table 1. Fecal samples were collected from all mice one week before diet switch to inulin-containing diet, then 1 week and 4 weeks after diet switch (week -1, 1 and 4, respectively). On the day of fecal sample collection, each mouse was placed in autoclaved cage in the morning and fresh fecal samples collected (at least 6 pellets), snap frozen and stored at -80ºC. Whole body composition was assessed by qNMR (Echo Medical Systems, Houston, TX); data from all mice were collected on week -1, 1, 3 and 5 (Fig 1). Food intake was measured per week, and the amount of food eaten were divided per number of mice per cage. ~~Fecal~~

~~pellets were collected from each mouse, flash frozen and stored at −80° C until further processing.~~ Plasma samples were collected a day before diet switch to inulin-containing diet and after 5 weeks of supplementation, in EDTA-coated tubes under fed condition in the morning between 8-10:00 AM and stored at -80°C until further analysis. All mice were subjected to indirect calorimetry in the week before diet switch to inulin supplement, and after 5 weeks of supplement (Fig. 1). Metabolic parameter data were obtained using Promethion indirect calorimetry system at Jackson Lab. For each session, mice were individually housed in Promethion metabolic cages equipped with running wheels, with *ad libitum* access to food and water, and recorded for 5 days to allow for acclimatization. Only data recorded from day 4-5 were used in the analysis. At the end of recording session, mice were returned to their home cages, with their original littermates. Fecal and plasma samples were shipped on dry ice to Texas A&M University for downstream analyses.

2.3 Fecal SCFA Levels Measurements

Levels of SCFAs (acetate, propionate, and butyrate) and branched chain fatty acids (BCFAs: isobutyrate, isovalerate, and valerate) in feces were measured using gas chromatography-mass spectrometry (GC-MS) following the Integrated Metabolomics Analysis Core's standard analysis protocol. The fecal samples were lyophilized over-night, powdered, and weighed. 50 mg of each fecal sample was diluted in 800 µL extraction solution (30 mM hydro-chloric acid) and spiked with 200 mM heptadeuterated butyric acid (d7-Butyric acid) internal standard to the final concentration of 0.1mM. Samples were homogenized using a Precellys homogenizer, then centrifuged for 10 min

at 15,000 x g at 4°C. Supernatants were collected and mixed with equal volume of ethyl acetate, vortexed for 10 s to emulsify, incubated on ice for 5 min, then centrifuged for 1 min at 15,000 x g at 4°C. From each sample, 150 µL of supernatant were transferred to sample vials and maintained at room temperature on an autosampler before injection. 1 µL of extracted sample was injected with a split ratio of 20:1, into a gas chromatography triple quadrupole mass spectrometer (TSQ EVO 8000, Thermo Scientific, Waltham, MA) for chromatographic separation and quantification. The ionization was carried out in the electron impact (EI) mode at 70 eV. Separation was achieved using a ZB WAX Plus capillary column (30 m x 0.25 mm, 0.25 µm film thickness, Phenomenex). The MS data and retention times were acquired in full scan mode from mass-to-charge ratios (m/z) 40-500 for the individual target compounds. The target compounds were quantified in the Selected Ion Monitoring (SIM) mode using the following product ions in positive-ion mode (compound: product ions in m/z): acetic acid: 43, 45, 60; propionic acid: 43, 73, 74; isobutyric acid: 41, 43, 73; butyric acid: 42, 60, 73; isovaleric acid: 43, 60, 87; valeric acid: 41, 60, 73; D7-butyric acid: 45, 63, 77. The injector, MS transfer line and ion source were maintained at 230°C, 240°C and 240°C, respectively. The flow rate of helium carrier gas was kept at 1 mL/min. Absolute levels of SCFAs in µM were calculated, then normalized to mg dried fecal weights. The internal standard d7-Butyric acid, an isotopically labeled butyric acid, was spiked in each sample and used to normalize for extraction efficiency. The samples were extracted in Ethyl Acetate, and the standard curve was prepared in Ethyl Acetate as well. Sample acquisition and analysis was performed with TraceFinder 3.3 (Thermo Scientific).

2.4 Fecal DNA Extraction, 16S rRNA Sequencing and Data Processing

Fecal microbiome library preparation and sequencing was done at the Molecular Genomics Core, following the core's established protocols. Briefly, microbial DNA was extracted using the FastDNA Spin Kit for feces (MP Bio-medicals, Irvine, CA). DNA library was prepared using the Swift Amplicon 16S+ITS Panel (Swift Biosciences, Ann Harbor, MI), targeting the V1-V9 hypervariable regions of 16S rRNA. Amplicons were sequenced on an Illumina MiSeq platform (Illumina, San Diego, CA) with 2 × 250 bp paired end reads. Data processing was performed using QIIME 2.

2.5 Cytokine analysis

The plasma levels of pro-inflammatory cytokines were measured using the MILLIPLEX mouse cytokine multiplex panel (EMD Millipore, Darmstadt, Germany). Samples and standards were prepared following the manufacturer's protocols. Each cytokine level was calculated based on its own standard curve and expressed as mean concentration (pg/ml) ± SEM.

2.6 Statistical Analysis

Alpha diversity and beta diversity were calculated using the web-based tool MicrobiomeAnalyst [27]. Analysis of Bray-Curtis dissimilarities was calculated using the relative abundance of Amplicon Sequence Variants (ASVs), to analyze beta diversity among samples. Significance of community composition differences among sample groups was determined by analysis of similarities (ANOSIM). Comparisons between

groups were made at various taxonomic levels. Linear discriminant effect size (LEfSe) analysis was performed at the family and genus levels to find features differentially represented between different groups; all groups were compared together. LEfSe combines the standard tests for statistical significance (Kruskal-Wallis test and pairwise Wilcoxon test) with linear discriminate analysis; it ranks features by effect size, putting features that explain most of the biological difference at the top [28]. LEfSe analysis was performed at the α value of 0.05 for the Kruskal-Wallis test and the threshold of 2 on the logarithmic LDA score for discriminative features. A dot map of discriminant features was generated using the LefSe module in MicrobiomeAnalyst.

Two-way repeated-measures ANOVA followed by Tukey's post-hoc test was used to analyze the body composition and metabolic data, using the Prism 9.0 software (GraphPad software, La Jolla, CA, USA). For SCFA data, since only samples that meet the required amount (50 mg lyophilized feces) for extraction were processed, data were analyzed using two-way ANOVA with diet and age as independent factors. For multiplex cytokine data, since some measurements were below detection level and therefore considered missing values, data were analyzed using two-way ANOVA with diet and age as independent factors, following by Tukey's post-hoc tests. Data were presented as mean ± SEM. Significance was assumed whenever $P < 0.05$.

3. Results

In this study, we compared the impact of inulin supplement on body composition, food intake, fecal SCFA levels and microbial composition in young-, middle- and old-age

mice. C57BL/6J male mice at 5-, 11- and 26-monts (M) of age (n = 7, 8 and 5, respectively) were put on control purified diet (RD) for 2 weeks, then switched to inulin-containing purified diet (high fiber, HF) for 6 weeks as shown in the schematic in Fig. 1. ~~These mice were born and raised at Jackson Laboratory, fecal and plasma samples were collected on the same day and kept at -80°C until processing at Texas A&M University.~~ To address whether the capacity to ferment inulin differ between different ages, we first measured microbially derived SCFAs levels in the feces from these mice on purified control diet, and 4 weeks after inulin supplementation (Fig 5). The control and inulin-containing diets are matched in macronutrient and micronutrient, differing only in fiber contents, with 4.7% cellulose in control and 17.3% inulin in inulin-supplement diet, respectively (Table 1). Four weeks of inulin supplement significantly increased fecal butyric, propionic, acetic and valeric acid levels, as well as the branched-chain SCFAs isobutyric and isovaleric acids in all age groups, as indicated by significant diet effects (Fig. 2). Of note, fecal acetic acids from mice on control diet were below detection level in our system, hence only data after inulin feeding were shown (Fig. 2C). Interestingly, significant age and diet interaction effects were detected in fecal butyric and propionic acid levels. The increase in butyric acids were more prominent in the young- and middle-age groups, and non-significant in the old-age group (Fig. 2A). Furthermore, inulin-induced increase in propionic acid levels showed an age-dependent decline (Fig. 2B).

~~Previously we showed age-associated changes in microbial composition [29]. As diet is a major modulator of the gut microbiota~~ Next, we assessed the modifications in microbiota composition in the different age groups in response to inulin supplementation.

We performed 16S rRNA gene sequencing in fecal samples collected from young-, middle- and old-age groups before (RD), 1 week (HF1) and 4 weeks (HF2) after diet switch to inulin supplement. As shown in Fig. 3A and B, inulin-containing diet had a significant impact on the evenness (Shannon index) and the richness (Chao1 index) measures of α-diversity [30]. On the other hand, age had no significant effect on the richness of the microbial composition, while it had a significant effect on the evenness as indicated by the Shannon index (Fig. 3B). There were no significant age and diet interactions. Inulin-containing diet significantly decreased α-diversity of microbial communities in all age groups. Taxonomic analysis at the phylum level showed that there is an increase in *Bacteroidetes* after 1 week of inulin intake in all age groups, which diminished slightly at 4 weeks of inulin intake (Fig. 3C). Consistently, analysis of *Firmicutes/Bacteroidetes* (F/B) ratio showed that inulin intake significantly decreased F/B ratio in all age groups (Fig. 3D).

Analysis of β-diversity of the microbial communities by principal coordinates analysis (PCoA) showed distinct separation of the microbial communities by diet and age (Fig. 4A). Diet-dependent separation between the communities was observed at PC1 axis, which explained 39.4% of the variance, while age-dependent separation between the communities was observed at PC2 axis, which explained 16.8% of the variance. Inulin intake exerted a time-dependent shift towards the right, along PC1 axis, in all 3 age groups. Interestingly, 1 week of inulin intake has more profound impact on microbiota composition than 4 weeks of inulin intake, as indicated by the shift to the far right along the PC1-axis after 1 week, then towards left along the PC1-axis after 4 weeks of inulin

intake. In addition, an age effect was observed along the PC2 axis, at RD, 1 week and 4 weeks of inulin supplement. Taken together, these data suggest that diet has a stronger impact on the microbiota structure than age~~, and that short-term inulin intake impacted the microbiota composition more strongly than longer-term intake~~.

The alterations of the intestinal microbiota at genus and family levels among the 3 age groups at different time points were analyzed using linear discriminant analysis effects size (LEfSe), with a linear discriminant analysis threshold score set at 2. Ten significant bacterial families were identified (Fig. 4B), and 43 significant features at genus level were identified (data not shown). Based on LEfSe analysis, box and whisker plots of selected bacteria are shown in Figs 5 and 6. Ruminococcaceae and Streptococcaceae families were significantly reduced by inulin supplementation (Fig. 5A and B, respectively). At the genus level, inulin supplement decreased relative abundance of *Lactococcus*, and *Ruminiclostridium_9* in all age groups (Fig. 5C and D). Interestingly, *Bifidobacterium* genera were most abundant in the middle-age group on purified control diet, and remain relatively unchanged by inulin intake, while they were increased by inulin intake time-dependently in the old-age group (Fig. 6A). Inulin supplementation increased the relative abundance of *Akkermansia* in all age groups, albeit the effect was more prominent in the young- and middle-age group (Fig. 6B). For the butyrate-producing bacteria *Roseburia*, the relative abundance was lower in the old-age group than in the young- or middle-age group on control diet (Fig. 6C). Inulin intake significantly increased the abundance of *Roseburia* in all age groups; notably, this increase rapidly dwindled in the young- and old-age groups, but persisted in the middle-age group after 4

weeks of inulin supplement (Fig. 6C). Correlation analysis of *Roseburia* with butyric acid levels showed a trend for positive correlation after inulin supplement (Fig. 6F), but not on control diet (Fig. 6E). We found that 4 weeks of inulin intake increased the relative abundance of *Bacteroides* (Fig. 6D)~~, a recently reported dominant inulin-responder that leads to production of abundant succinate in mouse intestine~~. Interestingly, this bacterial population was already high in the old-age group on control diet compared to other age groups. ~~Taken together, the microbiota composition analysis demonstrated that inulin intake has diet-, age- and time-dependent impact on microbiota composition, suggesting that the host-microbe interactions reach a new equilibrium upon long-term diet change, and these adaptations differ in different age groups.~~ Overall, the fecal microbiome and metabolome data suggested that while inulin intake exerted stronger impact on microbiome structure than age, the extent of microbial response may be diminished in the old-age group compared to other age groups.

To address whether the extent of host metabolic response to inulin supplement differs in different age groups, we assessed several metabolic parameters before and after inulin supplement in the same mice. We found that there is a trend for decreased food intake in young- and old-age groups in the first week of inulin intake; this decrease is transient in the old-age group and showed a rebound in the following weeks (Fig. 7C). While the food intake remained relatively unchanged in the middle-age group, consumption of inulin-containing diet was similar in all age groups after 5 weeks (Fig. 7A-C). Inulin supplement decreased body weight and fat mass in all age groups (Fig. 7D-L). Notably, the fat-reducing effect of inulin was most prominent in the middle-aged group, with a

significant decrease in fat mass as early as one week after inulin supplement (Fig. 7H and K). ~~This contrasts with a recent study showing that inulin did not alter the body weight nor the adiposity in mice; these mice were 6 weeks-old at the start of the experiment and fed with inulin supplementation for 11 weeks. This age is considerably younger than the mice used in the current study. Hence, our data highlight the age-associated difference in metabolic response to inulin~~. To characterize the metabolic parameters, all mice were subjected to indirect calorimetry in the week before diet switch, and 5 weeks after inulin supplement (experimental timeline in Fig. 1); data from middle- and old-age groups were shown in Fig. 8. After acclimatization period, data from day 5 of each metabolic recording session showed that body weights of middle- and old-aged mice were significantly reduced after 5 weeks of inulin supplement (Fig. 8A-B). Notably, inulin supplement significantly increased total energy expenditure in the night cycle in middle-aged mice, but not in old mice (Fig. 8C and D). In addition, physical activities including both home cage activities (XY-beam breaks) and voluntary activities (wheels running distance) were assessed. Inulin intake significantly reduced XY-beam breaks in both day and night cycles, and significantly increased wheels running distance in night cycles, suggesting a shift in activities from home age activities to voluntary wheels running in middle-aged mice (Fig. 8E and G). These changes in physical activities after inulin supplement weren't apparent in old mice (Fig. 8F and H). Taken together, these data suggest that inulin supplement reduced adiposity in middle-aged mice, likely attributed to increased energy expenditure via increased voluntary exercise, and these metabolic adaptations are diminished in old mice.

Next, we examined the plasma levels of pro-inflammatory cytokines IL-6, IL-1α, TNFα, and CXCL1 (also known as KC) in mice before and 5 weeks after inulin supplement (Fig. 9). We found that 5 weeks of inulin intake had no significant impact on IL-6, IL-1α, or TNFα levels. There was age-associated increase in TNFα levels, but not in IL-6, IL-1α, or CXCL1. Interestingly, inulin significantly reduced CXCL1 levels in all age groups. These results suggest that while 5 weeks of inulin supplement did not significantly alter the pro-inflammatory cytokines associated with aging, it had a lowering effect on the chemoattractant CXCL1.

DISCUSSION

In this study, we showed that inulin intake decreased body weight and fat mass, changed the composition of the gut microbiota and increased SCFAs production; notably, these changes are most prominent in the middle-aged mice. Previously we and others have reported that there are distinct age-dependent changes in the gut microbiome that are related to aging-induced inflammation [29, 31-33]. Here we showed a strong effect of diet in all age groups, as early as 1 week of inulin feeding, in decreasing the Firmicutes to Bacteroidetes (F/B) ratio, which is a strong indicator of functioning gut/metabolic health [34]. This coincides with a decrease in alpha diversity with the inulin supplement. Importantly, bacterial groups associated with dysbiotic conditions were shown to decrease in abundance by inulin supplement. These include Ruminococcaceae, Streptococcaceae, *Lactococcus*, and *Ruminiclostridium 9*. Increased abundance of bacteria in the Ruminococcaceae and Streptococcaceae families have been found in patients experiencing inflammatory bowel syndrome and inflammatory arthritis

conditions, respectively [35, 36]. Higher levels of *Ruminococcus* spp. (belonging in the Ruminococcaceae family) have also been observed in elderly humans and associated with high frailty scores [37]. Interestingly, *Ruminococcus 2* abundance has been found to be positively correlated with composite adipogenic, pro-inflammatory, and obese phenotype [38]. In addition, *Ruminiclostridium 9* abundance has been found to be positively correlated with non-alcoholic fatty acid liver disease [39]. Taken together, our gut microbiota data suggest a beneficial effect of inulin in reducing dysbiotic bacteria populations.

It is well known that aging results in a predisposition to inflammation as well as to infections; this decrease of the immune response is termed immunosenescence [40, 41]. In this study, we observed age-associated increase in systemic TNFα levels, suggesting increased pro-inflammatory states in aging mice. However, despite the reduction in the pro-inflammatory bacteria groups after inulin supplement, we did not observe a significant impact of inulin intake on the aging-associated pro-inflammatory cytokines IL-6, IL-1α and TNAα. Of note, 5 weeks of inulin supplement led to decrease in systemic levels of CXCR1 in all age groups. CXCR1 is a member of the CXC chemokine family, and together with its receptor CXCR2, play a crucial role in host immune response by recruiting and activating neutrophils for microbial killing at the tissue site [42, 43]. Moreover, recent studies suggest that neutrophil aging (from the time neutrophils are released into the blood to the time they disappear from the circulation) is regulated in a circadian fashion, by cytokines or metabolites whose levels follow diurnal changes (e.g., those produced by the microbiota)[44]. Future studies are required to address the causal

relationship between reduced dysbiotic microbiota and CXCR1 levels by inulin feeding, and the potential functional impact on neutrophil aging and immune system.

Previous studies have reported that inulin consumption increases *Bifidobacterium* and *Akkermancia muciniphila* in humans and mice [7, 18, 45, 46]. The bifidogenic effect of inulin has also been demonstrated *in vitro* in a simulator of human intestinal microbial system [47]. In this study, we found that inulin intake significantly increased *Bifidobacterium* time-dependently, while the increase in *Akkermancia* and *Roseburia* peaked at 1 week of inulin intake then declined. Notably, this increase in *Bifidobacterium* was most prominent in the old-age group, which had lower abundance of *Bifidobacterium* under control diet compared to other age groups, and this effect persisted after 4 weeks of inulin intake. While the mechanisms for such differential changes in microbiota composition across age and diet response are currently unclear, the gut microbiota are known to display interesting cross-feeding interactions [5, 48]. For example, the acetates and lactates produced by Bifidobacteria can serve as substrates for butyrogenic taxa [48]. A recent study, by employing a single-cell genomics approach, demonstrated that inulin feeding increased *Bacteroides* spp., resulting in the production of abundant succinate in the mouse intestine [49]. Furthermore, the authors obtained the genomes of this inulin-responder, identified their polysaccharide utilization loci to produce succinate through the glycolytic system and a reductive partial TCA cycle for release into the intestinal environment. Interestingly, our 16s rRNA data showed that inulin supplement increased the abundance of the *Bacteroides* genera in an age-specific manner. Surprisingly, this genus was most abundant in the old-age group compared to young- and middle-age

groups on control diet, and the increase in abundance in *Bacteroides* in response to inulin feeding was most prominent in the old-age mice. While our study used C57BL/6J mice at 3 different ages, the study by Chijiiwa et al. [49] used wild-type 6-week-old male mice from the BALB/c background. Whether the succinates produced by *Bacteroides* is used by other bacteria in cross-feeding effects, or taken up by host colonocytes as metabolic fuels are currently unknown. Future work in measuring the *Bacteroides* strains, succinate and lactate levels in the lumen and colonocytes of different aged mice, in response to dietary fiber, may provide further insights into the host-microbe interactions in the intestinal environment in aging mice.

Body compositions analysis showed that inulin supplement exerted the strongest effect in the middle-aged mice in reducing body weight and adiposity; this is likely attributable in part to the increase in voluntary wheel running activities during metabolic recording sessions. Previous study has shown that inulin did not alter the body weight nor the adiposity in mice; these mice were 6 weeks-old at the start of the experiment and fed with inulin supplementation for 11 weeks [50]. While this age is considerably younger than the mice used in the current study, our experimental design in including metabolic cage studies may also have impacted the results. Nevertheless, body weights measured during metabolic recording sessions showed that inulin supplement, while significantly reducing body weights of both middle- and old-aged mice, significantly increased energy expenditure in middled-aged mice but not old mice. Hence, our data highlight the age-associated difference in metabolic response to inulin.

Previous studies have shown that SCFAs contribute to improved metabolism in diet-induced obesity [24, 25]. Propionate has been shown as ~~is primarily a precursor for intestinal gluconeogenesis, and~~ an inhibitor of hepatic lipid synthesis [21, 51]. Propionate can be synthesized by Bacteroidetes, several Firmicutes belonging to the Negativicutes class, and *Akkermansia municiphilla* [5, 52, 53]. On the other hand, butyrate-producers include *Bifidobacterium*, *Faecalibacterium*, *Roseburia* and *Akkermansia*. Butyrate supplementation has been shown to reduce diet-induced obesity in C57BL/6 mice via promotion of energy expenditure and induction of mitochondria function in brown adipocytes and skeletal muscle [54]. Moreover, Kasahara et al [55], using germ-free mice, showed that *Roseburia Intestinalis* is a butyrate-producer. They demonstrated that colonization of a synthetic core microbial community with *Roseburia Intestinalis* in germ-free *ApoE* knockout mice fed a diet with high plant polysaccharide diet (TD.2918) significantly increased cecal butyrate levels but not acetate or propionate levels compared to mice colonized with core microbiota without *Roseburia*. Thus, we performed Pearson correlation analysis of butyric acid levels with *Roseburia* abundance, and found a significant negative correlation when the mice were on regular control diet, but a trend for positive correlation when the mice were on inulin-containing diet. These data are consistent with previous reports showing that cellulose, the fiber in the control diet, has low fermentability compared to soluble fibers such as inulin [56]. Overall, we observed an age-dependent decline in inulin-induced propionic acid as well as butyric acid production, consistent with the reduced efficacy of inulin in reducing adiposity in old mice. ~~Future work assessing liver and adipose tissue lipid metabolism in mice of different age in response to propionate or butyrate supplementation is required to determine~~

~~relative contribution of the metabolites to the fat-reducing effects of inulin.~~ Apart from potential effects of propionate or butyrate on modifying liver and adipose tissue lipid metabolism in mice, metabolic analysis revealed a significant impact of inulin supplement on increasing voluntary physical activities, leading to increased total energy expenditure and likely contributing to prominent decrease in adiposity in middle-aged mice. Whether the increase in voluntary activity is due to direct effects of SCFAs on the brain, or due to effects via enteric or vagal nervous systems signaling to the brain is currently unknown. Interestingly, previous reports have demonstrated that inulin supplement reduced neuroinflammation in old mice [33] and in transgenic mice carrying apolipoprotein epsilon4, a genetic risk factor for Alzheimer's Disease [57]. Further work is required to investigate the molecular mechanisms underlying the age-associated decrease in host response to inulin in terms of physical activity.

In summary, our data corroborate with previous studies supporting a role of high fermentable fiber in altering the microbiome and SCFA production, leading to beneficial metabolic adaptation in mice. The observation that the efficacy of inulin is reduced in old age highlights the importance of including age as a variable in studies determining host-microbe response to diets. ~~While our data demonstrated that the reduction in response occurred at the microbiome, SCFA production and metabolic level, more work needs to be performed to determine the molecular mechanisms underlying the age-dependent response to high fiber supplementation.~~ Additional adjunctive supplements, together with high fermentable fiber, may be required in the older adults to achieve efficacious SCFA levels and promote beneficial metabolic adaptation.

ABBREVIATIONS

ASVs: Amplicon Sequence Variants; BCFAs: branched-chain fatty acids; F/B: *Firmicutes*/*Bacteroidetes*; HF: high fiber; GC-MS: gas chromatography mass spectrometry; LEfSE: Linear discriminant analysis Effect Size; M: months; PCA: Principal Component Analysis; PCoA: Principal Coordinate Analysis; SCFAs: short-chain fatty acids.

AUTHOR CONTRIBUTIONS

C.-S.W. conceived, designed, performed the experiments, analyzed the data, and prepared the figures; S.S., and C. K. performed experiments and analyzed data; J.C.B. analyzed data, A. H. performed experiments, C.-S.W. and S.D.V.M. wrote the manuscript with input from all co-authors. All authors reviewed and approved the manuscript.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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TABLE 1. Composition of semi-purified diets used in this study.

Diets	**D12450J**	**D13081108**
Protein source	Casein	Casein
Fiber source	Cellulose	Inulin
Protein (kcal%)	20	20
Carbohydrates (kcal%)	70	70
Fat (kcal%)	10	10
Kcal/gm	3.8	3.5
Ingredient (%)		
Casein	19	17.3
L-Cystine	0.3	0.3

Corn Starch	48	39.5
Maltodextrin 10	11.8	10.8
Sucrose	6.5	6
Cellulose	4.7	0
Inulin	0	17.3
Soybean Oil	2.4	2.2
Lard	1.9	1.7
Mineral Mix, S10026	0.9	0.9
DiCalcium Phosphate	1.2	1
Calcium Carbonate	0.5	0.5
Potassium Citrate, 1 H_2O	1.6	1.4
Vitamin Mix, V10001	1	0.9
Choline Bitartrate	0.2	0.2

FIGURE LEGENDS

Figure 1. Experimental timeline.

Mice at 5-, 11- and 26 months of age ((n = 7, 8 and 5, respectively) were fed regular control diet (RD) for two weeks, then switched to inulin-containing diet (HF) for 6 weeks. Diet switch to HF was denoted as week 0. Data and samples were collected at the times indicated.

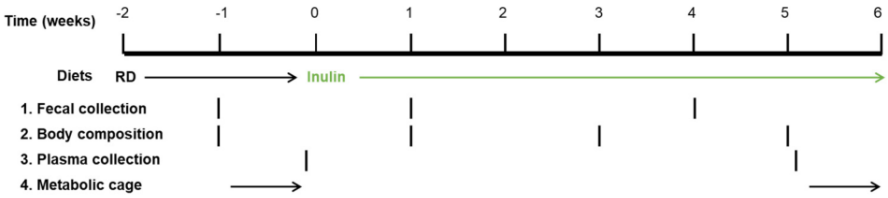


Figure 2. Inulin supplement increased fecal short-chain fatty acid levels.

Fecal samples collected from 5M, 11M and 26M mice at week -1 and 4 were processed for SCFA measurements. 5M: n=6, 6; 11M: n=7, 7; 26M: n=5, 5 for week -1 (RD diet)

and week 4 (inulin diet), respectively. (**A**) butyric acid, (**B**) propionic acid, (**C**) acetic acid, (**D**) valeric acid, (**E**) isobutyric acid, (**F**) isovaleric acid. Data were analyzed with two-way ANOVA with diet and age as independent factors (except for acetic acid, where levels were below detection limit on control diet), followed by Tukey's post-hoc tests. Acetic acid levels after inulin supplement were analyzed using one-way ANOVA. Data were presented as mean ± SEM. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$.

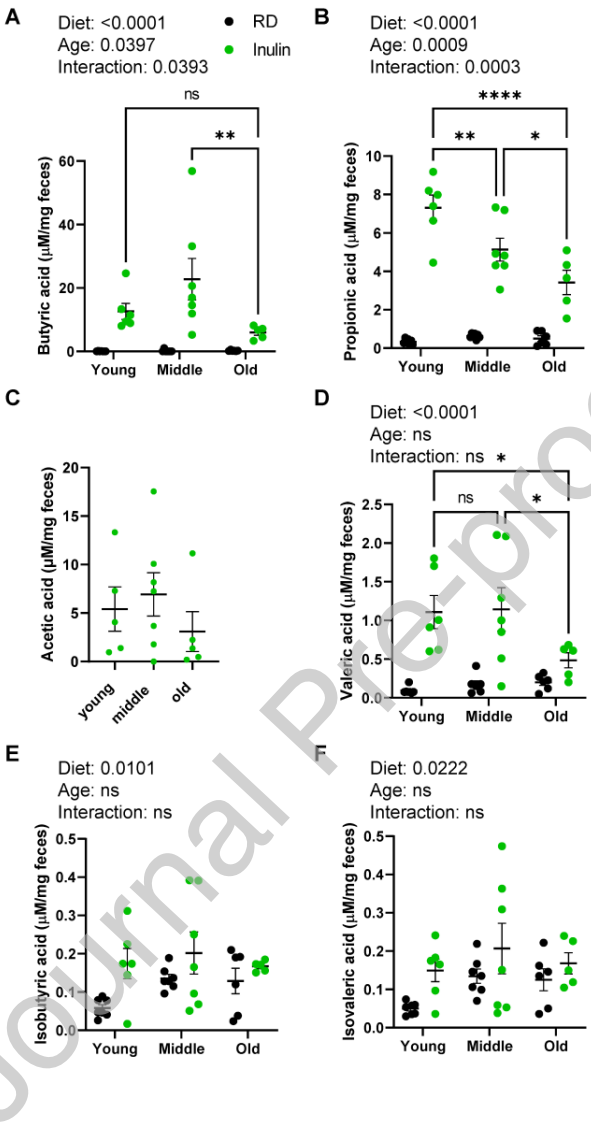


Figure 3. Effects of inulin on fecal microbiome diversity in mice of different ages.

Fecal samples collected from 5M, 11M and 26M mice at week -1 (RD), week 1 (HF1) and week 4 (HF2) were processed for microbiome analysis. 5M: n=3, 5, 4; 11M: n=4, 7, 5; 26M: n=4, 5, 4 for RD, HF1 and HF2, respectively. (**A-B**) Effects of inulin feeding on bacterial α-diversity, including richness (CHAO1, **A**), and overall sample diversity measured according to Shannon metrics (**B**). Data were analyzed with two-way ANOVA with age and diet as independent factors and presented as mean ± SEM with individual values shown. (**C**) Microbiome composition at the level of major phyla. (**D**) Firmicutes/Bacteroidetes (F/B) ratio.

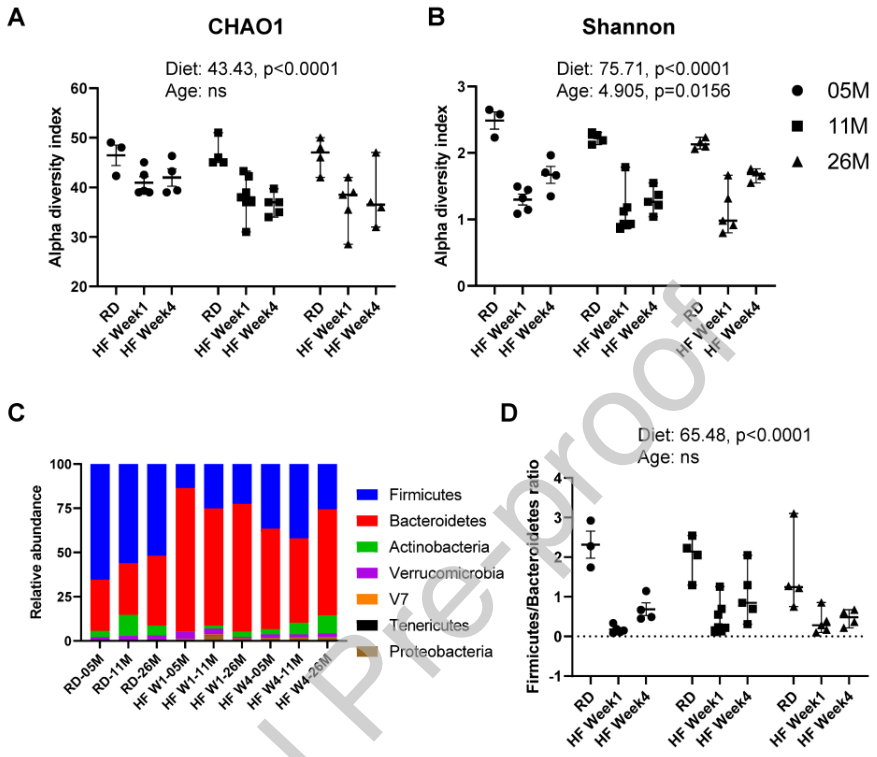


Figure 4. Inulin supplement changed relative abundance of specific bacterial populations.

(**A**) PCoA analysis plot representing microbial β-diversity. (**B**) The dot plot showed the Linear discriminant analysis (LDA) effect size (Lefse) scores computed for bacterial families significantly impacted by diet in different age groups. The right heatmap shows the relative abundance (log10 transformation). Data were analyzed and plots generated using MicrobiomeAnalyst.

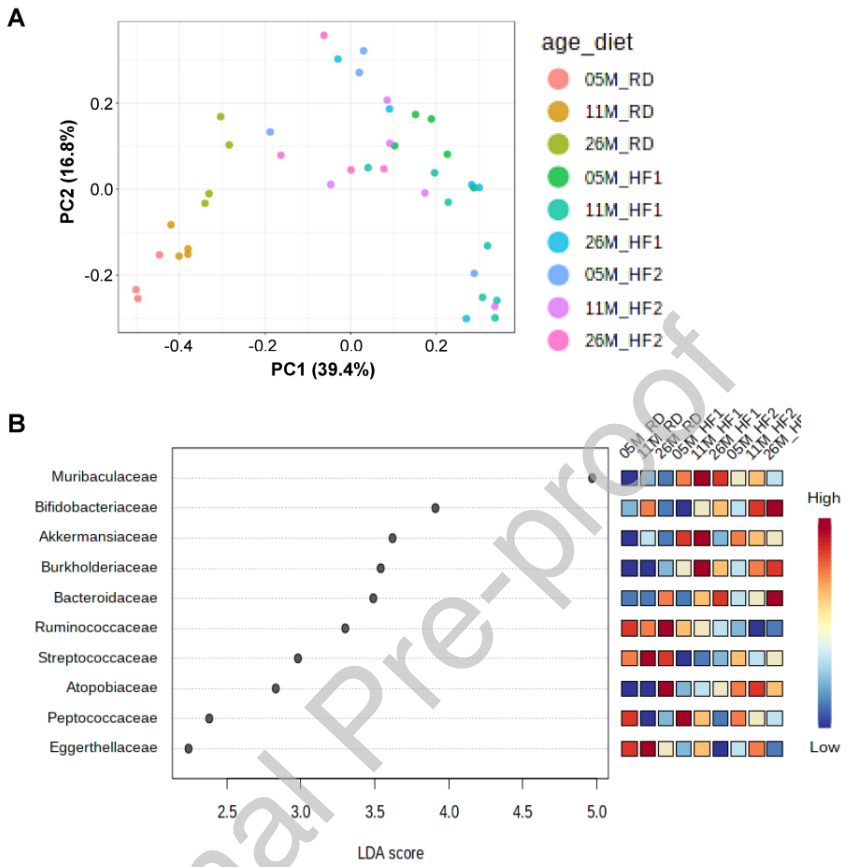


Figure 5. Inulin supplement decreased the relative abundance of pro-inflammatory bacterial families and genera.

Inulin feeding significantly decreased the relative abundance of specific bacterial populations, at Family level (**A**, **B**), and at Genus level (**C**, **D**). Data were analyzed and plots generated using MicrobiomeAnalyst.

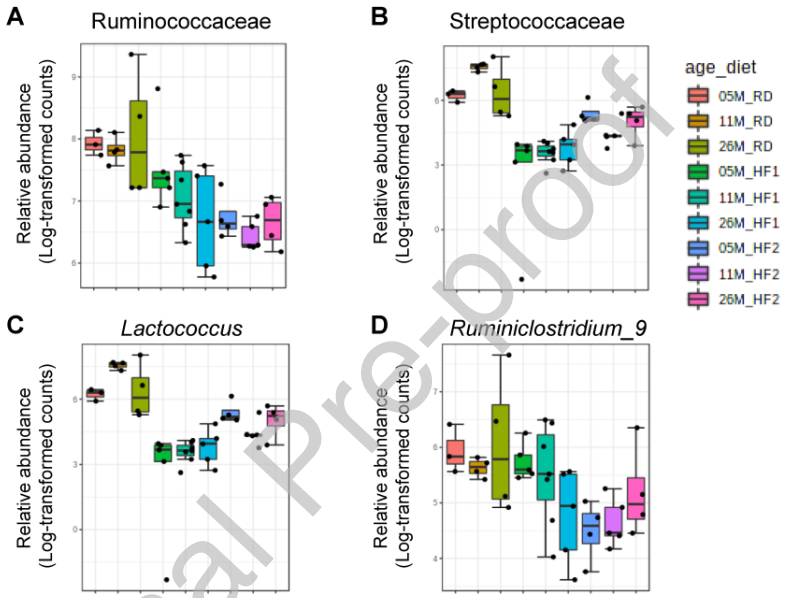


Figure 6. Inulin supplement increased the relative abundance of some bacterial genera in age-specific manner.

(**A**-**D**) Inulin feeding significantly increased the relative abundance of specific bacterial genera. Data were analyzed and plots generated using MicrobiomeAnalyst. (**E**, **F**) Pearson correlation analysis of Roseburia and butyric acid levels in mice on RD (**E**) and on inulin-containing diet (**F**).

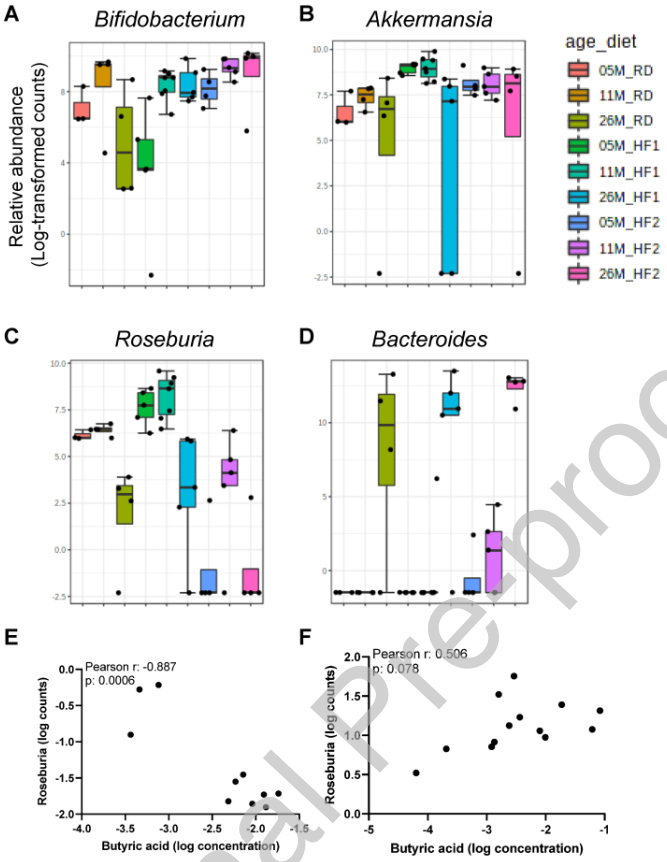


Figure 7. Inulin supplement decreased body weight and adiposity.

Mice at 5-, 11- and 26 months of age ((n = 7, 8 and 5, respectively) were fed regular control diet (RD) for two weeks, then switched to inulin-containing diet (HF), denoted as week 0. Mice were monitored for food intake (**A-C**), body weight (**D-F**) and fat mass (**G-I**). Since food intake was monitored weekly per cage then divided by number of mice housed in the cage, food intake data were analyzed with two-way ANOVA followed by Tukey's post-hoc tests. Body weight and fat mass data were analyzed with two-way ANOVA with repeated measures, followed by Tukey's post-hoc tests. Data were presented as mean ± SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

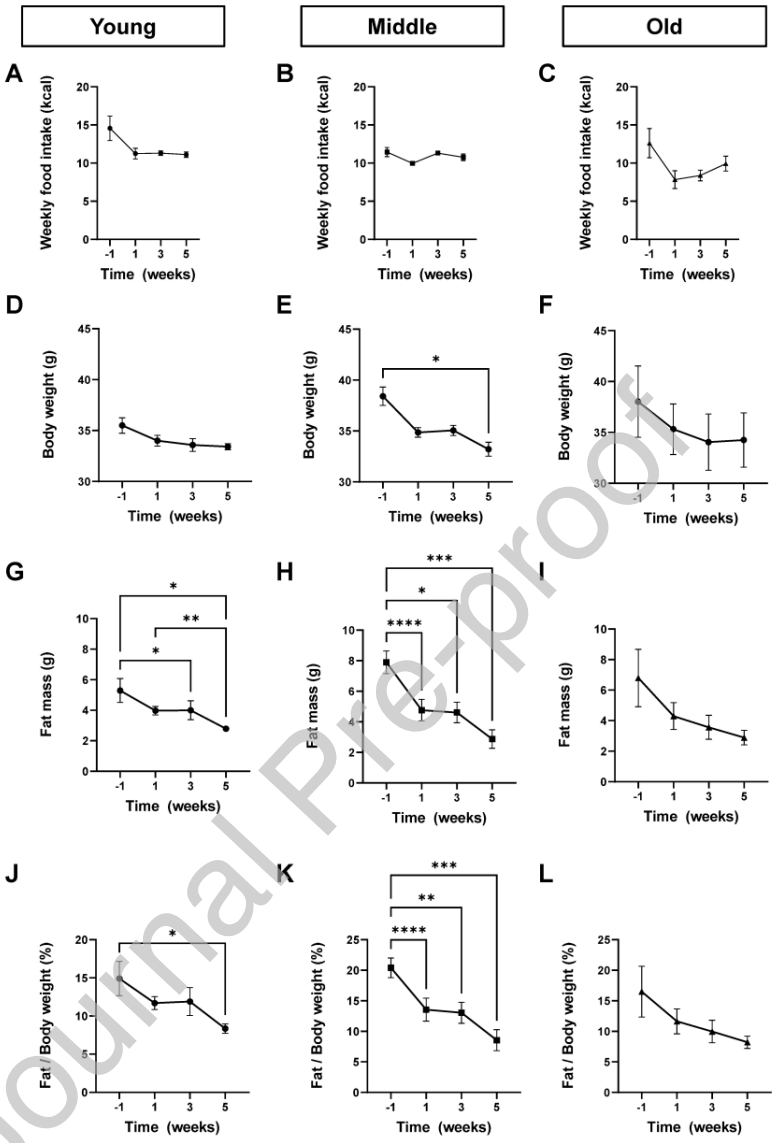


Figure 8. Inulin supplement increased total energy expenditure and voluntary activity in middle-aged mice.

Mice in the middle- and old-aged groups (n = 8 and 5, respectively) were subjected to indirect calorimetry in the week before diet switch to inulin-containing diet (RD), then after 5 weeks of inulin supplement (inulin). (**A-B**) body weights, (**C-D**) total energy expenditure (EE), (**E-F**) home cage activity (X-Y beam breaks), (**G-H**) wheels running distance. Data were analyzed with two-way ANOVA with repeated measures followed by Tukey's post-hoc tests. Data were presented as mean ± SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, ns: non-significant.

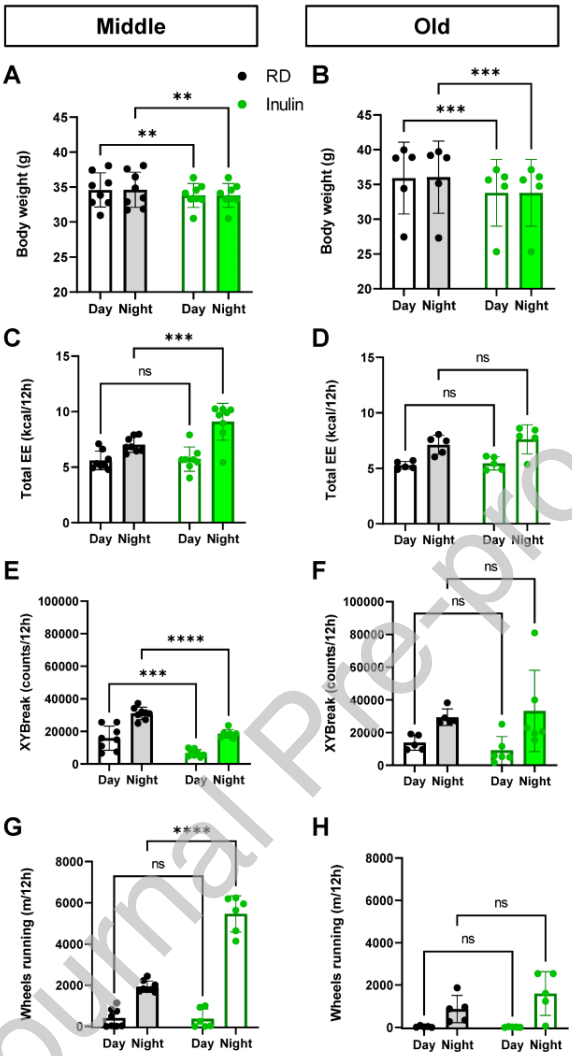
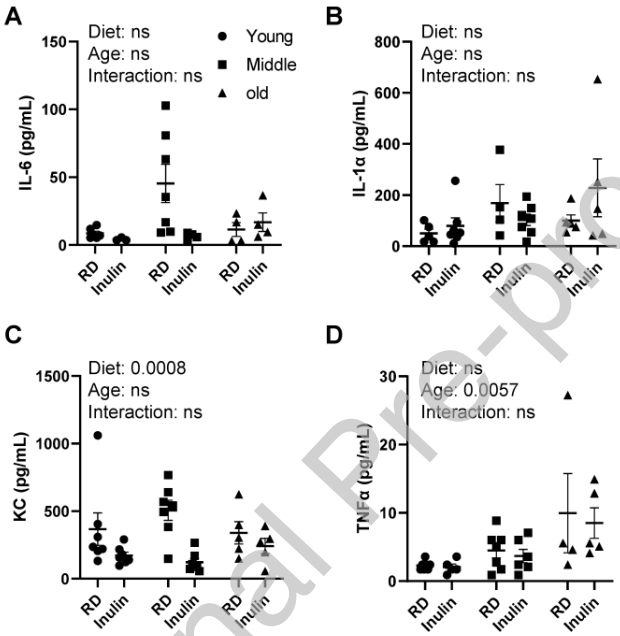


Figure 9. Effects of inulin supplement on plasma cytokine levels.

Plasma samples collected from 5M, 11M and 26M mice at week -1 and 5 were used for cytokine measurements. Each point denoted datum from a sample. (**A**) IL-6, (**B**) IL-1α,

(**C**) KC (CXCL1), (**D**) TNFα. Data were analyzed with two-way ANOVA with age and diet as independent factors, followed by Tukey's post-hoc tests. Data were presented as mean ± SEM.



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