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TITLE: Gulf War Illness Susceptibility and Gut Microbiome Dysbiosis: A Search for Biomarkers to Boost Resilience

PRINCIPAL INVESTIGATOR: Mariana Angoa-Perez, Ph.D.

RECIPIENT: Wayne State University

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Mariana Angoa-Perez

E-Mail: maperez@med.wayne.edu

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Wayne State University
5057 Woodward STE 13001
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14. ABSTRACT

Treatment with Gulf War Illness (GWI) agents resulted in gut microbiome alterations that translated into a differential expression of bacterial metabolites. Furthermore, these differences were evident within the GWI-susceptible group when compared to controls and with GWI-resilient subjects. Among the overall changes, a decrease in the amount of 3 major short chain fatty acids (SCFAs) including butyrate was found in GWI-susceptible animals, along with decreases in bile acids. Supplementation of butyrate was tested for its hypothesized capacity to balance the gut microbiome homeostasis and we evaluated its effects at the behavioral and bacterial composition levels. Butyrate was administered to animals that developed GWI symptoms and showed to be effective in ameliorating gastrointestinal alterations, pain sensitivity and cognitive impairments. This capacity of butyrate was associated with its ability to restore the gut microbiome composition to the one of controls.

15. SUBJECT TERMS
Gut microbiome, susceptibility, resilience, gut bacteria, microbiota transplantation, bacterial byproducts, butyrate

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

Epidemiological data suggest some level of susceptibility in the 25-30% of Veterans who become diagnosed with GWI. Animal models of GWI have shown a gut microbiome dysbiosis that is accompanied by depression- and anxiety-like phenotypes. We hypothesize that GWI may be influenced by the gut microbiome and key metabolites. Thus, mice exhibiting GWI symptoms (i.e., susceptible) will show imbalances in specific gut bacteria and their metabolites (i.e., biomarkers). GWI could be ameliorated with transplantation of the gut microbiome from animals without GWI, and with treatment with bacterial metabolites such as butyrate a short chain fatty acids (SCFA).

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

Gut microbiome, susceptibility, resilience, gut bacteria, microbiota transplantation, bacterial byproducts, butyrate.

3. **ACCOMPLISHMENTS:** The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer 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 goal 1: Identification of key microbial metabolites characteristic of the GWI-susceptible and GWI-resilient population that could serve as functional biomarkers of each condition.
Milestone 1: Key bacterial metabolites associated with GWI susceptibility were established via metabolomics analyses.
Major goal 2: Assessment of the ability of gut microbiota from GWI-resilient mice to ameliorate the gut dysbiosis present in GWI-susceptible subjects.
Milestone 1: FMT procedures have been carried out but samples are in the process of being sequenced. Adjustments to the preparation/storage of samples for FMT procedures were necessary to assure viable bacteria (mostly from the anaerobic types) were present for engrafting. Pooling of fecal samples from GWI-resilient mice was performed to generate enough volume of homogenates for FMT. Oxygen removal from homogenates as well as different concentrations of glycerol prior to freezing them were used. In light of this lag, and to optimize the completion of every objective, we moved onto Major goal 3.
Major goal 3: Examination of the capacity of butyrate to ameliorate gut microbiome dysbiosis and attenuate symptom severity in GWI-susceptible mice.
Milestone 1: The capacity of butyrate supplementation to reduce GWI susceptibility was determined at the both the behavioral and gut microbiome composition level.

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.

Major activities:

Major Task 1 specific objectives:

1.- Split caecum samples in half to perform metabolomics analyses on bacteria byproducts with LC-MS.

2.- Perform statistical analyses of the metabolomics data.

Significant results and key outcomes:

Gut microbiome metabolite profiles were overall different in GWI-treated subjects at 6 and 12 weeks compared to age-matched controls (see Fig. 1A). The majority of these microbial metabolites included bile acids and fatty acids. Given that 12 weeks showed more conspicuous changes compared to controls, a subsequent a partial least-square discriminant analysis (PLSDA) was performed in this group. This PLSDA revealed that controls and GWI-treated subjects cluster separated from each other, with the GWI group displaying a greater dispersion and 2 outliers that could be indicative of a separation between resilient and susceptible (Fig 1B). Changes in metabolites at 12 weeks post-treatment were more abundant and trended toward decreases compared to control levels. Subsequent analyses were carried out to differentiate GWI subsets for bacterial metabolites that were altered. For specific gut microbial byproducts, including short chain fatty acids (SCFAs) and bile acids, significant differences were subsequently found between GWI-susceptible subjects compared to GWI-resilient and controls (see Fig. 2 below).

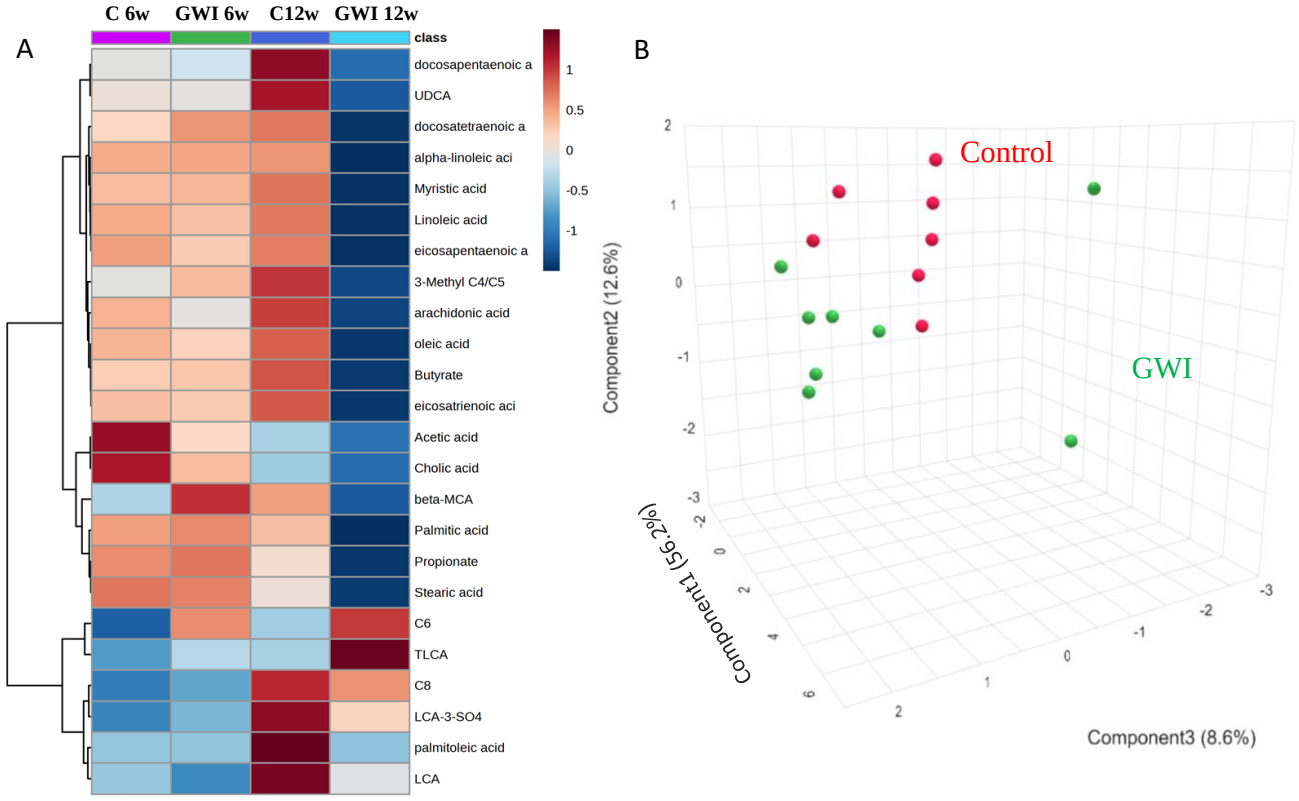

Figure 1. Heatmap (A) and PLSDA (B) displaying the differences in bacterial metabolomics profiles in GWI-treated subjects vs controls. Heatmap clustering displays an early and late time points of 6 and 12 weeks post-GWI, with more differences located at 12 weeks. Clustering was done using the Ward algorithm. PLSDA displays a differential clustering of metabolites for controls (red dots) and GWI (green dots) at 12 weeks.

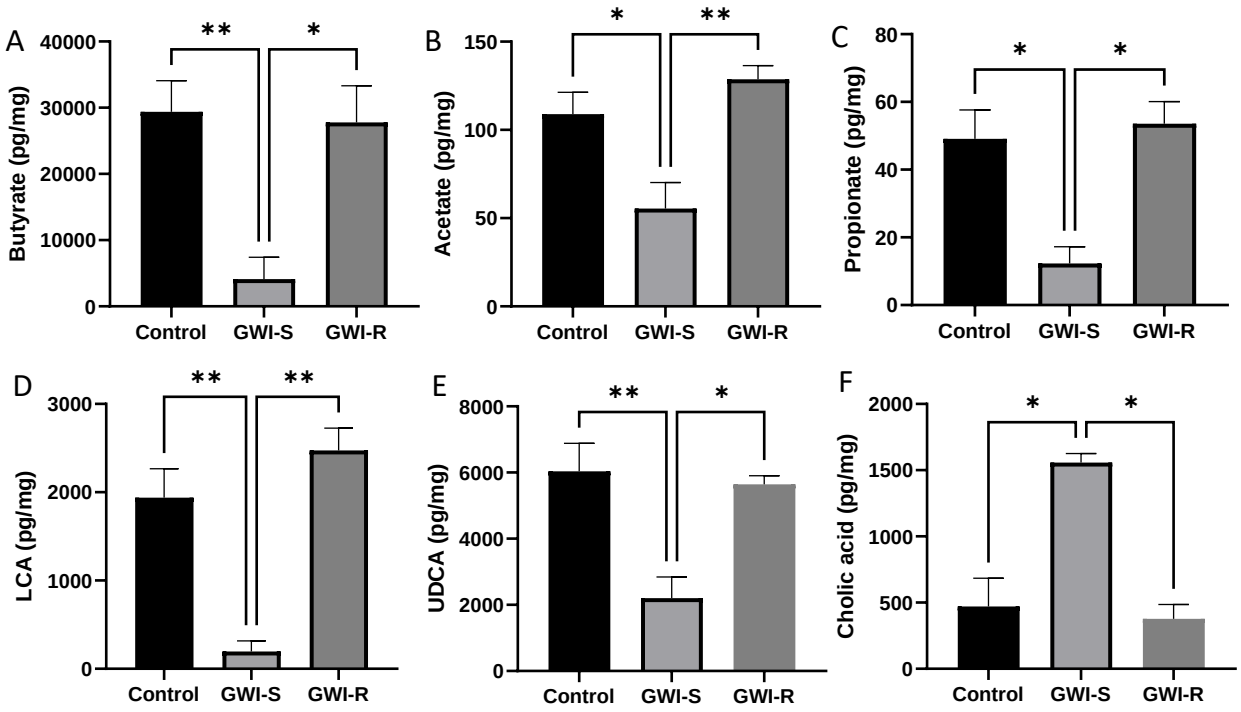

*Figure 2. Levels of the short-chain fatty acids butyrate (A), acetate (B) and propionate (C), and the bile acids LCA (D), UDCA (E), and cholic acid (F) in controls, GWI-susceptible and GWI-resilient subjects (Mean + SEM) measured in caecum by LC-MS. One-way ANOVA with Tukey's post hoc tests, * p< 0.05, ** p< 0.01.*

Results from specific bacterial metabolites are shown in Figure 2 for 3 major SCFAs and bile acids. For all these microbial byproducts, with the exception of cholic acid (Fig. 2F), levels were significantly reduced in GWI-susceptible subjects compared to both controls and GWI-resistant mice. No statistical differences were detected between controls and GWI-resistant animals. These patterns were inverse in the case of cholic acid, and this metabolite needs to be further investigated as a biomarker metabolite for potential GWI susceptibility.

These results fit our initial hypothesis regarding a decrease in the levels of SCFAs with GWI given that a reduction of these compounds has been associated with some of the symptoms of GWI, including gastrointestinal disturbances and cognitive impairments.

We have previously shown that GWI-susceptible subjects display more behavioral alterations consistent with greater Kansas criteria than controls and GWI-resilient mice.

Major Task 2 specific objectives:

1.- Treatment of C57BL/6 mice with Gulf War agents and screening of GWI symptoms at the behavioral level to divide them into GWI-susceptible and GWI-resilient.

2.- Collection of fecal samples from GWI-resilient subjects and preparation of homogenates for FMT procedures into GWI-susceptible animals were carried out. Sequencing of the bacterial 16S rRNA genes is pending.

Major Task 3 specific objectives:

1.- Treatment of C57BL/6 mice with Gulf War agents to subsequently classify them by their behavioral GWI symptoms.

2.- Divide GWI-susceptible and GWI-resilient subjects into two groups: a) A water control and b) a butyrate supplemented group.

3.- Evaluate the effects of butyrate supplementation at the behavioral level to determine the capacity of butyrate to ameliorate symptom severity.

Significant results and key outcomes:

At the behavioral level, treatment with GWI agents cause increases in bead expulsion, which is interpreted as a reduction in gastrointestinal transit (Fig. 3A), with increased pain sensitivity as shown by increases in the time to withdraw the subject paw in the thermal plantar test (Fig. 3B), with reductions in the recognition of a new object in the novel object recognition test (Fig. 3C), which is associated with deficits in cognition.

GWI treatment is also associated with increases in depression- (Fig. 3D) and anxiety- (Fig. 3E)) like behaviors as evidenced by increases in grooming time in the splash test and reduced times in the open arms of the elevated plus maze. Butyrate supplementation was effective in counteracting these GWI symptoms for gastrointestinal, pain and cognitive impairments, but it was ineffective for depression- and anxiety-like behaviors.

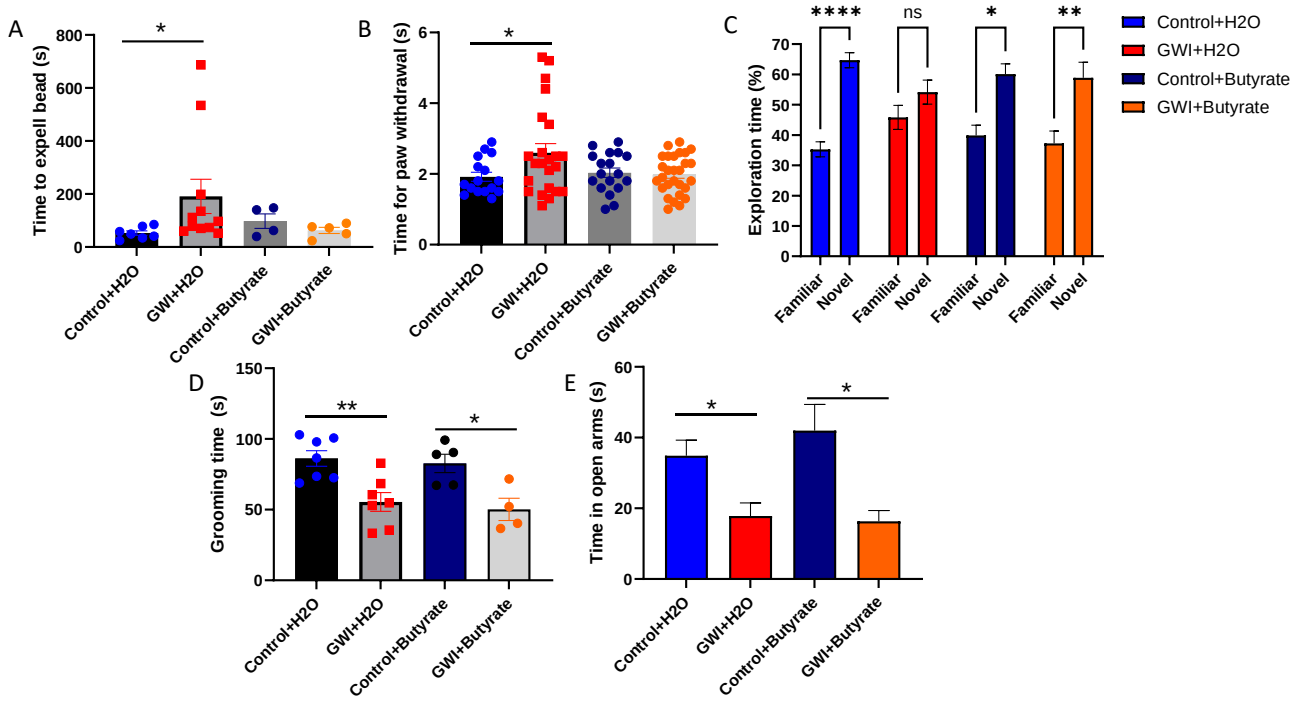

*Figure 3. Behavioral assessment of the effects of butyrate on GWI symptoms. Controls and GWI-treated animals were subsequently divided into water and butyrate. Gastrointestinal disturbances were evaluated using the bead test (A), pain was evaluated using the thermal plantar test (B), cognitive impairments were measured with the novel object recognition test(C), mood alterations were measured with the splash test (D) and anxiety was evaluated with the elevated plus maze (E). Mice treated with GWI agents and controls were each subdivided into water and butyrate. Mean + SEM, One-way ANOVA with Tukey's post hoc comparisons *p < 0.05, **p < 0.01, and ****p < 0.0001.*

Interestingly, an analysis of the gut microbiome composition with the Jaccard index revealed that treatment with butyrate (Fig. 4B) return the GWI profile to the one of controls (Fig 4A). While before butyrate the GWI group clustered apart from controls (p<0.01, NP-MANOVA), after supplementation with this SCFA the differences disappeared.

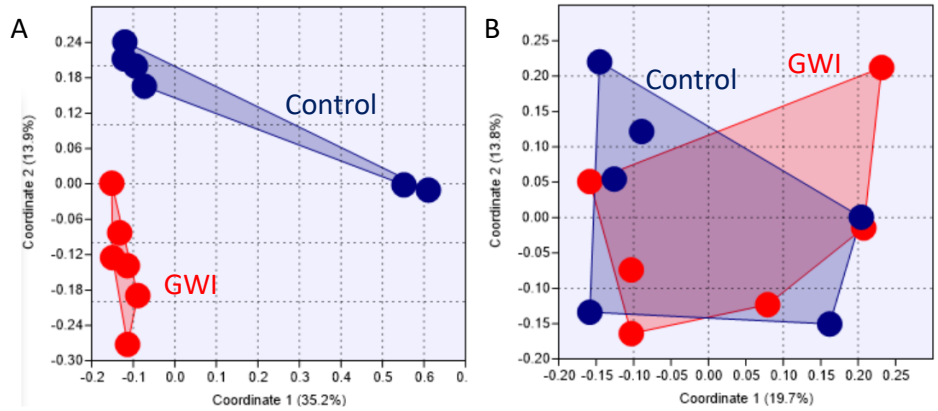

Figure 4. Gut microbiome composition (Jaccard index) in GWI-treated mice (red dots) and controls (navy blue dots) before (A) and after (B) butyrate supplementation. Controls cluster significantly apart from controls before butyrate supplementation (NP-MANOVA p< 0.01), whereas administration of this compound brings these two groups together and the statistical differences disappear.

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.

Nothing to Report

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.

For the next period, the outcomes of this project are planned to be presented at the Society for Neurosciences annual conference and data from this project will be published in an indexed Journal.

4. **IMPACT:** Describe 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).

This project has started to show the connection that exists between a multi-symptom disorder and the potential influence of the gut microbiome. While there are some reports of alterations in the gut bacteria with Gulf War Illness, the susceptibility to exhibit particular symptoms within the spectrum of this disorder and specific imbalances in the gut microbiome are novel. These results can set a precedent for a similar type of analyses that could shed light on other multi-symptom disorders.

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.

This project itself is an amalgamation of studies that involve peripheral mechanisms in the gut and the brain to try to understand the wide variety of symptoms in Gulf War Illness. Thus, the results will be pertinent not only within the domain of neurobiology, but also within the field of host-bacteria interactions.

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 Project Director/Principal Investigator (PD/PI) is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer 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:

Changes in approach and reasons for change
Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.

No changes in the objectives or scope are reported, but the order of the tasks was slightly modified to perform studies efficiently. Implementation of fecal microbiota transplantation (FMT) procedures required longer and while it is in process, the studies with butyrate were started to maximize productivity. Both FMT and butyrate tasks are expected to be completed proficiently.

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.

FMT implementation took longer than expected because viability of bacteria had to be tested to assure a successful procedure. We are in the process of completing the FMT tasks and do not anticipate any further delays.

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.

Nothing to Report

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

Not applicable

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).*

Nothing to Report

  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.

Angoa-Perez M, Zagorac B, Francescutti DM, Kuhn DM. Gulf war illness: A case of dysbiosis of the gut microbiome? John D Dingell VA Research Day; May 9th, Detroit, MI, 2023.

- **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. In addition to a description of the technologies or techniques, describe how they will be 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. State whether an application is provisional or non-provisional and indicate the application number. 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;*
- *biospecimen 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:	Mariana Angoa-Perez
Project role:	Principal Investigator
Research identifier (ORCID ID):	0000-0002-9749-1197
Nearest person month worked:	2.4
Contribution to project:	Dr. Angoa-Perez participated in the assessment of behavioral symptoms of GWI, metabolomics, bioinformatics analyses and other statistical analyses.
Name:	Donald M Kuhn
Project role:	Collaborator
Research identifier (ORCID ID):	0000-0002-8546-9507
Nearest person month worked:	0.84
Contribution to project:	Dr. Kuhn participated in interpretation of behavioral symptoms of GWI and biodiversity analyses.
Name:	Kevin R Theis
Project role:	Collaborator
Research identifier (ORCID ID):	0000-0002-8690-7599
Nearest person month worked:	1.2
Contribution to project:	Dr. Theis participated in guiding some of the bioinformatics analyses.

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.*

Organization name: John D. Dingell VA Medical Center Localization of Organization: Detroit, MI Partner's contribution to the project: Facilities- Dr. Kuhn's behavioral equipment is located at the VA and it is used for this project.

8. **SPECIAL REPORTING REQUIREMENTS**
   COLLABORATIVE AWARDS: N/A
   QUAD CHARTS: N/A

9. **APPENDICES:** N/A