A. COVER PAGE

Project Title: Occupational Exposure and Health Risk from Dairy Microbiome and Resistome to Dairy Farm Workers	
Grant Number: 5R01OH011578-04	**Project/Grant Period:** 09/01/2018 - 08/31/2022
Reporting Period: 09/01/2021 - 08/31/2022	**Requested Budget Period:** 09/01/2021 - 08/31/2022
Report Term Frequency: Final	**Date Submitted:** 11/21/2023
Program Director/Principal Investigator Information: GAUTAM DANTAS , BA PHD **Phone Number:** 13143627238 **Email:** dantas@wustl.edu	**Recipient Organization:** WASHINGTON UNIVERSITY Campus Box 1054 1 Brookings Drive SAINT LOUIS, MO 631304862 **DUNS:** 068552207 **UEI:** L6NFUM28LQM5 **EIN:** 1430653611A1 **RECIPIENT ID:**
Change of Contact PD/PI: NA	
Administrative Official: TERI MEDLEY WASHINGTON UNIVERSITY 1 Brookings Drive Campus Box 8018 ST. LOUIS, MO 63130 **Phone number:** 3147474134 **Email:** researchgrants@wusm.wustl.edu	**Signing Official:** TERI MEDLEY WASHINGTON UNIVERSITY 1 Brookings Drive Campus Box 8018 ST. LOUIS, MO 63130 **Phone number:** 3147474134 **Email:** researchgrants@wusm.wustl.edu
Human Subjects: NA	**Vertebrate Animals:** NA
hESC: No	**Inventions/Patents:** No

B. ACCOMPLISHMENTS

B.1 WHAT ARE THE MAJOR GOALS OF THE PROJECT?

The goal of this research study is to comprehensively characterize the microbiota and resistome of dairy cows and workers on dairy farms who are subject to unique occupational exposures due to the use of antimicrobials to treat diseases or to increase the production of milk. We propose to achieve this goal through 4 independent aims. 1) Determine the shared and unique nasal and gut microbiota among dairy farm workers and dairy cows. 2) Understand how nasal and fecal microbial communities, virulence factors, and resistomes change temporally in dairy farm workers and dairy cows and examine the association between nasal/gut microbiome and seasonal incidence of influenza-like-illness (ILI), as well as gastrointestinal (GI) symptoms, in dairy workers. 3) Quantitative analysis of acquisition, persistence, and transmission of ARGs from dairy cows to farm workers. 4) Develop a grounded understanding of dairy farm workers' attitudes, beliefs, and behaviors regarding their shared microbiome with dairy cows (the cows, their manure, and the environment) utilizing semi-structured interviews and observations.

YR1 goals:
To achieve the major goals of this project, we are conducting a longitudinal study of the phylogenetic composition of the nasal and gut microbiota of dairy farm workers, dairy cows, and office workers with no occupational cow exposure. We will sample 88 dairy farm workers working in 22 dairy farms, 88 dairy cows from the 22 dairy farms, and 88 non-farm, office based workers. The non-farm office worker cohort will be age and sex-matched with the dairy workers' cohort. The human and animal samples will be collected three times a year (during fall (August/September), winter (January/February), and spring (March/April) for each of the three consecutive years) for a total of 4752 samples. The sampling times are designed to capture the three main seasons of substantial farm work activity.

We also obtained permission to collect and archive skin and saliva samples from cows and humans in addition to the nasal and fecal samples. Since accessing farms and sample collection are a challenging part of the project, we wanted to utilize this opportunity by collecting additional types of samples at the same time. These additional samples do not change the scope or budget of the project in any way.

During the first 6 months of the first year, our goals are to obtain IRB and IACUC approval for all proposed experiments; collect preliminary samples to validate methods; and begin recruitment, which will be conducted over a 6-month timeframe during the first year. During the second 6 months of the first year, our goals are to complete recruitment; continue method validation on preliminary samples, collect spring and fall season samples; and begin 16S rRNA sequencing and data analysis for spring and summer samples.

B.1.a Have the major goals changed since the initial competing award or previous report?

No

B.2 WHAT WAS ACCOMPLISHED UNDER THESE GOALS?

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B.3 COMPETITIVE REVISIONS/ADMINISTRATIVE SUPPLEMENTS

For this reporting period, is there one or more Revision/Supplement associated with this award for which reporting is required?

No

B.4 WHAT OPPORTUNITIES FOR TRAINING AND PROFESSIONAL DEVELOPMENT HAS THE PROJECT PROVIDED?

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B.5 HOW HAVE THE RESULTS BEEN DISSEMINATED TO COMMUNITIES OF INTEREST?

To ensure study participant satisfaction and continuous engagement, we generated an infographic describing our preliminary results in lay terms. Specifically, we provided a description of our methods and a visualization of the gut microbiome compositions. The infographic was disseminated among the farmer and non-farmer cohorts.

B.6 WHAT DO YOU PLAN TO DO DURING THE NEXT REPORTING PERIOD TO ACCOMPLISH THE GOALS?

Not Applicable

B.2 (B.2_Accomplishments.pdf)

B.2 Accomplishments

We have met all proposed objectives of the award. The results of our analyses are documented in a manuscript currently under review for publication and are discussed in brief below.

Aim 1: Determine the shared and unique nasal and gut microbiota among dairy farm workers and dairy cows.

Sub-aim 1.1: Recruitment and sample collection

Recruitment and sample collection

Up to the onset of the COVID-19 pandemic in March of 2020, we recruited farmers from 37 dairy farms, ranging in size from 24 to 1,700 milking cows (median = 110, mean = 195.5, SD= 270.1). Our sampling took place between 03/2019 and 03/2020, spanning five seasons (i.e., spring 2019 through spring 2020). A total of 712 fecal (farmer = 171, non-farmer = 114, cow = 427) and 726 nasal (farmer = 171, non-farmer = 137, cow = 418) samples were included in the subsequent analyses. These samples were the foci of the analysis described below and documented in a manuscript currently undergoing peer review.

Since the easing of the pandemic lockdowns, we have collected additional 1,376 fecal (farmer = 314, non-farmer = 351, cow = 711) and 1,323 nasal (farmer = 308, non-farmer = 323, cow = 692) samples, which are currently being processed and will undergo a similar analysis as the pre-pandemic sample set.

Sub-aim 1.2: Perform high-throughput 16S sequencing to analyze nasal and gut microbiome architecture

Sample preparation and analysis for high-throughput 16S sequencing

Nasal and manure samples were shipped to the Dantas Lab at Washington University School of Medicine. Using the DNeasy Powersoil Pro Kit (QIAGEN), genomic DNA (gDNA) was extracted directly from Eswab for nasal samples and from chipped and aliquoted fecal samples. In all samples, gDNA yield was high enough to prep for 16S amplification (Aim 1, Sub-aim 1.2) and for metagenomic sequencing (Aim 2).

To analyze the phylogenetic composition of the samples,1,305 fecal and nasal samples in the Dantas lab have gone through 16S rRNA gene profiling according to Earth Microbiome Project protocols. Briefly, we amplified the V4 region of the 16S rRNA gene using barcoded 515F/806R PCR primers. Libraries were submitted for multiplexed paired-end 2x250bp sequencing on the Illumina MiSeq High Output platform at the Center for Genome Sciences and Systems Biology (CGSSB) at WUSM, where the Dantas lab is a core member. Samples were pooled together and sequenced to obtain at least 1 million 2x250 bp reads. DNA extraction, sequencing and analysis protocols for dairy cow and human samples had been optimized as samples we collected.

Phylogenetic profiles of each of the fecal and nasal samples have been analyzed using the DADA2 pipeline. Demultiplexed reads were quality filtered and clustered into amplicon sequence variants (ASVs). Output sequences were assigned taxonomy. The resulting ASV table and their relative abundances are currently being used to characterize the differences in nasal and gut microbial communities between dairy farm workers and dairy cows with respect to non-farm office workers. To date, we have computed α-diversity (measure of species richness of a given sample) and β-diversity (measure of similarity between samples) to compare the diversity within each sample and group and between samples and groups. We further performed PCoA using UniFrac distances between samples to explore the clusters visually and tested the significance of such clustering using appropriate statistical tests. We have shown that Occupational exposures shift the farmer nasal microbiome to be compositionally more similar to the nasal microbiome of dairy cows. Among the bacterial families overrepresented in farmers are Lactobacillaceae, Aerococcaceae, and Enterococcaceae. In contrast to the observed difference in nasal communities, cows persistently harbored a more diverse gut community than both human groups, while the farmer and non-farmer gut microbiomes did not differ from each other significantly in diversity or richness.

Aim 2: Understand how nasal and fecal microbial communities, virulence factors, and resistomes, change temporally in dairy farm workers and dairy cows and examine the association between nasal/gut microbiome and seasonal incidence of influenza-like-illness(ILI), as well as gastrointestinal(GI)symptoms, in dairy workers.

Sub-aim 2.1: Characterization of nasal and gut microbial community and phylogenomic level analysis

B.2 (B.2_Accomplishments.pdf)

Whole metagenomic sequencing

We performed whole-metagenomic shotgun DNA sequencing on 712 fecal samples. DNA sequencing libraries were prepared using a modified Nextera-XT tagmentation protocol (Illumina) for sequencing fecal metagenomes following published work. Sequencing libraries were submitted for multiplexed paired end 2x150bp sequencing on the Illumina NextSeq High Output platform at the CGSSB at WUSM and S4 flow cell of the NovaSeq 6000 multiplexed paired-end 2x150bp sequencing platform at the McDonnell Genome Institute at WUSM. Preliminary analysis showed high levels of contaminating DNA in nasal DNA sequencing libraries, with 95.1% of the sequencing reads identified to be of human or cow origins. Thus, whole metagenomic sequencing was only conducted on fecal samples. In total, we generated 22.7 Gb of paired-end sequencing data, and profiled the species and metabolic pathway compositions using MetaPhlAn 4 and HUMAnN 3, respectively. We found that the differences between the gut communities of the two human groups emerge at the level of individual taxa. Namely, farmer guts were enriched in butyrate-producing Firmicutes. Lastly, we observed differences in the metabolic capacities of the farmer and non-farmer guts; the farmer microbiomes were enriched in degradation pathways for simple sugars (e.g., lactose, galactose), while the metabolic pathways for degradation of complex sugars (e.g., starch, glycogen) were overrepresented in the non-farmer guts.

Horizontal dissemination of microbes

To assess the extent of horizontal dissemination of microbes within dairy farm settings, we used inStrain to investigate the cooccurrences of microbial sub-species (i.e., lineages) in human and cow guts. We first assembled 15,005 metagenome-assembled genomes (MAGs), with 993 high- and 3,891 medium-quality assemblies. We found an enrichment in the number of lineages present in farmers and cows relative to the lineages present in cows and non-farmers and the frequency of lineage sharing was substantially higher in subject pairs residing in the same farms, suggesting that cohabitation with livestock is the predominant driver of acquisition of livestock-associated microbial lineages. The lineages cooccurring in both farmer and cow guts represent 11 genera, 3 of which (Prevotella, Holdemanella, and Blautia) are overrepresented in the farmer gut relative to that of non-farmers. Notably, all these genera were found to be enriched in both farmer and cow nasal microbiomes, relative to the nasal community of non-farmers.

Aim 3: Quantitative analysis of acquisition, persistence, and transmission of ARGs from dairy cows to farm workers.

Sub-aim 3.1: Expand existing ARG databases by incorporating functionally identified ARGs enriched in dairy cows and dairy farm farmers

Preparation of functional metagenomic libraries

To maximize our chances of identification of novel ARGs, we selected 284 fecal samples, representing the latest sampling event from each unique study subject (cows, farmers, and office workers). We pooled the total metagenomic DNA from these samples in sets of ~20 in accordance with the subject type of origin. The DNA pools were fragmented to 2-5 kb in length. Sheared DNA fragments will be size-selected, cloned into expression vector, and transformed into *E. coli*, generating ~13 libraries, each containing at least 10^6 unique clones and 5-10 Gb of metagenomic DNA. The resulting transformant libraries will then be assayed for AR by plating on media containing antimicrobials at clinically-relevant concentrations to which our screening strain of *E. coli* (DH10B) is susceptible. Plasmids from transformants that survive antimicrobial selection will then be extracted en masse, sheared, barcoded, and pooled for sequencing on a NovaSeq 6000 platform. Sequencing reads will be assembled and annotated for AR functions using PARFuMS (Parallel Annotation and Reassembly of Functional Metagenomic Selections), a pipeline developed in the Dantas lab to enable efficient identification of thousands of ARGs from diverse metagenomes.

Characterize resistance gene profiles using updated database

We combined the 2,049 functionally screened ARGs with those present in the Comprehensive Antibiotic Resistance Database (CARD) and the NCBI antimicrobial resistance gene catalogue. To this combined set, we also added 17,292 ARGs resulting from past functional selections on samples of diverse origins (preterm infants, soil, non-human primates). This combined set allowed us to find evidence for transfer of cow-enriched ARGs by microbes shared between cows and farmers, and these ARGs were overrepresented in the farmer gut relative to that of non-farmers. Notably, we find examples of these

transferred ARGs encoded within mobile genetic elements and identify these cassettes in human clinical isolates reported elsewhere, demonstrating the connection between agriculture and public health.

B.4 (B.4_Professional_Development.pdf)

B.4 What opportunities for training and professional development has the project provided?

All graduate students and postdocs supported on this grant have completed an individual development plan (IDP), using the tool myIDP hosted by Science Careers at Washington University. New graduate students and postdocs are introduced to IDPs and the myIDP tool during orientation and workshops are offered throughout the year. Dr. Dantas reviews IDPs with the respective graduate students and postdocs at their annual review. IDPs are reviewed and updated at least annually. We recognize that graduate students and postdocs need both information and opportunities to explore the variety of career outcomes pursued by our alumni. The Washington University Division of Biology and Biomedical Sciences (our umbrella PhD program) and the Office of Postdoctoral Affairs (OPA) have an Education Coordinator and the University employs a full-time career strategist to provide career and professional development training along with Career Talks for graduate students and postdocs. Training and professional development activities for trainees also include monthly teleconferences between the two project teams, weekly lab-wide research-in-progress meetings, biweekly journal club, and weekly one-on-one mentoring meetings with Dr. Dantas.

C. PRODUCTS

C.1 PUBLICATIONS

Are there publications or manuscripts accepted for publication in a journal or other publication (e.g., book, one-time publication, monograph) during the reporting period resulting directly from this award?

Yes

Non-compliant Publications Previously Reported for this Project

Public Access Compliance	Citation
N/A: Not NIH Funded	VanWormer JJ, Bendixsen CG, Shukla SK. Dairy Farm Work and Protection from Gastrointestinal Illness. Journal of agromedicine. 2023 October;28(4):640-646. PubMed PMID: 37128886; DOI: 10.1080/1059924X.2023.2209091.

C.2 WEBSITE(S) OR OTHER INTERNET SITE(S)

NOTHING TO REPORT

C.3 TECHNOLOGIES OR TECHNIQUES

NOTHING TO REPORT

C.4 INVENTIONS, PATENT APPLICATIONS, AND/OR LICENSES

Have inventions, patent applications and/or licenses resulted from the award during the reporting period? No

If yes, has this information been previously provided to the PHS or to the official responsible for patent matters at the grantee organization? No

C.5 OTHER PRODUCTS AND RESOURCE SHARING

Category	Explanation
Data or Databases	We employed functional metagenomic selections to identify novel antibiotic resistance genes (ARGs) encoded in the human and cow guts. Our functional screens yielded We 2,049 ARGs, more than half originating from cows and most not present in the existing ARG databases. By combining the ARGs from our functional screens with the reference genes from existing ARG catalogues, we generated a reference set with 31,333 sequences, which, to our knowledge, is the largest ARG dataset in existence. As part of the upcoming manuscript detailing our work (currently in review), we will be making our extended ARG dataset, along with the detailed instructions for its use, available to the research community.

D. PARTICIPANTS

D.1 WHAT INDIVIDUALS HAVE WORKED ON THE PROJECT?

Commons ID	S/K	Name	Degree(s)	Role	Cal	Aca	Sum	Foreign Org	Country	SS
GDANTAS	Y	Dantas, Gautam	BA,PHD	PD/PI	0.8	0.0	0.0			NA
SHUKLAS	Y	SHUKLA, SANJAY K	PHD	PD/PI	2.2	0.0	0.0			NA
VANWORMERJJ	N	VANWORMER, JEFFREY J.	PHD	Co-Investigator	0.5	0.0	0.0			NA
BENDIXC	N	BENDIXSEN, CASPER G.	PHD	Co-Investigator	0.3	0.0	0.0			NA
BEJANMAHMUD	N	Mahmud, Bejan	BS,MS,PHD	Graduate Student (research assistant)	1.7	0.0	0.0			NA
ESSEKHAN	N	Aigbokhan, Esse	BS	Graduate Student (research assistant)	2.8	0.0	0.0			NA
	N	Shrestha, Ram Babu		Research Associate	2.4	0.0	0.0			NA
	N	Thao, Le		Research Associate	3.1	0.0	0.0			NA
	N	Kronholm, Erik		Programmer	2.6	0.0	0.0			NA
	N	Kitchner, Terri		Technician	3.3	0.0	0.0			NA
	N	Patel, Sanket		Technician	1.3	0.0	0.0			NA
	N	Koshalek, Kyle		RC/PM	1.1	0.0	0.0			NA
	N	Presson, Martha		Technician	4.6	0.0	0.0			NA

Glossary of acronyms:
S/K - Senior/Key
Cal - Person Months (Calendar)
Aca - Person Months (Academic)
Sum - Person Months (Summer)
Foreign Org - Foreign Organization Affiliation
SS - Supplement Support
RS - Reentry Supplement
DS - Diversity Supplement
OT - Other
NA - Not Applicable

D.2 PERSONNEL UPDATES

D.2.a Level of Effort

Not Applicable

D.2.b New Senior/Key Personnel

Not Applicable

D.2.c Changes in Other Support

Not Applicable
D.2.d New Other Significant Contributors Not Applicable
D.2.e Multi-PI (MPI) Leadership Plan Not Applicable

E. IMPACT

E.1 WHAT IS THE IMPACT ON THE DEVELOPMENT OF HUMAN RESOURCES?

Not Applicable

E.2 WHAT IS THE IMPACT ON PHYSICAL, INSTITUTIONAL, OR INFORMATION RESOURCES THAT FORM INFRASTRUCTURE?

NOTHING TO REPORT

E.3 WHAT IS THE IMPACT ON TECHNOLOGY TRANSFER?

Not Applicable

E.4 WHAT DOLLAR AMOUNT OF THE AWARD'S BUDGET IS BEING SPENT IN FOREIGN COUNTRY(IES)?

NOTHING TO REPORT

G. SPECIAL REPORTING REQUIREMENTS SPECIAL REPORTING REQUIREMENTS

G.1 SPECIAL NOTICE OF AWARD TERMS AND FUNDING OPPORTUNITIES ANNOUNCEMENT REPORTING REQUIREMENTS

NOTHING TO REPORT

G.2 RESPONSIBLE CONDUCT OF RESEARCH

Not Applicable

G.3 MENTOR'S REPORT OR SPONSOR COMMENTS

Not Applicable

G.4 HUMAN SUBJECTS

G.4.a Does the project involve human subjects?

Not Applicable

G.4.b Inclusion Enrollment Data

NOTHING TO REPORT

G.4.c ClinicalTrials.gov

Does this project include one or more applicable clinical trials that must be registered in ClinicalTrials.gov under FDAAA?

G.5 HUMAN SUBJECTS EDUCATION REQUIREMENT

NOT APPLICABLE

G.6 HUMAN EMBRYONIC STEM CELLS (HESCS)

Does this project involve human embryonic stem cells (only hESC lines listed as approved in the NIH Registry may be used in NIH funded research)?

No

G.7 VERTEBRATE ANIMALS

Not Applicable

G.8 PROJECT/PERFORMANCE SITES

Not Applicable

G.9 FOREIGN COMPONENT
No foreign component
G.10 ESTIMATED UNOBLIGATED BALANCE
Not Applicable
G.11 PROGRAM INCOME
Not Applicable
G.12 F&A COSTS
Not Applicable

I. OUTCOMES

I.1 What were the outcomes of the award?

Globally, half a billion people are employed in animal agriculture and are directly exposed to zoonotic microorganisms. However, the extent to which such exposures affect the resident human microbiomes remain unknown. Here, we address the above gap in knowledge by conducting an in-depth investigation of the nasal and gut microbiomes of dairy farmers and their cows. To determine the microbial signatures associated with livestock farming, we included in our investigation a comparator cohort of age-, sex-, and ZIP code-matched people with no occupational exposures to farm animals. Lastly, our sampling was longitudinal in nature, covering five seasons; in all, we analyzed 712 fecal and 726 nasal samples from 66 farmers, 60 non-farmers, and 166 cows, representing, to our knowledge, the most comprehensive study and dataset of its kind. We applied a combination of both 16S and shotgun metagenomic sequencing to this extensive sample set to demonstrate the acquisition of cow-associated microbes by farmers, with this acquisition being most apparent in the nasal ecosystem. To profile gut resistomes, we first employed functional metagenomic selections to identify novel and known antibiotic resistance genes (ARGs) that are amenable to horizontal gene transfer. We identified 2,049 functionally screened ARGs, more than half originating from cows and most not present in the existing ARG databases. By combining the ARGs from our functional screens with the reference genes from existing ARG catalogues, we generated a reference set with 31,333 sequences, which, to our knowledge, is the largest ARG dataset in existence. Using this reference set, we show that the acquired livestock microbes introduce encoded ARGs into the farmer gut, where the genes subsequently disseminate to new bacterial hosts via mobile genetic elements. In one notable example, we find a resistance-encoding mobile cassette that is enriched in the cow gut but has disseminated to resident microbes of the farmer gut; notably, this same cassette is also found in clinical bacterial isolates reported elsewhere, directly tying the selection for antimicrobial resistance in farm animals to human health.