AWARD NUMBER: W81XWH-18-1-0749

TITLE: Mammary Tissue Host-Microbiome Interactions in Breast Cancer Development

PRINCIPAL INVESTIGATOR: Leah T. Stiemsma

CONTRACTING ORGANIZATION: Pepperdine University
Malibu, CA 90263

REPORT DATE: September 2019

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland, 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
Sep 2019	Annual	30 Sep 2018 –29 Sep 2021

4. TITLE AND SUBTITLE

Mammary Tissue Host-Microbiome Interactions in Breast Cancer Development

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-18-1-0749

5c. PROGRAM ELEMENT NUMBER

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

6. AUTHOR(S)

Leah T Stiemsma

E-Mail: leah.stiemsma@pepperdine.edu

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Pepperdine University
24255 Pacific Coast Hwy
Malibu CA, 90263

8. PERFORMING ORGANIZATION REPORT

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Through this project we will determine the role of the mammary tissue microbiome in breast cancer development using 16S ribosomal RNA sequencing and dual-transcriptomic sequencing. In the first year of this project we have selected and received 165 samples from the Susan G. Komen and Indiana University Simon Cancer Center Tissue Banks. Isolation of DNA for 16S rRNA sequencing is well underway and we anticipate submission of DNA for sequencing in December 2019 and results from this portion of this project in March 2020. We are currently analyzing pilot dual-RNA sequencing data, which will inform any procedural modifications we should make to our RNA isolation protocols. We will use the 16S data to inform the depth of coverage necessary to conduct this type of novel transcriptomic sequencing. As such, the RNA sequencing analysis will be delayed until late Spring/early Summer 2020. Thus far, the project is well underway. Results from this work will be key in characterizing host-microbiome cross-talk in the pathogenesis of breast tumor development.

15. SUBJECT TERMS

Mammary tissue, microbiome, bacteria, breast cancer

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	15	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

1. INTRODUCTION

This study aims to characterize the microbiome of mammary tissue donated from three groups of women: healthy women (healthy tissue, n = 50), women who were healthy at the time of tissue donation but later developed breast cancer (pre-diagnostic tissue, n = 15), and women with breast cancer. From the women with breast cancer we will study two tissue types: tissue adjacent to the malignant tumor (adjacent normal, n = 50) and tumor tissue (n = 50). We will employ 16S ribosomal RNA gene sequencing to analyze the composition of the mammary tissue microbiome among these four tissue types. In addition to studying the composition of the mammary microbiome, we also aim to study its function and interaction with its human host. We will perform dual-transcriptomic sequencing, whereby we extract and sequence mammary tissue microbial community RNA and human RNA from the same tissue. Using a multivariate linear modeling strategy, we aim to determine which bacterial taxa and genes are associated with the human transcriptome. Further, we will analyze whether these associations are modified by cancer status. Collectively, these analyses will determine the mechanistic role of the mammary tissue microbiome in breast cancer development.

2. KEYWORDS

Mammary tissue, microbiome, bacteria, breast cancer, pre-diagnostic, adjacent normal, tumor, 16S ribosomal RNA, meta-transcriptome, next generation sequencing

3. ACCOMPLISHMENTS

3a. What were the major goals of the project?

Training:
Major Task 1: Training and educational development in breast cancer research

- Subtask 1: Present research at Pepperdine Natural Sciences Divisional Seminars, attend UCLA microbiome club meetings (quarterly), attend seminars at the Jonsson Comprehensive Cancer Center. **- Ongoing**
- Subtask 2: Attend one national scientific meeting in a relevant scientific field in Y2 and in Y3. **- Ongoing**
- Subtask 3: Maintain regular mentorship meetings with Dr. Michels at UCLA (twice monthly via teleconference or in person); Dr. Binder at UCLA (once monthly via teleconference or in person), and Dr. Marino at IU (once quarterly via teleconference). I will also travel to IU in the Summer of Y2 and Y3 for collaborative meetings with Dr. Marino. - **Ongoing**
- Subtask 4: Audit relevant courses in cancer epidemiology and computational biology in Y2 and Y3. **– To do**

- Subtask 5: Attend an RNA-sequencing workshop in Summer of Y1 **– Completed: month 11**

Research:

Specific Aim 1: There is a distinct mammary tissue microbiome compositional signature associated with breast cancer development. - Ongoing

Major Task 1: Selection and shipping of samples - Complete

- Subtask 1: Selecting and requesting breast tissue samples from the Komen Tissue Bank (KTB), ordering supplies and equipment. – **Complete,** Projected timeline: months 1 – 7; **completed: month 8**
- Ship tissue specimens to Dr. Stiemsma and prepare for DNA/RNA isolation (from IU to Pepperdine). – **Complete,** Projected timeline: months 1 – 8; **completed: month 9**

Major Task 2: 16S rRNA sequencing of breast tissue samples - Ongoing

- Subtask 1: DNA will be isolated from breast tissue samples at Pepperdine. – **Ongoing**, projected timeline: months 8 – 10; **anticipated completion: month 15**
- Subtask 2: 16S rRNA sequencing of DNA samples at UCLA microbiome core – **To Do**, projected timeline: months 10 – 12; **anticipated completion: month 17**
- Subtask 2: Preprocessing and analysis of 16S rRNA gene sequencing data – **To Do**, projected timeline: months 12 – 15; **anticipated completion: month 18**
- Subtask 4: Manuscript preparation and submission – **To Do**, projected timeline: 18; **anticipated completion: month 21**

Specific Aim 2: Distinct microbial functional variations are associated with healthy, pre-cancerous, and cancerous tissue. - Ongoing

Major Task 1: RNA extraction – Ongoing, projected timeline: months 8 – 10; **anticipated completion: month 19.** Further details discussed in '*Actual or anticipated problems or delays and actions or plans to resolve them*'.

Major Task 2: Dual-RNA sequencing – To Do

- Subtask 1: Dual-RNA sequencing performed at USC Molecular Genomics Core – **To Do**, projected timeline: months 10 - 12; **anticipated completion: month 21**
- Subtask 2: Sequence preprocessing and filtering to identify the microbial transcriptomic profile – **To Do**, projected timeline: months 15 - 18; **anticipated completion: month 23**
- Subtask 3: Analysis of associations of microbial functional groups and cancer status – **To Do**, projected timeline: months 18 - 22; **anticipated completion: month 25**
- Subtask 4: Integration and analysis of microbial transcriptome with microbiome 16S data using MaAsLin (multivariate analysis with linear modeling) and analysis of effect modification by cancer status - **To Do**, projected timeline: 26 – 30; **anticipated timeline: month 27**

- Subtask 5: Manuscript preparation and submission – **To Do**, projected timeline: month 30; **on track**

Specific Aim 3: The microbiome interacts with the host transcriptome to drive cancer development - To Do

Major Task 1: Selection of genes associated with immune, anti-apoptotic, and pro-proliferative pathways and association with cancer status - To Do, projected timeline: months 26 - 30; **on track**

Major Task 2: Integration and analysis of microbial and host transcriptomic profiles using MaAsLin and analysis of effect modification by cancer status - **To Do,** projected timeline: months 30 - 33; **on track**

- Subtask 1: Manuscript preparation and submission – **To Do**, projected timeline: 36, **on track**

3b. What was accomplished under these goals?

All major accomplishments are bolded.

AIM I: There is a distinct mammary tissue microbiome compositional signature associated with breast cancer development.

Sample Selection: **We selected and have received 165 tissue samples from the Susan G. Komen (KTB) and Indiana University Simon Cancer Center (IUSCC) tissue banks.** We used a cutoff of 100mg of fresh, frozen tissue as our primary requirement before applying additional selection criteria.

The following criteria were used to identify samples from 50 healthy control and 15 pre-diagnostic subjects from the KTB: no prior breast surgery within three months of donating, no lactation or pregnancy at the time of sample collection, no personal history of cancer or benign breast disease, and no antibiotic use at the time of sample collection. Similar criteria were used to identify 50 adjacent normal and 50 tumor samples from the IUSCC (no lactation or pregnancy at the time of sample collection, no prior breast disease (where possible), and no antibiotic use at the time of sample collection) with the exception of lack of breast surgery and no personal history of cancer. Of these 100 samples, 46 are from the same women (matched adjacent normal and tumor samples). We preferentially selected samples from women with both adjacent normal and tumor samples available. However, these matched pairs were limited given our selection criteria and samples with enough tissue available for our planned analyses.

Dr. Marino (Indiana University) was key in identifying these samples and was responsible for shipping the samples to Dr. Stiemsma at Pepperdine University in Malibu, California. Dr. Stiemsma received all the samples by mid-June 2019. In addition to the tissue samples identified from these women, the following metadata

was requested (received in August 2019) from the tissue banks: age, prior breastfeeding, prior breast surgery, BMI category, parity, menopausal status, cancer therapy, medications, BRCA1/2 positive, age at menarche, age at first birth, alcohol consumption, postmenopausal hormone therapy, diet, and physical activity.

DNA Isolation: **From April – June 2019, DNA isolation protocols were established for appropriate isolation of DNA from the breast tissue. In Summer 2019, metagenomic DNA was isolated from 50mg of 130 of 165 of these tissue samples (remaining samples are currently being processed)** using the AllPrep PowerFecal DNA/RNA kit from Qiagen. We modified the lysis procedure to include 50uL of proteinase K, an hour-long heating step, and bead beating homogenization to ensure isolation of both human and microbial DNA. Once all of the DNA is isolated from these samples, we will submit the cohort for 16S ribosomal RNA gene sequencing (projected submission in December 2019).

AIM II: Distinct microbial functional variations are associated with healthy, pre-cancerous, and cancerous tissue.

RNA Isolation and meta-transcriptomic sequencing: To ensure the highest quality of RNA possible prior to submitting RNA samples from the full cohort for dual-transcriptomic sequencing, **we conducted a pilot dual-transcriptomic sequencing run in August 2019. This pilot sequencing run was conducted on 5 breast tissue samples and will be analyzed in Fall 2019**.

In addition, the dual-transcriptomic sequencing of host and microbial DNA is a novel approach to studying host-microbiome interaction. Specifically, this dual-transcriptomic technique involves a double ribosomal RNA depletion for both human and microbial RNA. Further, the depth of sequencing suggested is ~20 – 30 million reads for human gene expression and 10 – 15 million reads for microbial gene expression. To ensure we sequence to the appropriate depth, we will use the 16S RNA gene sequencing analysis to inform the depth of coverage needed to characterize the microbial community. For example, if the microbial community of mammary tissue is low in diversity, less reads may be needed for accurate coverage of the microbial meta-transcriptome. Upon analysis of the microbiome 16S data and RNA sequencing pilot data, we will begin isolating RNA from the remainder of the sample available (~50mg) for all samples in our cohort. We anticipate submitting RNA for meta-transcriptomic sequencing in late Spring/early Summer 2019.

qPCR analyses: Using the Qiagen AllPrep PowerFecal DNA/RNA kit, we can isolate DNA and RNA in parallel from the same tissue sample. RNA isolated in this way is not of high enough quality to conduct dual-transcriptomic sequencing; however, it is of high enough quality for quantitative PCR to analyze specific host and microbial genes. **Annie Kump, a student in my lab has already generated cDNA for 60 of these samples (15 per cancer category). She will use this cDNA to analyze the**

role of short chain fatty acids in host-microbiome cross-talk in the human breast via quantitative PCR.

AIM III: The microbiome interacts with the host transcriptome to drive cancer development.

All planned analyses for this aim require the 16S and meta-transcriptomic sequencing data. We are excited to discuss details of this analysis in future reports.

3c. What opportunities for training and professional development has the project provided?

Workshops: In August 2019, I attended the UC Davis Bioinformatics Core RNA Sequencing Workshop. This was an exceptional workshop, which provided me with invaluable information regarding RNA isolation and preparation for sequencing, sequence pre-processing, and analysis of RNA sequencing data. I was also able to discuss the goals of my project one-on-one with instructors at this workshop, which gave me valuable insight into the best approaches for sequencing these samples and the tools necessary to do so. Additionally, I learned the computational pipeline to preprocess meta-transcriptomic data and analyze the data for differential gene expression.

Conference Attendance in 2020: I will be attending one of the following conferences in 2020 (The American Association for Cancer Research – The Microbiome and Viruses in February 2020 or The American Society of Microbiology – ASM Microbe in June 2020) to present our 16S ribosomal RNA sequencing data from this project. In addition, I plan to attend the Center of Microbiome Innovation International Meeting in March 2020.

Student Mentoring: I was also able to engage a number of undergraduate students in this research over the past year. Specifically, in Summer 2019 I participated in Pepperdine's Summer Undergraduate Research in Biology Program (SURB). Through this program, I mentored and supervised three undergraduate students, all of whom contributed to this research project through DNA/RNA isolation, establishment of protocols, or quantitative PCR of host and microbial genes. This Fall, two of these summer students will continue to work on this project and one new student is joining the lab. All three students will work with me over the following academic year to complete the tasks outlined in the sections above and will attend at least one of the conferences listed above to present their data with me. This experience has served as professional development not only for these students, but for me as well. As I further develop my own research program, these mentoring/supervisory experiences are crucial to my development as an independent investigator.

3d. How were the results disseminated to communities of interest?

Dissemination of Research Through the Summer Undergraduate Research in Biology Program at Pepperdine (Summer 2019): In Summer 2019, I participated a mentor in the Summer Undergraduate Research in Biology Program (SURB) at Pepperdine. Through this program, undergraduates gain experience working in a research lab; developing hypotheses, writing proposals, giving presentations, and conducting lab work. I was a mentor to three undergraduate students (Annie Kump, Daniel Herrera, and Courtney Hoskinson) through this program.

Throughout the summer, these students engaged with 10 other SURB students, Pepperdine faculty, and with non-Pepperdine scientists (guest speakers), discussing their results and receiving feedback/troubleshooting support. In addition, at the end of the Summer (July 2019), my students gave oral presentations open to the Pepperdine community, and presented posters at the SURB Poster Session and Summer Undergraduate Research Banquet. Through this banquet, our research was disseminated to Pepperdine faculty, research students, family members, local ecological staff/scientists, Pepperdine staff, and Pepperdine administrators.

Dissemination of Research Through Conference Attendance in 2020: My current research students and I plan to attend one of the following conferences in 2020 (The American Association for Cancer Research – The Microbiome and Viruses in February 2020 or The American Society of Microbiology – ASM Microbe in June 2020) to present our 16S ribosomal RNA sequencing data from this project. In addition, I plan to attend the Center of Microbiome Innovation International Meeting in March 2020.

3e. What do you plan to do during the next reporting period to accomplish the goals?

AIM I: There is a distinct mammary tissue microbiome compositional signature associated with breast cancer development.

DNA Isolation: We will continue isolating DNA from the remaining breast tissue samples. Once all of the DNA is isolated from these samples, we will submit the cohort for 16S ribosomal RNA sequencing (projected submission in December 2019).

Analysis and Manuscript preparation/submission: Anticipated date for manuscript submission in June 2019.

AIM II: Distinct microbial functional variations are associated with healthy, pre-cancerous, and cancerous tissue.

RNA Isolation and meta-transcriptomic sequencing: We will wait for the 16S rRNA sequencing results (anticipated completion - March 2020) and analysis of our pilot RNA sequencing run (anticipated completion – December 2019) to inform the depth of coverage needed to perform the dual-meta-transcriptomic sequencing we

propose. We anticipate submitting RNA for sequencing in late Spring/early Summer 2020.

Analysis and Manuscript preparation/submission: We will analyze the RNA sequencing data in Summer 2020, with an anticipated date for manuscript submission in Fall 2020.

AIM III: The microbiome interacts with the host transcriptome to drive cancer development.

All planned analyses for this aim require the 16S and meta-transcriptomic sequencing data. We are excited to discuss details of this analysis in future reports.

4. IMPACT

4a. What was the impact on the development of the principal discipline(s) of the project?

Nothing to report for this reporting period. Below we discuss the future impact of this project.

Using these highly innovative techniques, this work will inform future studies focusing on breast cancer development, specifically studies of the breast tissue microbiome. If we identify particular breast tissue bacteria or bacterial/human genes that interact to promote breast cancer, future studies can address further, how these interactions contribute to breast cancer risk. Alternatively, if we identify any bacteria or bacterial/human genes that interact to protect against breast cancer, future studies can address further the mechanisms by which these interactions protect against this disease. Future clinical applications of our work include targeting breast tissue bacteria or genes prior to or after breast cancer onset. As this has not been previously investigated, our work represents a crucial step toward ending breast cancer.

4b. What was the impact on other disciplines?

Nothing to report for this reporting period. Below we discuss impacts on other disciplines following completion of this study.

Impact on clinicians: In the last decade, the role of the microbiome has been highlighted in many human diseases. Through research of the human microbiome, new potential avenues for treatment and protection from diseases have been developed. However, the role of the mammary microbiome in breast cancer and general breast health has not been fully elucidated. Our study will play a key role in determining how

these microbes are involved in this disease, which will impact future treatment options and preventive strategies used by clinicians in this field.

Impact on fields of computational biology and bioinformatics: Through this project, we will be employing a novel sequencing technique (dual-transcriptomic sequencing) by which we sequence the host and microbial transcriptome. In this way, we sequence all of the host and microbial genes and differentiate them *in silico.* This technique has yet to be performed in the context of the breast tissue microbiome, and is primarily used to study host-pathogen interactions rather than host-microbiome interactions. Thus, our execution of this technique will inform the fields of computational biology and bioinformatics of the technical/computational requirements necessary to perform this type of analysis.

4c. What was the impact on technology transfer?

Nothing to report.

4d. What was the impact on society beyond science and technology?

Nothing to report.

5. CHANGES/PROBLEMS:

5a. Changes in approach and reasons for change

Nothing to report.

5b. Actual or anticipated problems or delays and actions or plans to resolve them

There are no changes to the scope of this project and the project is well underway. However, the time needed to isolate nucleic acids from the tissue samples is taking longer than anticipated. The samples for this project did not arrive until mid-Summer 2019, affecting our ability to complete DNA isolations according to the original timeline (September 2019). We expect to have DNA isolated and submitted for 16S rRNA sequencing by December 2019. To ensure we apply the appropriate amount of coverage to our meta-transcriptomic analysis, are waiting to isolate RNA for dual-transcriptomic sequencing until we review the 16S rRNA sequencing results and the meta-transcriptomic pilot sequencing run. For this reason, the RNA sequencing of these samples will likely not take place until late Spring/early Summer 2020. This delay however, will not result in any additional expenses.

5c. Changes that had a significant impact on expenditures

We do not anticipate completing the 16S rRNA sequencing until March 2020 and the RNA sequencing until Fall 2020. Thus, the funds set aside for these portions of the project in year 1, will not be spent until year 2.

5d. Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Significant changes in use or care of human subjects

Nothing to report.

Pepperdine Institutional Review Board Approval Dates: August 2018 – end of study, assuming no significant changes.

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:

Publications, conference papers, and presentations

Journal publications

Nothing to report.

Books or other non-periodical, one-time publications

Nothing to report.

Other publications, conference papers and presentations.

Two students (Annie Kump and Daniel Herrera) presented posters on this project at the Summer Undergraduate Research in Biology Program Summer Poster Session at Pepperdine University – July 2019.

Website(s) or other Internet site(s)
Nothing to report.

Technologies or techniques

Nothing to report.

Inventions, patent applications, and/or licenses

Nothing to report.

Other Products

Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

7a. What individuals have worked on the project?

Name:	Leah Stiemsma
Project Role	PI
Nearest Person Month Worked:	1.12 calendar months & 2.5 summer months
Contribution to the Project:	Dr. Stiemsma directs the overall project. Dr. Stiemsma works alongside the undergraduate students identified below to prepare all samples for microbiome and transcriptome analysis (DNA and RNA extraction). She will also analyze the microbiome sequencing data and, with guidance from Dr. Marino (Indiana University) and Dr. Binder (UCLA), will analyze associations between the breast tissue transcriptome and microbiome.
Funding Support:	Breakthrough Fellowship Award – Department of Defense

Name:	Karin B. Michels
Project Role	Mentor
Nearest Person Month Worked:	0.24 calendar months
Contribution to the Project:	Mentor

Funding Support:	U01 from NIH/NCI/NIEHS

Name:	Alexandra M. Binder
Project Role	Mentor
Nearest Person Month Worked:	0.24 calendar months
Contribution to the Project:	Mentor, statistical support
Funding Support:	K07 from NIH/NCI

Name:	Natascia Marino
Project Role	Mentor
Nearest Person Month Worked:	0.24 calendar months
Contribution to the Project:	Mentor, bioinformatic support to analyze the RNA sequencing data, and assisted with sample selection.
Funding Support:	

Name:	Annie Kump
Project Role	Undergraduate Student
Nearest Person Month Worked:	6.00 Calendar Months
Contribution to the Project:	Annie contributes by isolating DNA and RNA from the tissue samples.
Funding Support:	Undergraduate research fellowship

Name:	Courtney Hoskinson
Project Role	Undergraduate Student
Nearest Person Month Worked:	6.00 calendar Months
Contribution to the Project:	Courtney contributes by isolating DNA and RNA from the tissue samples. She will assist Dr. Stiemsma with the RNA sequencing pilot data analysis.
Funding Support:	Undergraduate research fellowship

Name:	Daniel Herrera
Project Role	Undergraduate Student
Nearest Person Month Worked:	3.00 calendar Months
Contribution to the Project:	Daniel contributed by isolating DNA and RNA from the tissue samples.
Funding Support:	Undergraduate research fellowship

Name:	Jaelyn Gabel
Project Role	Undergraduate Student
Nearest Person Month Worked:	6.00 calendar Months
Contribution to the Project:	Jaelyn contributes by isolating DNA and RNA from the tissue samples. She will also assist Dr. Stiemsma with the 16S rRNA

	sequencing analysis.
Funding Support:	Undergraduate research fellowship

7b. Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Dr. Alexandra Binder is now funded at 75% effort on a KO7 grant from the National Cancer Institute, National Institutes of Health (award number: K07 CA225856). She was previously funded by a U01 grant from the National Cancer Institute/National Institute of Environmental Health Sciences, National Institutes of Health (U01ES026130). This change does not affect her effort level as a mentor on this project.

7c. What other organizations were involved as partners?

Organization Name: Indiana University
Location: Indiana, United States
Partner's Contribution: Collaboration/Sub-contract

Organization Name: University of California, Los Angeles
Location: California, United States
Partner's Contribution: Collaboration/Mentorship

8. SPECIAL REPORTING REQUIREMENTS

Not applicable.