AWARD NUMBER: W81XWH-18-1-0557

TITLE: Composition, Function, and Role of the Intestinal Microbiome in Pediatric Heart Failure and Heart Transplantation

PRINCIPAL INVESTIGATOR: Joseph A. Spinner, MD

CONTRACTING ORGANIZATION: Baylor College of Medicine

REPORT DATE: JUNE 2022

TYPE OF REPORT: Final Progress Report

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

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
JUNE 2022	Final Report	09/01/2018 – 02/28/2022

4. TITLE AND SUBTITLE

Composition, Function, and Role of the Intestinal Microbiome in Pediatric Heart Failure and Heart Transplantation

5a. CONTRACT NUMBER

W81XWH-18-1-0557

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Joseph Spinner, MD

E-Mail: spinner@bcm.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

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

Baylor College of Medicine
One Baylor Plaza
Houston, TX 77030

8. PERFORMING ORGANIZATION REPORT NUMBER

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

U.S. Army Medical Research and Development 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

The role of the intestinal microbiome has gained substantial interest as a novel marker for prognosis of disease and as a potential target for therapeutic intervention. There is evidence that the microbiome exerts a fundamental influence on immunity, can be altered in heart failure, and can be further disrupted by immunosuppressive medications. This indicates the potential significant impact that alterations to the intestinal microbiome may play in the care of children who have undergone heart transplant. The composition, function, and role of the intestinal microbiome in children that undergo heart transplant is not currently known. The main objective of this research is to characterize and investigate the role of the intestinal microbiome in this population and generate the preliminary data necessary to determine effect estimates that will be used to power large prospective randomized studies of targeted microbial restoration. Over 300 stool samples were collected and analyzed from 105 patients. Post-transplant samples had significantly less overall bacterial diversity compared to pre-transplant samples. Patients that underwent heart transplant during the study period had significant microbial compositional shifts early post-transplant. Furthermore, heart transplant patients with both early post-transplant rejection and later post-transplant rejection had specific microbial compositional changes compared to those without rejection. As this study is prospective in nature, there is still on-going long-term follow up of the patients from whom stool samples were collected.

15. SUBJECT TERMS

Intestinal Microbiome, Pediatric Cardiology, Congenital Heart Disease, Heart Transplant, Diarrhea

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRDC
Unclassified	Unclassified	Unclassified	Unclassified	13	19b. TELEPHONE NUMBER *(include area code)*

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

TABLE OF CONTENTS

1. INTRODUCTION:

The role of the intestinal microbiome has gained substantial interest as a novel marker for diagnosis and prognosis of disease and as a potential target for therapeutic intervention. There is evidence that the microbiome exerts a fundamental influence on innate and adaptive immunity, can be altered in heart failure, can shift rapidly during intestinal ischemia and reperfusion, and can be further disrupted by immunosuppressive medications. This indicates the potential significant impact that alterations to the intestinal microbiome may play in the care of children who have undergone heart transplant. Furthermore, the success of fecal microbial transplant in patients with *Clostridium difficile* diarrhea has demonstrated that the microbiome is potentially modifiable and indicates the therapeutic potential of microbiome restoration to improve the duration and severity of diarrheal disease. The composition, function, and role of the intestinal microbiome in children and young adults with congenital heart disease or heart transplant is not currently known. Our **long-term goal** is to identify modifiable risk factors and develop innovative treatment strategies to improve outcomes for these patients. The **main objective** of this research is to characterize and investigate the role of the intestinal microbiome in this population and generate the preliminary data necessary to determine effect estimates that will be used to power large prospective randomized studies of targeted microbial restoration. Characterizing the intestinal microbiome in this patient population offers significant potential to greatly impact and improve the health outcomes of individuals with congenital heart disease. Improving post-heart transplant outcomes can also ameliorate the supply-and-demand mismatch crisis of donor organ allocation by reducing the need for re-transplantation.

KEYWORDS:

Intestinal Microbiome, Pediatric Cardiology, Congenital Heart Disease, Heart Transplant, Diarrhea

2. ACCOMPLISHMENTS:

What were the major goals of the project?

The three major goals of this project are to:

1) Prospectively document and compare alterations in the composition and diversity of the intestinal microbiome and associated metabolome in children and young adults listed for heart transplant to the intestinal microbiome and associated metabolome in healthy, age- and sex-matched controls.
2) Prospectively document and compare alterations in the composition and diversity of the intestinal microbiome and associated metabolome in children and young adults before and after placement of a ventricular assist device or heart transplant.
3) Evaluate the association of alterations in the composition and diversity of the intestinal microbiome and associated metabolome in children and young adults with the following post-heart transplant outcomes: diarrhea, systemic infection, coronary allograft vasculopathy, graft rejection, graft failure, and re-transplant or death.

What was accomplished under these goals?

The main objective of this proposal is to characterize and investigate the role of the intestinal microbiome in children that undergo heart transplantation and generate the preliminary data necessary to determine effect estimates that will be used to power large prospective randomized studies of targeted microbial restoration. The target enrollment for the study was projected to be 145 patients. There were a total of 154 patients that were enrolled in the study, which exceeded our goal. From these patients, 316 stool samples were analyzed from 105 patients. Not all patients underwent heart transplant (some are still waiting or died while waiting; those samples have not yet been analyzed). This includes prospective sequential samples from 25 patients with pre-transplant samples who have undergone transplant and from whom sequential post-transplant samples were obtained and analyzed. There were 12 patients that received a ventricular assist device then underwent heart transplant during the study period.

Patient characteristics that were recorded included age, gender, race/ethnicity, type of congenital heart disease or cardiomyopathy, and medications at time of stool collection. In addition, prospective clinical outcomes were collected. Multiple patient outcomes including diarrhea, infection, coronary allograft vasculopathy, graft rejection, graft failure, and death have occurred.

During the study period, 316 stool samples underwent DNA extraction, processing, and sequencing. As stated in the approved SOW, the goal was to have 120 pre-heart transplant samples, 40 post-ventricular assist device samples, and 195 post-heart transplant samples. Despite receiving a no-cost extension in order to permit more time to process and analyze stool samples and continue to perform data analysis with biostatistical support, we did not analyze all pre-transplant specimens nor were we able to complete analysis on 195 post-heart transplant specimens due to issues encountered during the COVID-19 pandemic described below. Metabolomics analysis is still not yet complete as well.

In response to the continuation of the COVID-19 Pandemic, Baylor College of Medicine continued college-wide measures to help limit the spread of the virus and perform responsible conduct of research. Starting in March 2020, the College limited access to research facilities. Following the OMB Flexibility guidelines, researchers were retained on grants during this period when they had both continuity support and direct activities in support of the grant. Research staff continued to have limited access to perform in-person patient enrollment, survey collection, and stool collection, DNA extraction, processing, and sequencing due to Baylor College of Medicine and Texas Children's Hospital restrictions and social distancing mitigation policies.

As per the approved SOW, the plan was to perform data analysis during months 15-18. Performing microbial DNA extraction and sequencing in large batches at the same time allows for quality control to most optimally analyze the stool samples. However, due to the concerted effort at Baylor College of Medicine and Texas Children's Hospital to follow social distancing recommendations, limited access to campus facilities (including research facilities) and a transition to "virtual" patient encounters, the number of stool samples and further bacterial DNA extraction, processing, sequencing, and analysis was significantly affected. Data analysis therefore was performed during the period of the no-cost extension to improve quality control.

Despite the restrictions encountered during the COVID-19 Pandemic, we still analyzed a total of 316 stool samples, and we continued to clinically followed the enrolled patients. During the study period, over 75 clinically significant "events" occurred during the follow-up time.

In total, 316 stool samples underwent bacterial DNA extraction and processing through an Illumina MiSeq 16S sequencing pipeline. Two separate regions of the highly variable areas of the 16S rRNA gene, V1V3 and V4, were targeted for sequencing. Resulting raw sequences were analyzed via the standard analysis pipeline, which utilizes the UPARSE algorithm for clustering of sequences into operational taxonomic units (OTUs) and the SILVA database for taxonomic classification of each OTU. Metabolomic analyses was performed using p180 Kits (Biocrates) on the Ultra-Performance Liquid Chromatography tandem mass spectrometer (AbSciex 6500). At this time of this report, metabolomic analysis is not yet complete.

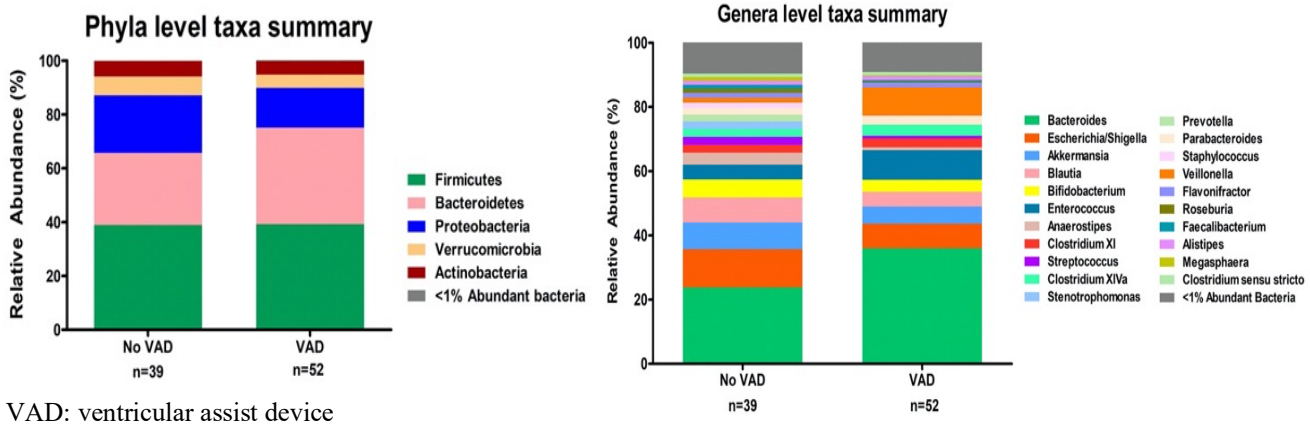
The analysis of stool samples can be broken down into 2 cohorts:

1) prospective cohort: patients with pre-transplant samples that underwent heart transplant during the study period and had multiple post-transplant samples analyzed

2) cross-sectional cohort of patients > 1 year post-transplant (113 samples from 80 patients analyzed; 7 of which obtained during active heart transplant rejection)

When assessing samples obtained from pre-transplant patients, we identified compositional differences at both the phyla and genus levels between patients supported on a ventricular assist device (VAD) vs those patients not supported on a VAD (Figure 1A and Figure 1B). Notable differences included the differences in the abundance of *Bacteroides, Veillonella*, and *Anaerostipes*.

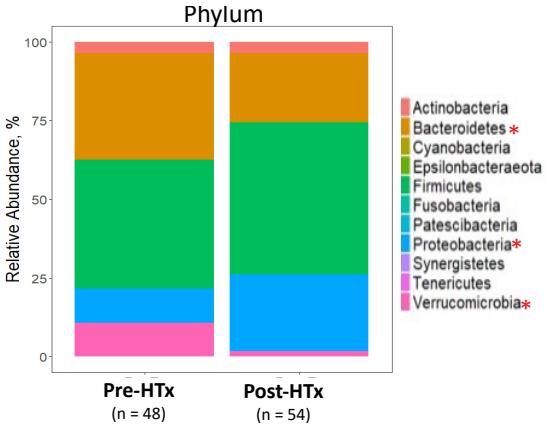
Figures 1A and 1B: Compositional Differences Among Patients Waiting for Heart Transplant: VAD vs no VAD



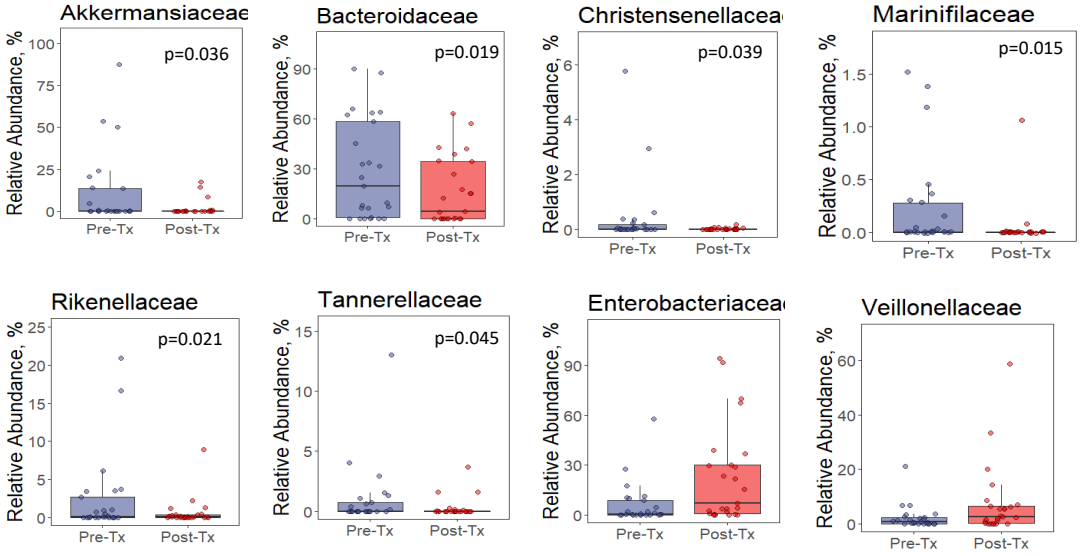
VAD: ventricular assist device

When comparing the pre-transplant samples to the early post-transplant samples (Prospective Cohort 1), we identified compositional differences at both the phyla and family levels (Figure 2A and Figure 2B). At the phylum level, notable differences included a decreased abundance of *Bacteroidetes* (orange), an increased abundance of *Proteobacteria* (blue), and a decreased abundance of *Verrucomicrobia* (pink) in the post-transplant samples. At the family level, compared to the pre-transplant samples (blue), we identified a decreased in the abundance of *Akkermansiaceae*, *Bacteroidaceae*, *Christensenellacea*, *Marinifilacea*, *Rikenellaceae*, and *Tannerellaceae*, and an increase in the abundance of *Enterobacteriaceae* and *Veillonellaceae* in the post-transplant samples (red).

Figures 2A (Phylum) and 2B (Family): Compositional Differences Among Patients Undergoing Heart Transplant: Pre-Transplant vs Post-Transplant

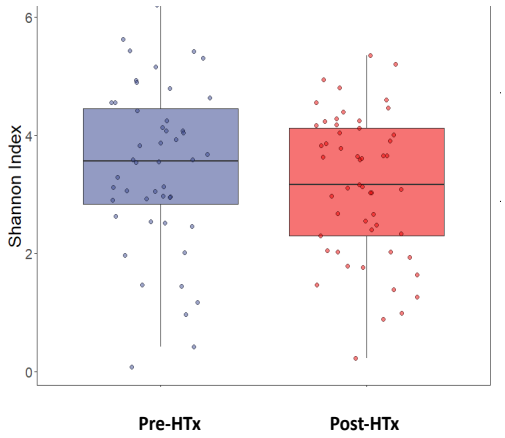


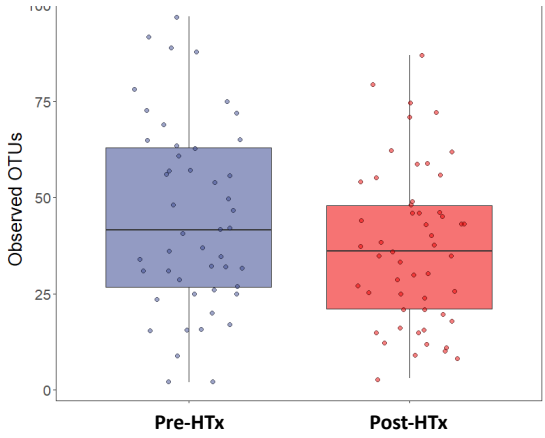
Phylum	Coef	Std Err	p-value
Bacteroidetes	- 0.17	0.08	0.044
Proteobacteria	+ 0.21	0.08	0.015
Verrucomicrobia	- 0.15	0.06	0.035



We also compared the overall level of bacterial diversity between pre-transplant and post-transplant stool samples, as a lower level of bacterial diversity has been associated with worse outcomes among bone marrow transplant recipients. When measuring the level of bacterial diversity either by the alpha diversity measured by the Shannon Diversity Index (Figure 3A) or by the number of operational taxonomic units (OTUs; Figure 3B), we found that post-transplant specimens had lower overall bacterial diversity compared to the pre-transplant specimens.

Figures 3A (Shannon) & 3B (Operational Taxonomic Units): Bacterial Diversity Pre- vs Post-Heart Transplant





	Pre-HTx n=48	Post-HTx n=54	p-value
Days Pre or Post-HTx (med)	44.9 days pre	29.8 days post	
Shannon	3.46 ± 1.38	3.18 ± 1.21	0.046

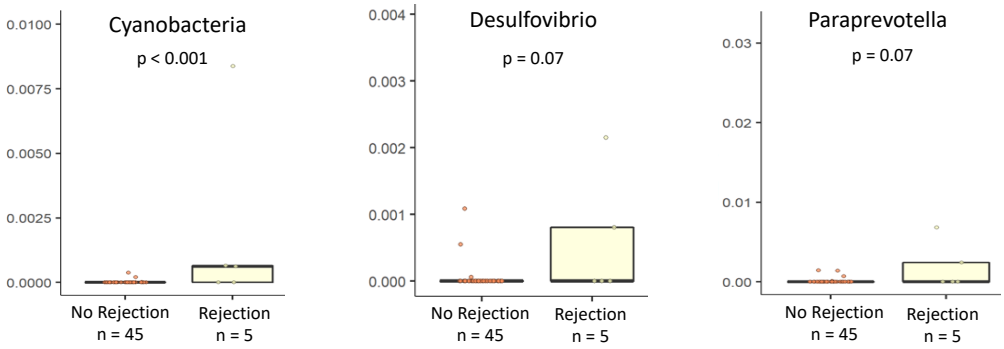
Lme model with patient as random effect

	Pre-HTx n=48	Post-HTx n=54	p-value
Days Pre or Post-HTx (med)	44.9 days pre	29.8 days post	
Observed OTUs	44.9 ± 24.2	37.5 ± 40.4	0.0034

Lme model with patient as random effect

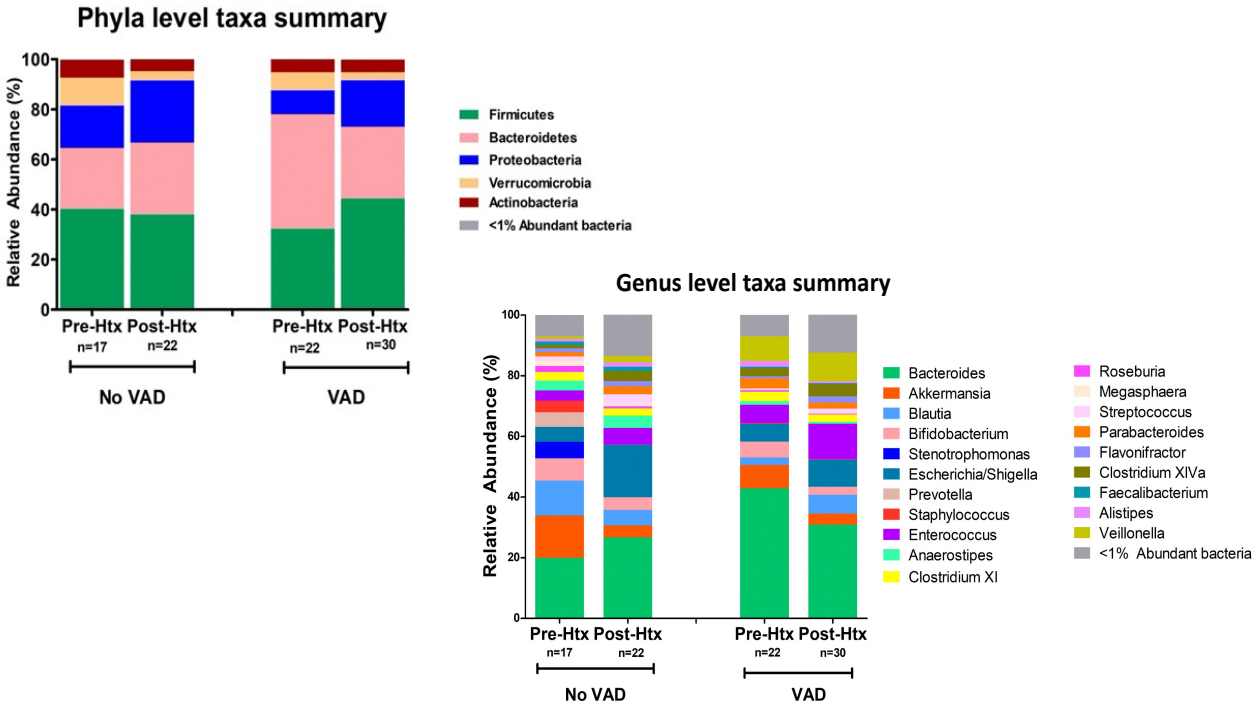
We also evaluated if the degree of bacterial diversity at the immediate pre-transplant sample was associated with a composite outcome of post-transplant infection or rejection ("event") within 1-year post-transplant. Pre-transplant bacterial diversity was not predictive of early post-transplant events (Shannon index "no event" 3.25 +/- 1.09 vs "event" 3.06 +/- 1.52; p = 0.4). However, we did find specific bacterial compositional differences between patients with early heart transplant rejection within 1-year post-transplant vs those that did not (Figure 4)

Figure 4: Specific Pre-HTx Genera and Acute Rejection Within 1-YEAR Post-HTx



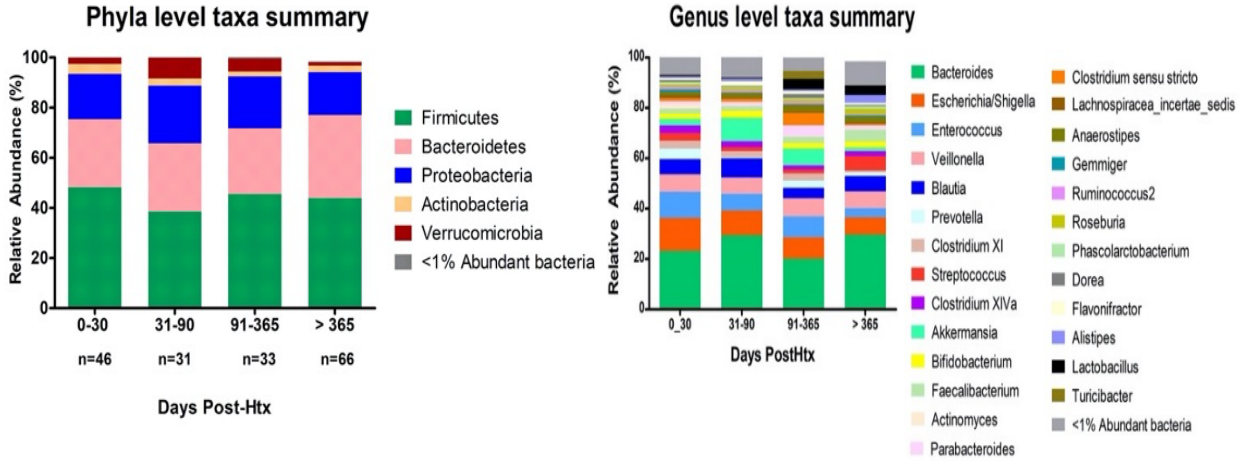
Considering the previous findings of compositional differences existing between pre-transplant patients on a VAD vs pre-transplant patient that were not on a VAD, we also compared the post-transplant samples specifically between those with prior VAD support vs those without prior VAD support. Again, we found compositional differences at the phyla and genus levels (Figures 5A and 5B).

Figures 5A and 5B: Compositional Differences Among Pre-HTx vs Post-HTx Patients Based on VAD support



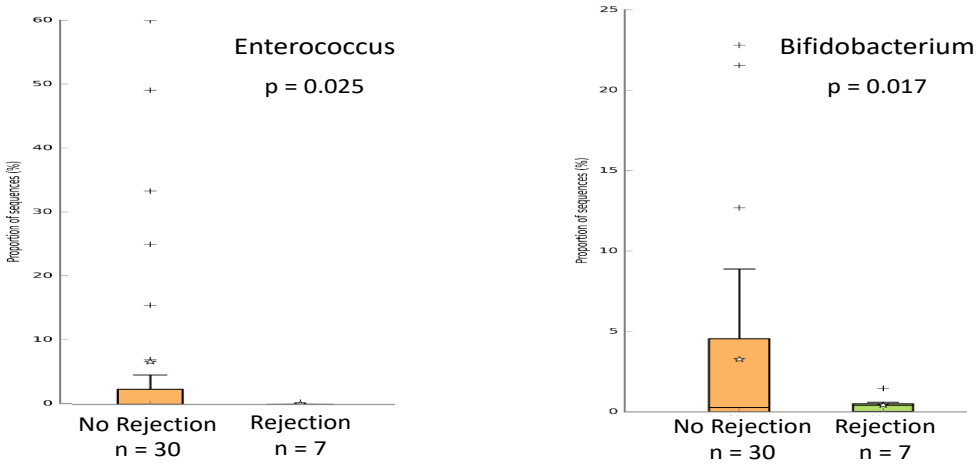
As part of the study, pre-transplant patients were prospectively enrolled pre-transplant and serial stool samples were collected post-transplant. There appear to be compositional shifts depending on time elapsed from transplant (Figures 6A and 6B).

Figures 6A and 6B: Serial Microbial Compositional Changes Post-Transplant



Lastly, we analyzed stoom samples from patients that were more than 1-year post-transplant from the cross-sectional cohort to determine if there were any bacterial compositional changes associated with post-transplant rejection. We identified a significantly higher abundance of *Bifidobacterium* and higher abundance of *Enterococcus* among patients without post-transplant rejection (Figure 7) compared to patients with post-transplant rejection.

Figure 7: Compositional Differences: NO Rejection vs Rejection



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

This project provided me the opportunity to learn the techniques and skills to perform intestinal microbiome stool analysis. This also was an excellent opportunity to improve skills with database management and biostatistics. These skills will permit me the opportunity to seek further funding to investigate the intestinal microbiome in pediatric heart transplant. This has also significantly contributed to my professional development on to the track to becoming an independent investigator.

How were the results disseminated to communities of interest?

We presented multiple abstracts of the work produced by this award in the form of both poster and oral presentations at regional, national, and international scientific meetings. These abstracts were presented at the 2020 International Society for Heart and Lung Transplantation Annual Meeting and Scientific Sessions, 2021 International Society for Heart and Lung Transplantation Annual Meeting and Scientific Sessions, and 2022 International Society for Heart and Lung Transplantation Annual Meeting and Scientific Sessions. There are currently several manuscripts in process that were not published at the time of this submitted report.

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

Not applicable

IMPACT:

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

These data are the first reported investigations of the intestinal microbiome in pediatric heart transplantation. These pilot data will now permit us to seek future funding to complete larger studies and studies that are multi-institutional. Work is also now being completed to determine the functional role of intestinal microbial metabolites produced/metabolized by intestinal bacteria. These future studies may lead to the targeted therapies for microbial restoration (such as with pre- or probiotics) among children with heart failure or undergoing heart transplantation in the future.

What was the impact on other disciplines?

Nothing to report at this time – although some findings may be extrapolated to other populations of children receiving immunosuppression.

What was the impact on technology transfer?

Nothing to report.

What was the impact on society beyond science and technology?

Nothing to report at this time.

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

After the study was initiated, we amended the protocol to include patients under 1 year of age. This permitted us to enroll more patients during the COVID-19 Pandemic. This will also provide us the opportunity in the future to perform a sub-group analysis of patients less than 1 year of age to determine if the intestinal microbial compositional changes are different among infants compared to older children.

Actual or anticipated problems or delays and actions or plans to resolve them

As mentioned previously, in response to the COVID-19 Pandemic, Baylor College of Medicine instituted college wide measures to help limit the spread of the virus and perform responsible conduct of research. Beginning in March 2020, limited access to research facilities was implemented with phased increases of access. Due to the concerted effort at Baylor College of Medicine and Texas Children's Hospital to follow social distancing recommendations, there was limited access to campus facilities (including research facilities) for research staff, and there was a transition to more "virtual" patient encounters. This resulted in fewer stool samples being obtained than was the initial goal as specified in the SOW. Performing DNA extraction, sequencing, and analysis was also delayed and limited during this time. We therefore requested and received a no cost extension and previously expanded our patient population to include children under 1 year of age.

Changes that had a significant impact on expenditures

Nothing to report.

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

During a prior reporting period, we amended the protocol to include patients under 1 year of age. There were no other changes.

Significant changes in use or care of vertebrate animals

Not applicable.

Significant changes in use of biohazards and/or select agents

Not applicable.

6. PRODUCTS:

Publications, conference papers, and presentations

We presented multiple abstracts of the work produced by this award in the form of both poster and oral presentations at regional, national, and international scientific meetings. These abstracts were presented at the 2020 International Society for Heart and Lung Transplantation Annual Meeting and Scientific Sessions, 2021 International Society for Heart and Lung Transplantation Annual Meeting and Scientific Sessions, and 2022 International Society for Heart and Lung Transplantation Annual Meeting and Scientific Sessions. There are currently several manuscripts in process that were not published at the time of this submitted report.

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

Nothing to report.

Other publications, conference papers and presentations.

I have presented our work on multiple occasions at the Baylor College of Medicine Cardiovascular Research Institute and at Texas Children's Hospital research symposiums.

Website(s) or other Internet site(s)

Nothing to report.

Technologies or techniques

Nothing to report.

Inventions, patent applications, and/or licenses

Nothing to report.

Other Products

Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name: Joseph Spinner, MD
Project Role: PI
Researcher Identifier (e.g. ORCID ID): https://orcid.org/0000-0001-9539-6252
Nearest person month worked: 1
Contribution to Project: Dr. Spinner is the project lead & is responsible for the design, implementation, & deliverables.
Funding Support: Baylor College of Medicine Pediatric Cardiology covers salary & protected time for project

Name: Sridevi Devaraj
Project Role: Co-Investigator
Researcher Identifier (e.g. ORCID ID): https://orcid.org/0000-0001-9189-7914
Nearest person month worked: < 1
Contribution to Project: Dr. Devaraj is a co-investigator responsible for sample extractions, sequencing, & metabolomics testing. She also interprets the statistical analysis performed by the biostatisticians at the TCH Microbiome Center.
Funding Support: None

Name: Kelli Noon
Project Role: Research Nurse
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: 1
Contribution to Project: Ms. Noon assisted with clinical data collection and helped keep the database up to date.
Funding Support: None

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period? No

What other organizations were involved as partners?

Nothing to Report.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: Not applicable.

QUAD CHARTS: Not applicable.

9. APPENDICES: None.