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The Role of the Human Gut Microbiome and Inflammation in Heat Related Illness Among Migrant Farmworkers

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B.S. Biobehavioral Health
Pennsylvania State University
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Abstract

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By Christopher Carr

Prolonged exposure to hot environments can lead to a continuum of conditions known as heat related illness (HRI). It has been well documented that the driving force behind the severity of HRI is septicemia. To examine the relationship between the gut microbiome and the body's inflammatory response caused by septicemia, farmworkers exposed to at least two weeks of working in a hot environment were enrolled in a cross-sectional study. Levels of lipopolysaccharide-binding protein (LBP), C-reactive protein, proinflammatory cytokines IL-1-beta, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, TNF-alpha, and IFN-gamma in both the serum and stool of subjects were measured along with gut microbiome alpha and beta diversity. It was hypothesized that individuals with higher levels of LBP would be better adapted to experiencing heat stress and would be protected against HRI. It was also hypothesized that the makeup of the gut microbiome would explain differences between the cases and controls. The results of the analysis found that stool CRP, stool IL-1-Beta, and stool IL-8 levels were significantly higher among cases. There were no significant differences between cases and controls among gut microbiome alpha or beta diversity. These results indicate cases had an increased inflammatory response to heat exposure meaning those with lower levels of CRP, IL-1-Beta, and IL-8 in their stool were less likely to experience HRI. There was also a significant difference between gender for serum LBP, serum IL-8, serum and IL-13. The results of this study highlight the need for further investigate the differences between those that experience HRI and those that do not.

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1. BACKGROUND/LITERATURE REVIEW

A. Introduction the Heat Related Illness

The thermoregulatory system is a key function that humans possess which allows the body to adapt to environments of varying temperatures. When this system fails to regulate body temperature in particularly hot environments, the body can experience hyperthermia. This is where the body is absorbing more heat than it is dissipating (1). A Heat Related Illness (HRI) is the medical term for the health problems that arise from hyperthermia. Heat exhaustion, heat cramps, heat syncope, rhabdomyolysis, and heat stroke are all common forms of HRI's (1). Heat exhaustion occurs when the body is responding to excessive water and salt loss which can have symptoms of heat cramps if engaging in strenuous physical activity and heat rash. Heat syncope is a condition where dizziness occurs usually because of prolonged standing or sudden rising while exposed to a hot environment (2). Rhabdomyolysis is a condition where the muscle tissue within the body begins to break down due to prolonged heat stress (2). The most serious form of HRI is heat stroke, when this occurs the body is unable to cool via sweating and internal temperatures can rise to over 40 degrees Celsius which can lead to death if not promptly treated. Important factors that can play a role in the manifestation of HRI include high blood pressure, BMI, medications such as diuretics, dehydration status, and aging skin which prevents proper circulation and sweating (1).

B. Population Affected by Heat Related Illness

HRI's are a prevalent problem in the United States and disproportionally affect seasonal employees of the agriculture industry during the summer months. It is estimated that there are 3 million migrant farmworkers in the United States (3). Approximately 72% of this population is reported to be foreign born, with 68% coming from Mexico (3). This population is at a disadvantage in many key areas such as language barriers, with 35% reporting that they were not

able to speak any English and 8% being able to speak English "somewhat" (3). In terms of education, 28% report completing secondary education grades 10 through 12 and only 9% report completing some form of higher education. Health insurance is also an issue with this population, 39% report being covered by unemployment insurance, 54% report no coverage, and only 7% report being covered by employer-provided insurance. Undocumented immigration status is also common among this population; it is estimated that 52% would be deemed illegal immigrants (4). This population was widely exposed to heat stress, 60% report being seasonal workers with an average of 42 hours of work a week and 25% working 50 or more hours (3). These statistics indicate that migrant farmworkers in the United States are both a large and vulnerable population exposed to heat stress.

C. Epidemiology of Heat Related Illness

The CDC estimates that between 1979-2003 approximately 334 people died each year from HRI (5). In the United States, the leading cause of death among weather related exposures is heat (6). The HRI mortality rate among migrant farmworkers is 4 per 1,000,000 workers per year (7). This is 20 times higher than the HRI mortality rate among the general population which is .2 per 1,000,000 workers per year (7). The population of migrant farmworkers exposed is expected to increase due to farms needing to increase their production over time (7). The risk disparity is also expected to widen due to rising global temperatures and more severe heat waves as a result of climate change (7). The year of 2016 was the hottest year on record with global temperatures being 1.78 degrees Fahrenheit warmer than the mid-20th century mean (8).

D. Underlying Biological Processes of Heat Related Illness

There is a need for further research on what causes HRI but the current knowledge shows that are four major pathways through which the body attempts to adapt to heat stress. The cardiovascular thermoregulatory response (CVTR), the production of heat shock proteins (HSP's), an acute immune response, and a response to oxidative stress. The CVTR is the process that initiates sweating via cutaneous vasodilation to the periphery. This can cause a drop in blood pressure which must be controlled by splanchnic vasoconstriction. This response is heavily influenced by hydration and salt levels in the body. HSP's are a class of proteins that are upregulated in many types of stress not just heat exposure. HSP's stabilize new proteins or help refold proteins that were previously damaged by stress exposure (9). HSP's specifically can protect the tight junctions of endothelial cells in the human gut (10). The result of continued splanchnic vasoconstriction is an oxygen-deficient state which results in an increase in oxidative stress. The effect of oxidative stress on the human gut can lead to the formation of gaps in the tight junctions of the epithelium. This is a problem due to the presence of pathogenic bacteria in the gut entering the circulatory system (11). This bacterial translocation can cause sepsis and inflammation. The acute immune response is the fourth pathway through which the body responds to heat stress. It involves the increase of cytokine levels in the circulatory system (12). The pro-inflammatory cytokines that are produced in response to heat stress are TNF-α, IL-1β, IL-6, IL-8, IL-10, and IFN-γ which function to activate neutrophils (12). It is hypothesized that the physiologic process the body undergoes during HRI is affected by the composition of the gut microbiome (GM) and the inflammatory process.

E. Introduction to the Gut Microbiome

According to the National Institutes of Health, approximately 1-3% of the total mass of the average human body is comprised of microorganisms (13). These microorganisms, known as the microbiota, live commensally, symbiotically, and sometimes pathogenically with the body. These organisms are involved in roles such as the functioning of our metabolism, defense against harmful pathogens, and the strengthening of the immune system. The different types of microbes found in the human body are archaea, bacteria, eukaryotes, and viruses (14). These microbes can be found in areas of the body such as the skin, nose, and gut. The human gut is defined as the small and large intestines which are parts of the human digestive tract. The gut extends from the pyloric sphincter of the stomach to the anus. Most of the microbes found in the gut belong to two bacterial phyla, Firmicutes and Bacteriodetes (14). Microbiota living in and on the human body is hypothesized to be greater than 10^{12} cells/mL and contribute approximately 8 million protein-coding genes (13).

The GM is believed to contain the most diverse intrapersonal microbiota (15). Diversity of the microbes means both the type of cells present and their unique genetic codes. Interpersonally, the GM can vary between humans due to varying diets, environmental exposures, antibiotic consumption, and genetics (16). The degree of diversity of the microbiota can play a role in diseases such as obesity, irritable bowel syndrome, type II diabetes, and colorectal cancer (16). This highlights how the composition of the GM can manifest observable diseases. Surprisingly, the GM is resilient despite the daily introduction of foreign bacteria and regular immune surveillance conducted by the body

F. The Gut Microbiome, Immune System, Inflammation, and Sepsis

A key process of the human body's ability to maintain homeostasis is for balance to exist between the GM and the innate and adaptive immune system (17). Current evidence shows the GM plays a critical role in the growth of both the systemic and mucosal immune system (18). The challenge for the mucosal immune system is to differentiate between symbiotic, commensal, and pathogenic bacteria in the gut to maintain a healthy balance in the body (19).

The importance of understanding the GM and its interaction with inflammatory processes and the relationship between inflammation and sepsis is ultimately going to show how the GM and inflammation affect HRI. Current knowledge shows that the development of heat stroke and ultimately death is driven by septicemia (20). Sepsis is a paradoxical three-stage condition where the body harms its own tissues in response to fighting infection. The three stages are the initial development of sepsis followed by severe sepsis, then septic shock, ultimately concluding with death (21). The inflammatory process is the body's response to harmful stimuli with the goal of removing what is detrimental to the body and repairing the affected area. It is a complicated chain of events that involves the immune system and the circulatory system. Inflammation can occur both acutely and chronically. Sepsis is related to inflammation in that once it develops it is accompanied by Systemic Inflammatory Response Syndrome (SIRS) (22). When the inflammatory response is dysregulated, sepsis results, this is highlighted by the body's shift towards an oxidative state. A surge in proinflammatory cytokines and chemokines during sepsis is what causes multi-organ failure and eventually death (20).

The finding that heat stroke can be driven by septicemia rests heavily on being able to detect if bacteria translocated from the gut into the blood stream. Lipopolysaccharide (LPS), which is found in the cell walls of gram-negative bacteria, can provide a measure of the level of

bacterial translocation occurring within a subject's body (20). During the acute immune phase response to heat stress, cytokines IL-1 and IL-6 are released and synthesize lipopolysaccharide-binding protein (LBP) (23). This binding protein has been found to bind and effectively neutralize LPS. LBP is stored in the endothelial cells of the intestine and measuring LBP can indicate the level of bacterial translocation (24). It also means that people that are better adapted to deal with exposure to heat may have higher levels of LBP (24). Another biomarker involved in the inflammatory response is C-reactive protein (CRP) which is released by the liver (25). The role CRP plays is specifically to bind to the Fc receptor, a protein found on the surface of cells that contribute to the protective abilities of the immune system, and enhance the inflammatory response (25). Due to HRI causing an inflammatory response, it is hypothesized that CRP levels will be elevated in the serum of study subjects, particularly in the study subjects that reported experiencing HRI.

G. Study Aims

This leads to the development of the hypothesis that certain biomarkers capable of being detected in both human serum and stool can highlight the role of the GM in HRI. It also introduces the question of whether the interpersonal differences in response to heat are explained by varying GM composition and that certain people may be better adapted to deal with heat exposure. The biomarkers that were examined include LBP, CRP, proinflammatory cytokines IL-1-beta, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, TNF-alpha, and IFN-gamma. GM diversity was also compared between study participants.

2. METHODS

A. Overall Study Design

This study was conducted in collaboration with the Girasoles Study, a community based biomonitoring study examining the relationship between occupational heat exposure and physiologic responses among farmworkers in Florida (26). The subjects and data in this study were collected in association with the Farmworker Family Health Program (FFHP). The FFHP is a program that through partnership with the Ellenton Farmworker Clinic, provides medical care to migrant farmworkers and their children. The clinic itself travels to farms so farmworkers can receive care after their workday. The actual data was obtained from Dr. Vicki Hertzberg from the Nell Hodgson School of Nursing at Emory University. The original goal of this study was to recruit 60 participants, ultimately 39 participants consented, of which 37 subjects provided both questionnaire data and biological specimens. Microbiome data was analyzed for 35 subjects. Subjects were recruited from the Girasoles study and met the study criteria of being a farmworker between the ages of 18-54 that is currently working in the field and has been for at least two weeks. Those that weighed less than 80 pounds, had a disease history of the esophagus, stomach, or intestine, had dysphagia, or a pacemaker were excluded from the study. The study subjects were given a heat exposure questionnaire that was a modification of the questionnaire administered by the Girasoles Study (26). The questionnaire was administered by bilingual clinical research assistants to ascertain heat exposure and demographics of the subjects. The questionnaire data was stored in Emory University's Research Electronic Data Capture (REDCAP) system. This study received IRB approval in April of 2016 (IRB#00086444).

B. Study Sample Collection and Processing

Two rectal swabs, blood, and urine samples were collected from study participants. Approximately 1000 microliters of blood was obtained per subject via fingerstick. Plasma was isolated from the blood samples for testing of Lipopolysaccharide Binding Protein (LBP), C-reactive Protein (CRP), and proinflammatory cytokines via the Emory Multiplexed Immunoassay Core (EMIC). Subjects provided a clean catch urine sample which was used to measure the urine specific gravity to determine dehydration status. Subjects provided two Catch-All rectal swabs. These samples were used to obtain stool levels of LBP, CRP, and proinflammatory cytokines via EMIC as well as DNA extraction for GM profiling via the Emory Integrated Genomics Core (EIGC). The proinflammatory cytokines (IL-1-beta, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, TNF-alpha, and IFN-gamma) present in the stool and serum samples were measured using a V-PLEX Proinflammatory Panel 1 human kit (MESO Scale Diagnostics, EMIC). Serum and stool levels of LBP were measured using the MESO Scale Human LBP kit (MESO Scale Diagnostics, EMIC). A Siemens 2161 Multistix 10SG reagent strip (CAT# AM-2166) was immersed in the urine samples to determine the specific gravity of the urine (values greater than 1.02 indicate dehydration). The GM was assayed from the stool with the help of EIGC facility. Microbial DNA was extracted from stool using the MoBio Power Soil kit. After QC, the V3-V4 region was amplified for each sample, then barcoded, pooled, and sequenced on a MiSeq (Illumina). The microbiome data was processed using a MoBio Powersoil DNA Isolation Kit. Illumina sequencing adapters and barcodes were used to amplify the 16s rRNA V3 and V4 regions. Sequence data was processed using Quantitative Insights into Microbial Ecology (QIIME) software (27). Downstream analysis was accomplished with the

PhyloSeq package, APE package, and using R studio and Statistical Analysis System (SAS) software (28, 29, 30, 31).

C. Data Processing Procedures

Using the questionnaire data, we created an outcome variable of HRI status. This was done by scoring survey questions on reported cramps, nausea, excessive sweating, confusion, dizziness, fainting, or headache. Every participant reported excessive sweating. We therefore defined HRI as the report of two or more symptoms other than excessive sweating. This definition is supported by existing literature on the topic (32).

After processing the raw DNA sequencing data through QIIME, the data was imported into R studio software using the Phyloseq package. Here the OTU table and phylogenetic tree files were merged with the questionnaire and laboratory metadata from SAS using the Phyloseq function (28). The rarefy even depth function was used in R studio to rarefy the microbiome data. Mean read depth was (171,771), with two outlier samples where reads were less than 15,000 reads. These samples were dropped and samples were rarefied to 90,000 reads. Before rarefaction, there were a total of 7,017 taxa. After rarefying there were 5,592 total taxa.

After the rarefaction step, the data were agglomerated to the genus level. Alpha diversity was calculated using the estimate richness function for both Chao1 measure and the Shannon index (28). Beta diversity of the data was also calculated and plotted using the ordinate function and Principle Coordinate Analysis in R. Both the Bray-Curtis and Weighted Unifrac distance plots were generated. The analysis of similarity function (anosim) in R was used to calculate the statistical significance of the Bray-Curtis distances. This was accomplished by converting the Phyloseq object to a Vegan object (34). This was achieved using the veganotu and vegdist functions (34). Relative abundance of each genus was calculated using the transform sample

count function. To identify the top ten most abundant genera, the data was sorted using the taxa sums function (28).

In SAS, the excel spreadsheets that contained the laboratory biomarker data were merged with the questionnaire data using the merge function within the data step procedure (31). For the values of the biomarker data that were below the detection range, the lower limit of detection of the specific assay, as found on the MESO Scale Diagnostic website, was divided by the square root of 2 (33). This data was exported from SAS for statistical analysis in R. Summary statistics were calculated for the samples using the means procedure (31).

D. Data Analysis Procedures

The biomarker data that was collected in this study was compiled and read into R statistical software from SAS. The specific variables were the serum and stool levels of LBP, CRP, and the proinflammatory cytokines. The values for each of the biomarkers from both the stool and serum samples were log transformed and a principle component analysis was conducted on the biomarker data using the prcomp function in R (30). Log transformation was done due to the small number of samples and a highly-skewed distribution of the data.

Bivariate analysis was conducted in SAS. The Chao1 Measure and Shannon Index values were exported from R and merged with the compiled dataset in SAS. Since the data was highly skewed and the sample size was not large, the Wilcoxon rank-sum test, a nonparametric test, was used to examine each of the continuous variables of interest with respect to the HRI outcome, urine specific gravity, and gender. Due to small sample size, Fisher's Exact Test was utilized for categorical variables.

Pearson's correlation analysis was conducted in R using the corrplot function (35). This was done to determine the correlations among the stool biomarkers and among the serum

biomarkers. Simple Linear Regression was conducted on the stool and serum biomarker variables with Wilcoxon rank-sum test p-values less than 0.10. Few variables were significant at the typical 0.05 level so for this study a higher p-value was used. The variables that were chosen were serum CRP, serum IL-2, stool CRP, stool LBP, stool IL-1 Beta, and Stool IL-8. The variables were regressed on the relative abundance of the top ten genera found in the gut. Since this analysis was part of a pilot study, simple linear regression was carried out even though assumptions for regression analysis were not met.

Utilizing the results of the univariate, bivariate, and simple linear regression results a final logistic model was built that aimed to quantify the risk of developing HRI based on a given set of predictors. Due to the small sample size of the study, overfitting the model was a concern. With a larger sample size, the collinearity between the predictor variables would be assessed using condition indices. Interaction and confounding would also be assessed while building the model. The variables that were selected to be predictors in the model with HRI status as the outcome were BMI and Stool CRP. BMI was selected because of its relationship with the GM and to comorbidities which can affect biomarker levels. BMI also takes into account lifestyle and diet of the study subjects. Stool CRP was selected because it is a biomarker of inflammation and was found to be significantly higher among subjects that had HRI compared to subjects that did not have a HRI.

3. RESULTS

A. Descriptive Statistics of Cohort

The study population included 37 total observations with 35 contributing microbiome data after rarefaction. Tables 1, 2, and 3 show demographic results, the nonparametric bivariate analysis, and the top ten genera found in the GM samples. Table 1A shows the descriptive statistics for the population that was analyzed. Approximately 83.7% of participants of the study were male. The average age of the participants ± the standard deviation (SD) was 33.5 ± 8.4 years old. Approximately 67% of subjects had a urine specific gravity of over 1.02 indicating that they were dehydrated. A total of 6 participants were classified as normal weight which is a BMI between 18.5 and 24.9. There were 20 participants classified as overweight which is a BMI between 25.0 and 29.9. The remaining 11 participants were classified as obese with a BMI greater than 30.0.

The subjects were predominantly immigrants, 91.2% reported that their nationality was Mexican, 1 subject reported being from the United States, and 2 were unknown. There were 45.9% of subjects that reported having smoked greater than 100 cigarettes in their lifetime. A majority of the farmworkers, 73.7%, reported working with vegetables in the field. Table 1A shows the number of participants that reported each of the HRI symptoms. All 37 of the participants reported excessive sweating, 25 reported having experienced a headache, 20 reported dizziness, 16 reported nausea, 14 reported cramps, 9 reported confusion, 2 reported fainting, and 4 reported having experienced another symptom. After creating the HRI variable, 23 subjects (62%) experienced 2 or more symptoms other than excessive sweating. This left 14 subjects that did not experience HRI by this definition. Table 1B summarizes the stool and serum biomarker variables LBP, CRP, proinflammatory cytokines IL-1-beta, IL-2, IL-4, IL-6, IL-8, IL-

10, IL-12p70, IL-13, TNF-alpha, IFN-gamma, and microbiome alpha diversity for the total samples. Table 1C shows these variables by HRI Status.

B. Bivariate Analysis and Microbiome Analysis

Tables 2 A, B and C show results for statistical tests. The results of the Wilcoxon rank-sum test and Fisher's exact test between cases and controls is shown in Table 2A. Based on an alpha of 0.05, there were two variables that were significantly different between those with HRI and those without. The mean value of stool CRP, stool IL-1-Beta, stool IL-8 were found to be significantly higher among cases of HRI when compared to controls (p-values = 0.033, 0.007, 0.042 respectively). Table 2B shows the Wilcoxon rank-sum test and Fisher's exact test results using the dichotomized urine specific gravity variable as the outcome variable. There were no statistically significant differences between those that were dehydrated and those that were not at an alpha of 0.05. Table 2C shows the results of the Wilcoxon rank-sum test and Fisher's exact test using biological gender as the outcome variable. Serum LBP, serum IL-8, serum IL-13, and serum TNF-alpha were all significantly higher in females than in males (p-values = 0.019, 0.005, 0.007, 0.024 respectively). The Fisher's exact test of BMI and gender was also statistically significant (p-value = 0.012).

Tables 3A, 3B, and 3C show the relative abundance of the top ten phyla and genera present in the GM of total the samples and then by HRI status. The top three in both cases and controls are Prevotella, Bacteriodes, and Faecalibacterium from the phyla Firmicutes and Bacteroidetes. Figure 1 shows the Chao1 Measure of GM alpha diversity based on HRI status. Figure 2 shows the Shannon Index of GM alpha diversity. No significant differences based on HRI status are seen. Figures 3 and 4 show the Bray-Curtis and Weighted Unifrac plots, respectively. These figures do not show a clustering of the points based on HRI status or gender.

Analysis of similarity conducted on the Bray-Curtis distances showed no significant difference based on HRI status (ANOSIM R Statistic = 0.038, p-value = 0.246). The results of the principle component analysis examining all the biomarkers with respect to HRI found that the first principle component was heavily weighted with the serum biomarkers and the second principle component was heavily weighted with the stool biomarkers. These first two principle components explained 47.71% of the variation between HRI and no HRI.

C. Correlation Analysis and Linear Regression

Correlation analysis is shown in Figure 5 and Figure 6. Those figures show the Pearson's Correlation Coefficient between the different biomarkers that were analyzed in this study. Among the correlations of the serum biomarkers, serum LBP levels were not significantly correlated with other serum biomarkers. Among the stool biomarkers, stool LBP levels were significantly associated with stool IL-8 levels and stool IL-4 levels (Pearson's correlation coefficient = 0.62, 0.79, P-value = <0.0001, <0.0001 respectively).

The simple linear regression of the biomarker variables and the relative abundance of the top ten genera present in the gut did not yield significant linear relationships. To further examine these variables, simple linear regression was carried out based on HRI status and these linear relationships were also not significant. The results of the logistic regression model with HRI status as the outcome variable and stool CRP and BMI as predictors was not significant. Stool CRP had an OR of 1.002 with 95% C.I. 0.999-1.004. BMI had an OR of 1.086 and a 95% CI of 0.908-1.299.

4. DISCUSSION

The original aims of this pilot study were to explore the potential differences between subjects that experienced HRI and subjects that did not among the migrant farmworker cohort. It was originally hypothesized that certain biomarkers may be protective against developing HRI. A set biomarkers were examined from the stool, serum, and urine of the study subjects along with GM data and other descriptive variables. This was to be carried with the goal of determining differences between subjects that experienced HRI and those that did not. The hypotheses of this study were based on the human body's response to heat stress which causes a proinflammatory state resulting in bacterial translocation from the gut into the bloodstream. This can result in sepsis and potentially death. The specific protein, lipopolysaccharide (LPS), is a hallmark of bacterial translocation from the gut. The body's response to combat levels of LPS is to synthesize lipopolysaccharide binding protein (LBP) which neutralizes LPS. This led to the hypothesis that subjects with higher levels of LBP would be better protected from the body's response to heat stress. LBP levels in both the stool and serum along with CRP, proinflammatory cytokines IL-1-beta, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, TNF-alpha, and IFN-gamma levels were all analyzed with respect to HRI status. The results did not show a significant difference in stool and serum LBP levels between those with HRI and those without. The biomarkers that were significantly different based on HRI status were stool CRP and stool IL 1 Beta levels. These biomarkers were significantly higher among subjects that experienced HRI (p-values = 0.033 and 0.007 respectively). This indicates that the HRI group had higher stool inflammatory biomarkers than those that did not experience HRI and potentially indicates those that experienced HRI had higher levels of proinflammatory cytokines, and CRP.

As part of the exploratory analysis, the biomarker predictors were examined with respect to gender and dehydration status as outcomes. No significant difference between dehydration status among the stool and serum biomarkers was found. Serum LBP, IL-8, and IL-13 levels were all significantly higher among females when compared to males. This indicates that females exposed to environmental heat could potentially be at a lower risk of experiencing HRI. Gender differences between LBP, CRP, and proinflammatory cytokines could be explored in future research studies.

Since bacterial translocation from the gut occurs during the body's response to heat stress, the other hypothesis of this study was that the makeup of an individual's microbiome can also play a role in developing HRI. This was addressed by analyzing both the alpha and beta diversity of the GM with respect to HRI status. There was no significant difference seen between subjects that experienced HRI and those that did not. Bray-Curtis and Weighted Unifrac plots showed no differences between HRI and no HRI. Alpha diversity measures Chao1 and Shannon Index differences between HRI and no HRI showed that the GM alpha diversity was not significantly different between the two groups. Based on the existing literature it is known that low diversity of the human GM can play a role in chronic diseases like Irritable Bowel Syndrome, and Crohn's Disease (14).

Simple linear regression looking at the potential linear relationships between serum and stool biomarker levels with relative abundance of the top ten genera of the GM did not find significant results. The R^2 values for these indicated that little variation was explained in these models. The logistic model created to determine the risk of developing HRI based on a set of predictor variables was also not significant. The odds ratios associated with Stool CRP and BMI were slightly higher than 1.0 (OR's = 1.002, 1.086 respectively). However, both confidence

intervals contained the 1.0, the null value. This indicates that among these samples higher BMI and higher Stool CRP levels were not significantly increasing the odds of developing HRI.

The data collected for this study provided a comprehensive biomarker and GM profile for each subject. This allowed for analysis of the inflammatory state and the microbiome with respect to the outcome variable which in this case was HRI. The limitations of this study were largely due to sample size and the selection of the samples. Enrollment of a subject required that they be a farmworker that had been working in the field for at least two weeks prior to selection. This resulted in every subject in the cohort being exposed to heat and reporting excessive sweating which can be a sign of HRI. This potentially decreased the differences that would be seen between the subjects that were designated as cases or controls. Those that were classified as not having HRI could have been experiencing HRI which could explain why no differences were seen in the GM and most of the biomarker results. Another limitation of the study is how difficult the microbiome is to analyze. It is highly influenced by many different factors which will result in greater variability of the results which is why it necessitates a large sample size to analyze.

More research is needed to explore the role inflammation and the GM plays in HRI. This is an issue that affects approximately 3 million people in the United States with many more millions around the world. It is an issue that is going to be exacerbated by climate change. The overall results of this study suggest that the differences between those that experienced HRI and those that did not could be explained by proinflammatory cytokines, gender, CRP, and LBP. These results do justify a larger study because multiple variables were just above statistical significance (serum CRP, serum IL-2, stool LBP, stool LBP) and a study with larger sample size would have more power to detect these differences. Future studies need to enroll an appropriate

group of controls that while they are representative of the case population they are not exposed to environmental heat and have a more even distribution of gender. The subjects of this study were also predominantly overweight and obese, future studies should seek to enroll subjects that have a more even distribution of BMI since it is known to influence the GM (36). This would provide a more accurate differentiation between those that experienced the outcome of interest in this study and those that did not. This could potentially be achieved by studying a cohort of farmworkers that work at a location with indoor and outdoor work environments and measuring the same variables as this study; LBP, CRP, proinflammatory cytokines IL-1-beta, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, TNF-alpha, and IFN-gamma along with the GM makeup and diversity. This would provide more accurate results on the affect heat exposure has within the human body.

REFERENCES

1. Hyperthermia: Too Hot for Your Health. *National Institutes of Health*. 2012;(https://www.nia.nih.gov/health/publication/hyperthermia).
2. HEAT STRESS. *Centers for Disease Control and Prevention*. 2016;(https://www.cdc.gov/niosh/topics/heatstress/default.html).
3. Home. *National Center for Farmworker Health*. 2012;(http://www.ncfh.org/fact-sheets--research.html).
4. Villarejo D. Occupational Injury Rates Among Hired Farmworkers. *Journal of Agricultural Safety and Health*. 1998;4(5):39–46.
5. Extreme Heat: A Prevention Guide to Promote Your Personal Health and Safety. *Centers for Disease Control and Prevention.* 2015; (http://emergency.cdc.gov/disasters/extremeheat/heat_guide.asp. September 22, 2015).
6. Gubernot DM, Anderson GB, Hunting KL. The epidemiology of occupational heat exposure in the United States: a review of the literature and assessment of research needs in a changing climate. *International Journal of Biometeorology*. 2013;58(8):1779–1788.
7. Jackson LL, Rosenberg HR. Preventing Heat-Related Illness Among Agricultural Workers. *Journal of Agromedicine*. 2010;15(3):200–215.
8. Northon K. NASA, NOAA Data Show 2016 Warmest Year on Record Globally. *NASA*. 2017;(https://www.nasa.gov/press-release/nasa-noaa-data-show-2016-warmest-year-on-record-globally/).
9. Li Z, Srivastava P. Heat-Shock Proteins. *Current Protocols in Immunology*, John Wiley & Sons, Inc. 2004;

10. Dokladny K. Physiologically relevant increase in temperature causes an increase in intestinal epithelial tight junction permeability. *AJP: Gastrointestinal and Liver Physiology*. 2006;290(2).
11. Hall DM, et al. Mechanisms of circulatory and intestinal barrier dysfunction during whole body hyperthermia. *American Journal of Physiology-Heart and Circulatory Physiology*. 2001;2:H509-H521.
12. Robins H, Kutz M, Wiedemann GJ, et al. Cytokine induction by 41.8 °C whole body hyperthermia. *Cancer Letters*. 1995;97(2):195–201.
13. MacDougall R, ed. NIH Human Microbiome Project defines normal bacterial makeup of the body. *National Institutes of Health*. 2015;(https://www.nih.gov/news-events/news-releases/nih-human-microbiome-project-defines-normal-bacterial-makeup-body).
14. Shreiner, AB, John YK, Vincent BY. The Gut Microbiome in Health and in Disease. *Current opinion in gastroenterology.* 2015;31(1):69–75.
15. Ursell LK, Clemente JC, Rideout JR, et al. The interpersonal and intrapersonal diversity of human-associated microbiota in key body sites. *Journal of Allergy and Clinical Immunology*. 2012;129(5):1204–1208.
16. Lozupone CA, Stombaugh JI, Gordon JI, et al. Diversity, stability and resilience of the human gut microbiota. *Nature*. 2012;489(7415):220–230.
17. Quigley EM. Gut Bacteria in Health and Disease. *Gastroenterology & Hepatology*. 2013;9(9):560-569.
18. Belkaid Y, Hand TW. Role of the Microbiota in Immunity and Inflammation. *Cell*. 2014;157(1):121–141.

19. Tanoue T, Umesaki Y, Honda K. Immune responses to gut microbiota-commensals and pathogens. *Gut Microbes*. 2010;1(4):224–233.

20. Leon LR, Helwig BG. Heat stroke: Role of the systemic inflammatory response. *Journal of Applied Physiology*. 2010;109(6):1980–1988.

21. O'Connell K, Higuera V. Sepsis. *Healthline*. 2017; (http://www.healthline.com/health/sepsis).

22. Bosmann M, Ward PA. The Inflammatory Response in Sepsis. *Trends in immunology*. 2013;34(3):129-136.

23. Schumann RR, Kirschning CJ, Unbehaun A, et al. The lipopolysaccharide-binding protein is a secretory class 1 acute-phase protein whose gene is transcriptionally activated by APRF/STAT/3 and other cytokine-inducible nuclear proteins. *Molecular and Cellular Biology*. 1996;16(7):3490–3503.

24. Hailman E, Albers JJ, Wolfbauer G, et al. Neutralization and Transfer of Lipopolysaccharide by Phospholipid Transfer Protein. *Journal of Biological Chemistry*. 1996;271(21):12172–12178.

25. Clos TWD. Function of C-reactive protein. *Annals of Medicine*. 2000;32(4):274–278.

26. Girasoles (Sunflowers) Study (R01-OH010657; L. McCauley, PI)

27. QIIME allows analysis of high-throughput community sequencing data. J Gregory Caporaso, Justin Kuczynski, Jesse Stombaugh, Kyle Bittinger, Frederic D Bushman, Elizabeth K Costello, Noah Fierer, Antonio Gonzalez Pena, Julia K Goodrich, Jeffrey I Gordon, Gavin A Huttley, Scott T Kelley, Dan Knights, Jeremy E Koenig, Ruth E Ley, Catherine A Lozupone, Daniel McDonald, Brian D Muegge, Meg Pirrung, Jens Reeder, Joel R Sevinsky, Peter J Turnbaugh, William A Walters, Jeremy Widmann, Tanya

Yatsunenko, Jesse Zaneveld and Rob Knight; Nature Methods, 2010; doi:10.1038/nmeth.f.303

28. Phyloseq: An R package for reproducible interactive analysis and graphics of microbiome census data. Paul J. McMurdie and Susan Holmes (2013) PLoS ONE 8(4):e61217.
29. Paradis E., Claude J. & Strimmer K. 2004. APE: analyses of phylogenetics and evolution in R language. Bioinformatics 20: 289-290.
30. RStudio Team (2015). RStudio: Integrated Development for R. RStudio, Inc., Boston, MA URL http://www.rstudio.com/.
31. SAS 9.4, (Cary, NC)
32. Arcury TA, Summers P, Talton JW, et al. Heat Illness Among North Carolina Latino Farmworkers. *Journal of Occupational and Environmental Medicine*. 2015;57(12):1299–1304.
33. Meso Sclae Diagnostics. V-Plex Proinflammatory Panel 1 (human) kit. Fact Sheet.
34. Jari Oksanen, F. Guillaume Blanchet, Michael Friendly, Roeland Kindt, Pierre Legendre, Dan McGlinn, Peter R. Minchin, R. B. O'Hara, Gavin L. Simpson, Peter Solymos, M. Henry H. Stevens, Eduard Szoecs and Helene Wagner (2017). vegan: Community Ecology Package. R package version 2.4-2. https://CRAN.R-project.org/package=vegan
35. Taiyun Wei, Viliam Simko (2017). R package "corrplot": Visualization of a Correlation Matrix (Version 0.82). Available from https://github.com/taiyun/corrplot
36. Haro C, Rangel-Zúñiga OA, Alcalá-Díaz JF, et al. Intestinal Microbiota Is Influenced by Gender and Body Mass Index. *PLOS ONE*. (http://journals.plos.org/plosone/article?id=10.1371%2Fjournal.pone.0154090).

TABLES:

Table 1A - Subject Demographics and Lab Results

	HIAMI Study Subjects (n=37)
Male Gender; n (%)	31 (83.7)
Age (years); mean (SD)	33.5 (8.4)
BMI	
Underweight <18.5; n (%)	0
Normal Weight 18.5-24.9; n (%)	6 (15.8)
Overweight 25-29.9; n (%)	20 (52.6)
Obese > 30; n (%)	11 (29.7)
Urine Specific Gravity; n (%)	
> or = 1.02	25 (67)
Nationality; n (%)	
United States	1 (2.7)
Mexico	34 (91.2)
Other	2 (5.4)
Smoking History (> 100 cigarettes); n %	17 (45.9)
Everyday Smoker; n %	7 (18.4)
Crops Worked With; n (%)	
Vegetables	27 (72.9)
Oranges	2 (5.4)
Other	8 (21.6)
Self Reported Heat Related Illness Symptoms; n (%)	
Cramps	14 (37.8)
Nausea	16 (43.2)
Excessive Sweating	37 (97.3)
Confusion	9 (24.3)
Dizziness	20 (54.1)
Fainting	3 (8.1)
Headache	25 (67.6)
Other	4 (23.5)
Heat Related Illness*; n (%)	
2 or more symptoms (other than excessive sweating)	23 (62)
3 or more symptoms	14 (37.8)
4 or more symptoms	5 (13.5)

*Defined as having two or more symptoms: cramps, nausea, excessive sweating, confusion, dizziness, fainting, headache.

Table 1B - Lab Results and Microbiome Alpha Diversity

	HIAMI Study Subjects (n=37)
Serum; mean (SD)	
CRP (ng/mL)	4917.77 (11442.22)
LBP (ng/mL)	2489.88 (1623.07)
TNF-alpha (pg/mL)	9.21 (12.70)
IL-1-Beta (pg/mL)	53.58 (75.18)
IL-2 (pg/mL)	0.23 (0.22)
IL-4 (pg/mL)	0.06 (0.05)
IL-6 (pg/mL)	4.08 (13.93)
IL-8 (pg/mL)	203.49 (567.26)
IL-10 (pg/mL)	0.64 (1.35)
IL-12p70 (pg/mL)	0.13 (0.15)
IL-13 (pg/mL)	0.66 (0.56)
IFN-gamma (pg/mL)	4.56 (2.91)
Stool; Mean (SD)	
CRP (pg/mL)	670.21 (1614.02)
LBP (ng/mL)	0.18 (0.39)
TNF-alpha (pg/mL)	0.96 (1.23)
IL-1-Beta (pg/mL)	10.97 (18.99)
IL-2 (pg/mL)	5.30 (11.80)
IL-4 (pg/mL)	0.08 (0.08)
IL-6 (pg/mL)	0.40 (0.78)
IL-8 (pg/mL)	124.20 (234.74)
IL-10 (pg/mL)	0.25 (0.42)
IL-12p70 (pg/mL)	0.52 (0.51)
IL-13 (pg/mL)	1.79 (3.07)
IFN-gamma (pg/mL)	4.22 (7.83)
Alpha Diversity; mean (SD)	
Choa1	101.96 (15.63)
Shannon	2.54 (0.27)

Table 1C - Lab Results and Microbiome Alpha Diversity by HRI Status

	No Heat Related Illness (n=14)	**Heat Related Illness (n=23)**
Serum; mean (SD)		
CRP (ng/mL)	2021.45 (2998.01)	6680.69 (14154.16)
LBP (ng/mL)	2536.97 (1089.07)	2461.22 (1899.38)
TNF-alpha (pg/mL)	8.82 (12.08)	9.44 (13.32)
IL-1-Beta (pg/mL)	38.65 (25.69)	62.67 (92.90)
IL-2 (pg/mL)	0.26 (0.19)	0.21 (0.24)
IL-4 (pg/mL)	0.05 (0.06)	0.05 (0.05)
IL-6 (pg/mL)	7.27 (22.61)	2.14 (2.32)
IL-8 (pg/mL)	317.83 (899.55)	133.89 (187.08)
IL-10 (pg/mL)	0.41 (0.35)	0.77 (1.69)
IL-12p70 (pg/mL)	0.12 (0.19)	0.13 (0.11)
IL-13 (pg/mL)	0.71 (0.69)	0.63 (0.47)
IFN-gamma (pg/mL)	5.20 (3.98)	4.18 (2.01)
Stool; Mean (SD)		
CRP (pg/mL)	192.38 (250.57)	961.05 (1998.0)
LBP (ng/mL)	0.20 (0.30)	0.16 (0.44)
TNF-alpha (pg/mL)	1.13 (1.93)	0.08 (0.47)
IL-1-Beta (pg/mL)	3.21 (5.07)	15.69 (22.66)
IL-2 (pg/mL)	6.94 (14.04)	4.29 (10.42)
IL-4 (pg/mL)	0.08 (0.03)	0.08 (0.10)
IL-6 (pg/mL)	0.23 (0.13)	0.50 (0.98)
IL-8 (pg/mL)	25.94 (33.10)	184.01 (282.20)
IL-10 (pg/mL)	0.33 (0.57)	0.20 (0.29)
IL-12p70 (pg/mL)	0.50 (0.33)	0.52 (0.60)
IL-13 (pg/mL)	1.73 (2.76)	1.83 (3.31)
IFN-gamma (pg/mL)	5.56 (11.89)	3.34 (3.84)
Alpha Diversity; mean (SD)		
Choa1	104.22 (13.29)	100.46 (17.16)
Shannon	2.52 (0.26)	2.55 (0.28)

Table 2A - Bivariate Analysis with HRI Status*

	Wilcoxon rank-sum test exact two-sided	Fisher's exact test
	P-value	
Age	0.647	
BMI		0.068
Gender		1.000
Smoking History (> 100 cigarettes)		0.744
Serum		
CRP	0.071	
LBP	0.298	
TNF-alpha	0.963	
IL-1-Beta	0.653	
IL-2	0.076	
IL-4	0.714	
IL-6	0.938	
IL-8	0.566	
IL-10	0.525	
IL-12p70	0.340	
IL-13	0.822	
IFN-gamma	0.987	
Stool		
CRP	0.033**	
LBP	0.060	
TNF-alpha	0.231	
IL-1-Beta	0.007**	
IL-2	0.546	
IL-4	0.486	
IL-6	0.632	
IL-8	0.042**	
IL-10	0.654	
IL-12p70	0.610	
IL-13	0.746	
IFN-gamma	0.632	
Urine Specific Gravity	0.909	
Alpha Diversity		
Choa1 Measure	0.428	
Shannon Index	0.829	

*Comparing 2 or less symptoms of HRI to greater than 2 symptoms (other than excessive sweating)

**Significant p-value (<0.05)

Table 2B - Bivariate Analysis with Urine Specific Gravity*

	Wilcoxon rank-sum test exact two-sided	Fisher's exact test
	P-value	
Age	0.822	
BMI		0.457
Gender		1.000
Smoking History (> 100 cigarettes)		1.000
Serum		
CRP	0.088	
LBP	0.101	
TNF-alpha	0.761	
IL-Beta	0.088	
IL-2	0.169	
IL-4	0.812	
IL-6	0.553	
IL-8	0.810	
IL-10	0.052	
IL-12p70	0.292	
IL-13	0.398	
IFN-gamma	0.910	
Stool		
CRP	0.061	
LBP	0.772	
TNF-alpha	0.737	
IL-Beta	0.377	
IL-2	0.327	
IL-4	0.471	
IL-6	0.713	
IL-8	0.360	
IL-10	0.377	
IL-12p70	0.761	
IL-13	0.835	
IFN-gamma	0.343	
Urine Specific Gravity		
Alpha Diversity		
Choa1 Measure	0.139	
Shannon Index	0.409	

*Comparing dehydrated to not (specific gravity > 1.02 is dehydrated)

**Significant p-value (<0.05)

Table 2C - Bivariate Analysis with Gender*

	Wilcoxon rank-sum test exact two-sided	Fisher's exact test
	P-value	
Age	0.930	
BMI		0.012**
Gender		
Smoking History (> 100 cigarettes)		0.188
Serum		
CRP	0.643	
LBP	0.019**	
TNF-alpha	0.024**	
IL-Beta	0.455	
IL-2	0.888	
IL-4	0.812	
IL-6	0.095	
IL-8	0.005**	
IL-10	0.053	
IL-12p70	0.452	
IL-13	0.007**	
IFN-gamma	0.214	
Stool		
CRP	0.247	
LBP	0.633	
TNF-alpha	0.856	
IL-Beta	0.532	
IL-2	0.888	
IL-4	0.059	
IL-6	0.385	
IL-8	0.673	
IL-10	0.230	
IL-12p70	0.104	
IL-13	0.673	
IFN-gamma	0.920	
Urine Specific Gravity		0.9589
Alpha Diversity		
Choa1 Measure	0.922	
Shannon Index	0.080	

* Male vs. Female

**Significant p-value (<0.05)

Table 3A - Top Genera by Relative Abundance Sums for Total Samples

All Samples		
Phylum (next taxanomic rank if unclassified)	**Genus (next taxanomic rank if unclassified)**	**Mean Relative Abundance**
Bacteroidetes	Prevotella	0.3186
Bacteroidetes	Bacteroides	0.0693
Firmicutes	Faecalibacterium	0.0466
Firmicutes	Unclassified (family Ruminococcaceae)	0.0371
Firmicutes	Unclassified (family Lachnospiraceae)	0.0357
Firmicutes	Sporobacterium	0.0345
Firmicutes	Finegoldia	0.0334
Firmicutes	Peptoniphilus	0.0311
Firmicutes	Unclassified (order Clostridiales)	0.0301
Firmicutes	Dialister	0.0287

Table 3B - Top Genera by Relative Abundance Sums among No HRI Subjects

No Heat Related Illness		
Phylum (next taxanomic rank if unclassified)	**Genus (next taxanomic rank if unclassified)**	**Mean Relative Abundance**
Bacteroidetes	Prevotella	0.3477
Bacteroidetes	Bacteroides	0.0682
Firmicutes	Faecalibacterium	0.0518
Firmicutes	Unclassified (family Ruminococcaceae)	0.0429
Firmicutes	Unclassified (family Lachnospiraceae)	0.0402
Firmicutes	Sporobacterium	0.0395
Firmicutes	Unclassified (order Clostridiales)	0.0333
Firmicutes	Finegoldia	0.0297
Firmicutes	Peptoniphilus	0.0265
Firmicutes	Dialister	0.0231

Table 3C - Top Genera by Relative Abundance Sums among HRI Subjects

Heat Related Illness		
Phylum (next taxanomic rank if unclassified)	**Genus (next taxanomic rank if unclassified)**	**Mean Relative Abundance**
Bacteroidetes	Prevotella	0.2992
Bacteroidetes	Bacteroides	0.0700
Firmicutes	Faecalibacterium	0.0432
Firmicutes	Finegoldia	0.0358
Firmicutes	Peptoniphilus	0.0343
Firmicutes	Unclassified (family Ruminococcaceae)	0.0333
Firmicutes	Unclassified (family Lachnospiraceae)	0.0327
Proteobacteria	Campylobacter	0.0324
Firmicutes	Dialister	0.0323
Firmicutes	Sporobacterium	0.0311

FIGURES:

Figure 1 - Shannon Index Alpha Diversity Plot by HRI Status

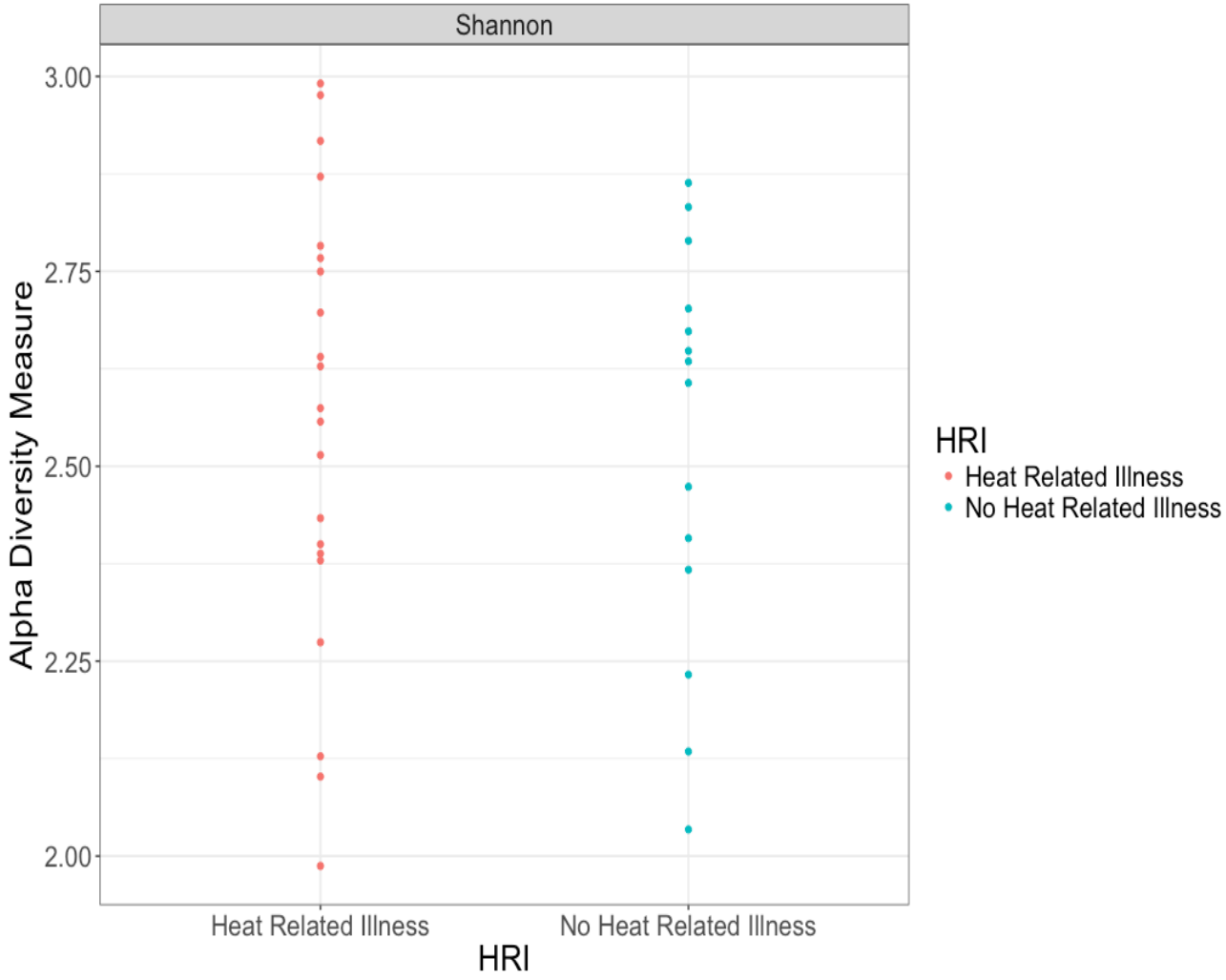

Figure 2 - Chao1 Measure Alpha Diversity Plot by HRI Status

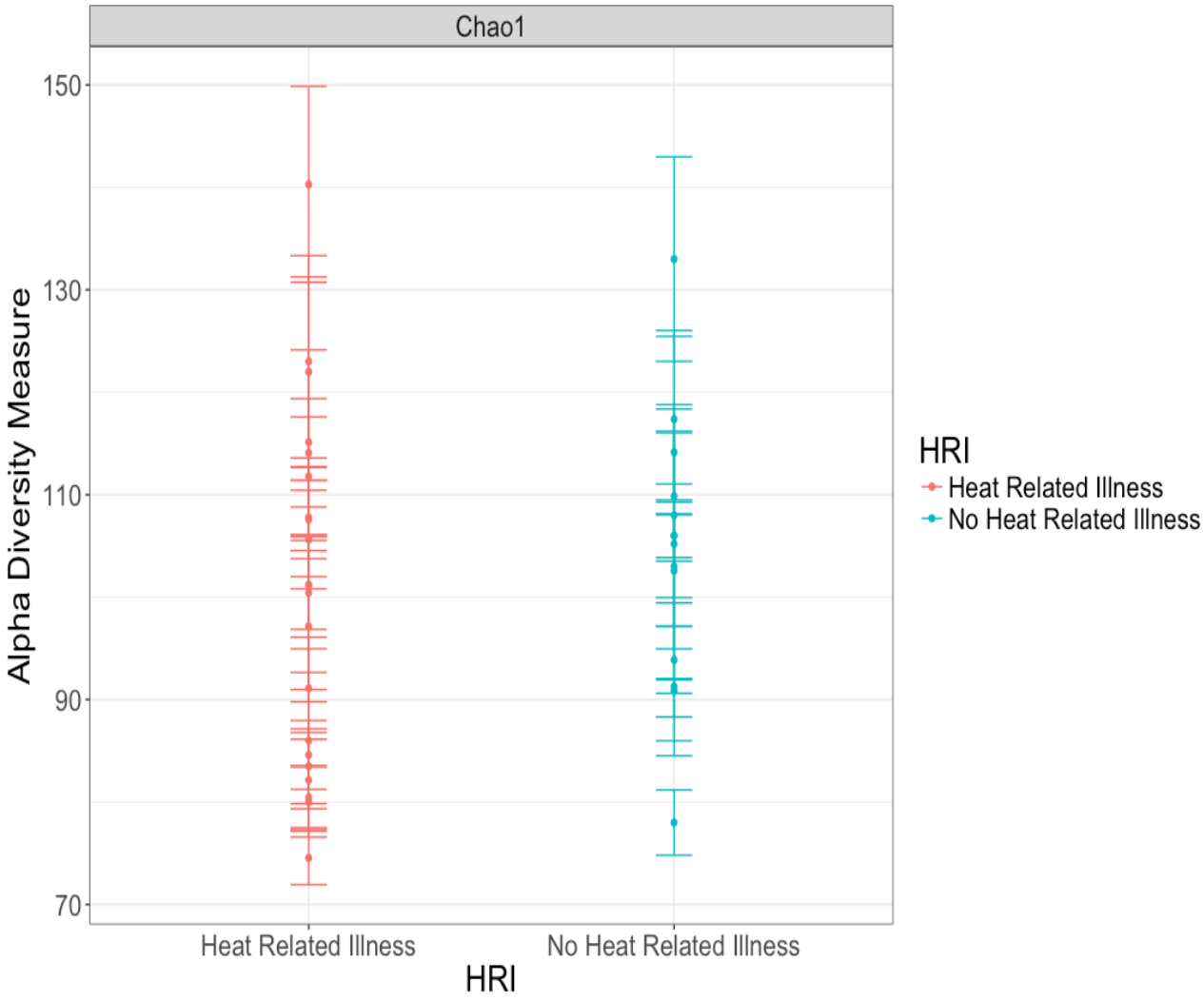

Figure 3 - Principle Coordinate Analysis Plot of Bray-Curtis Distances

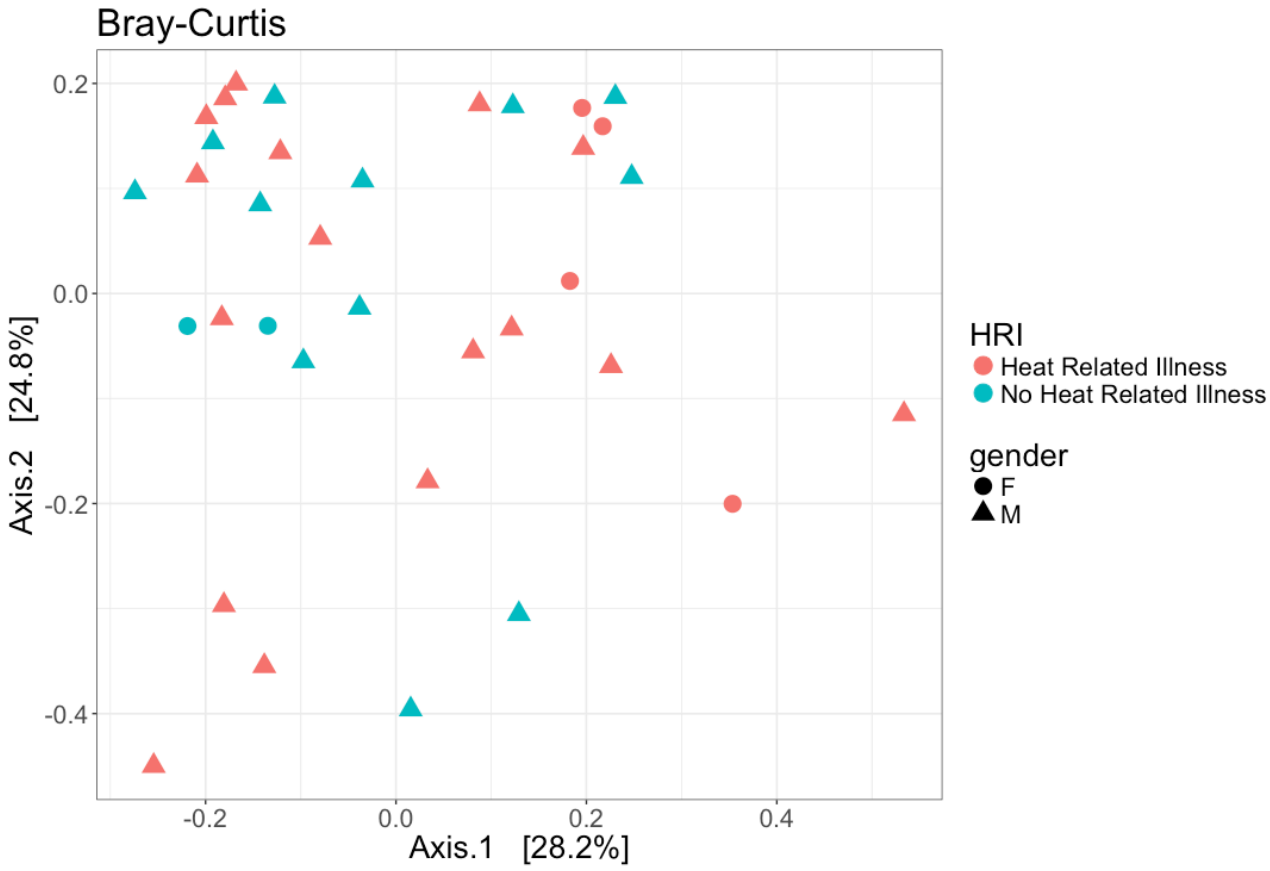

Figure 4 - Principle Coordinate Analysis Plot of Weighted Unifrac Distances

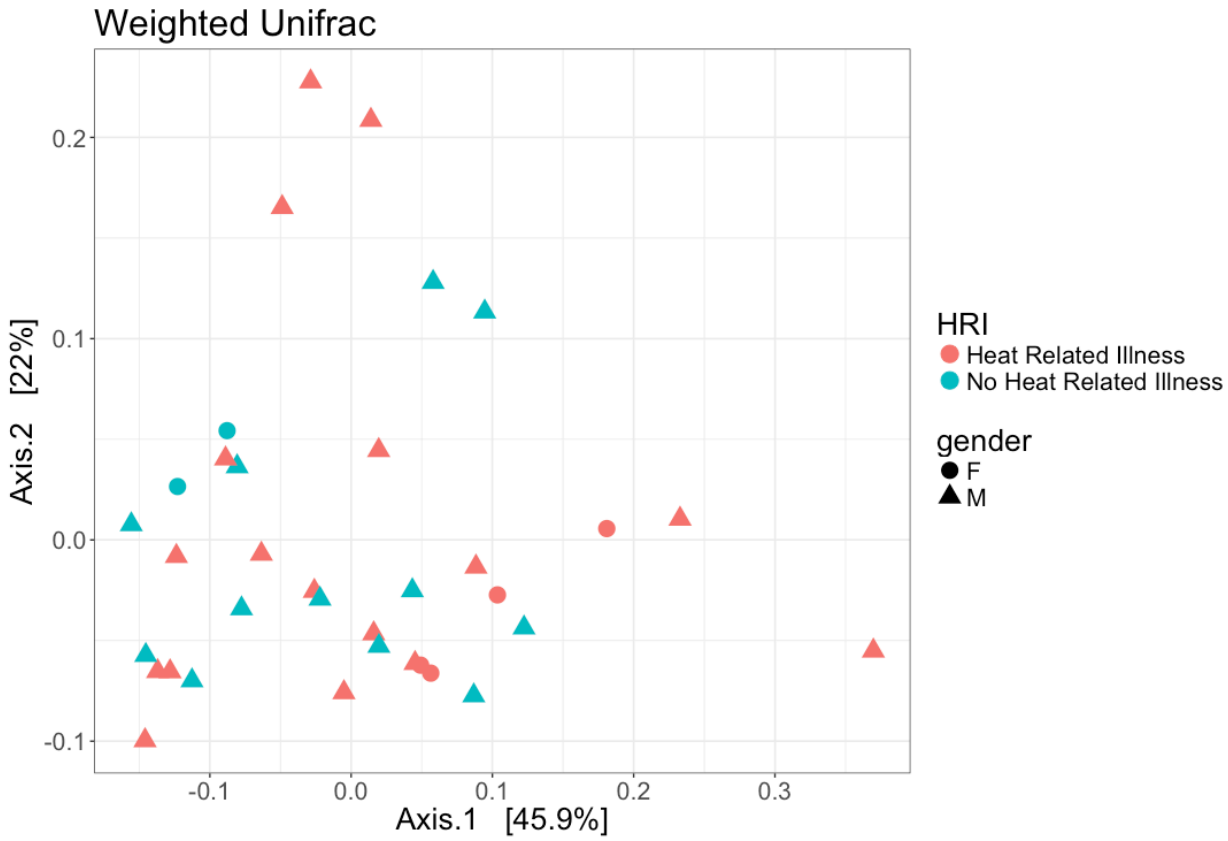

Figure 5 - Correlation Plot of Serum Biomarkers

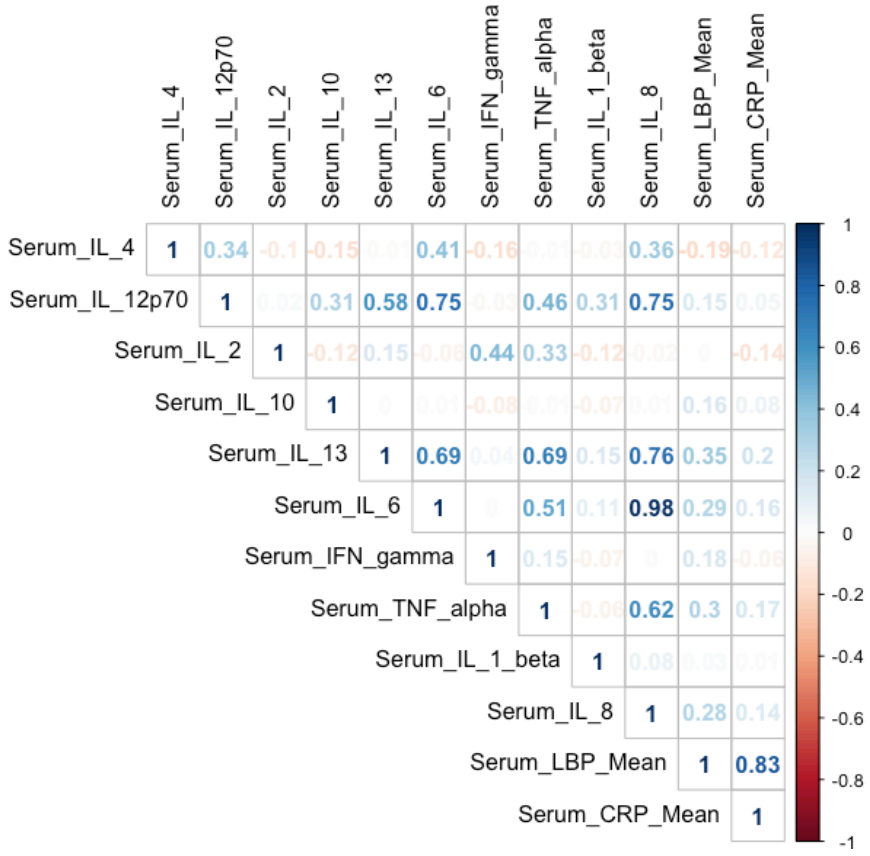

Figure 6 - Correlation Plot of Stool Biomarkers