Thanatomicrobiome Signatures in Drug Overdose Cases

by

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Abstract

Studies in forensic microbiology have been looking at the thanatomicrobiome as a new method for postmortem interval determination. The objective of this project is to investigate the thanatomicrobiome signatures of cadavers' internal organs (spleen and liver) from overdose victims. Overdose drugs were extracted from liver samples of 10 different human cadavers using a basic extraction method and extracts were analyzed to determine identity/concentration of the overdose drug(s). The most common drug found was Methadone. Bacterial DNA was extracted from postmortem samples from other human cadavers of overdose victims and identity of the bacteria determined using next-generation sequencing. Results show that *Clostridium spp.* was the most abundant genus found in the postmortem tissues and confirms the Postmortem Clostridium Effect in these criminal overdose cases. This effect will be used in the future to make predictive models of bacterial community types related to postmortem microbial communities in internal organs.

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INTRODUCTION

Postmortem interval (PMI) by definition, is elapsed time since a person has died and the postmortem investigations begins. PMI can scale from hours and days to months and years. Currently, there are multiple approaches used by investigators to determine PMI such as *algor, ligor and rigor mortis,* changes in biochemistry (Madea et al. 1994), insect activities (Meiklejohn et al. 2013), botanical processes (Lancia et al. 2013), skeletal evidence, and advanced decomposition (Bautista, 2012). However, these methods cannot give an exact time of death, but a range of time since death (Bautista, 2012). Additionally, as the PMI and range of time since death increases, the accuracy of the previously mentioned methods of PMI determination decreases (Bautista, 2012). This is an important issue that needs to be addressed in forensic science because PMI determination is a critical part of criminal and death investigations. Estimation of PMI can assist in victim identification and can potentially narrow the suspect list of an investigation (Catts, 1992). Forensic anthropologists and scientists stress the need for new objective and complete methods for PMI determination (Ferreira et al., 2013) through case studies and research.

Recent studies in forensic microbiology have been looking at the postmortem microbiome (the genetic material from community of microorganisms) as a potential new method for PMI determination (Javan et al., 2016b). These studies look at the organized changes in bacterial activity that happen after a person dies (termed the thanatomicrobiome) (Javan et al., 2016a). After death, bacteria from the gut migrate sequentially into other tissues in the human body (Javan et al., 2016a), such as the spleen, liver, heart and brain, and this spreading occurs through the invasion of bacteria into the

vascular, lymphatic, and respiratory systems (Can et al., 2014). Prior to death, these tissues should be sterile in the absence of disease (Ford, 1901; Stewart, 2012). Previous studies indicate that there are significant changes in the postmortem microbiome that are dependent on time since death and which organ is examined (Javan et al., 2016b). This suggests that knowledge of the microorganisms of the postmortem microbiome and the changes that happen in the body after death could be a useful new tool for forensic investigators for PMI determination (Javan et al., 2016b).

Several aspects of the postmortem microbiome have been examined in humans and other animals (Metcalf, J.L. et al., 2013). Studies have considered postmortem microbiome changes in human flora associated (HFA) mice (Heimesaat et al., 2012), submerged animal tissues (Dickson et al., 2011), people who died of assorted illness and diseases (Hauther et al., 2015), as well as many other conditions. However, in cases where the deceased was a drug overdose victim, the postmortem microbiome has not been explored. Drug abuse is an important topic in forensic science, due to an increasing death rate from drug overdose in the United States (Rudd et al., 2016). Just in one year (2014-2015), death due to drug overdose increased by 11.4%, which is a rate that has been consistently increasing since 1999 (Rudd et al., 2016). Drug overdose related deaths in the United States are most likely going to continue to rise in the following years, which is why forensic scientists need as many tools for analyzing drug overdose deaths as possible.

The initial objectives of my project were to determine if illicit and abused drugs affected the types of bacteria growing in the liver and heart in ten cases of fatal drug overdose. However, due to problems with the DNA extraction process, the objectives of this project were changed. The new objectives of this project were to test a method for

extracting overdose drugs from postmortem liver tissues and to look at the microbial diversity and species abundance in separate postmortem liver and spleen samples from human cadavers.

METHODS

I. Postmortem Samples - Cadaver samples were provided by Dr. Gulnaz Javan from Alabama State University. Her laboratory provided postmortem heart and liver tissue samples from ten different corpses of drug overdose victims with varying PMIs. These samples were stored in the laboratory of Dr. Frank Bailey at MTSU at -80°C until time of DNA extraction and chemical analysis. Human heart and liver tissue samples were sterilely separated into two portions, one for DNA extraction and the other for chemical analysis of the overdose drug(s).

II. Drug Extraction - A portion of each liver sample (~1-2 grams) was homogenized in a phosphate buffer (Margalho et al., 2011), and drugs were extracted and analyzed following Tennessee Bureau of Investigation Toxicology Quality Assurance and Procedures Manual 8.5 (Basic Drug Procedures). Briefly, tissue homogenates were made basic by addition of ammonium hydroxide and then extracted into an organic solvent (chloroform). Extracts were then analyzed by gas chromatography with flame ionization detection (GC/FID) followed by confirmation with gas chromatography with mass spectrometry (GC/MS) to determine the identity and concentration of the overdose drug(s) in each liver tissue.

III. DNA Extraction Methods - Preliminary tests were performed on portions of beef liver and chicken heart to determine the effectiveness of different techniques on extraction of bacterial DNA from tissue samples. Samples of the beef liver and chicken heart were infected with *Escherichia coli* (common bacterium found in the postmortem microbiome) and set out at room temperature for a few days to model the process of

decomposition.

PowerLyzer® PowerSoil® DNA Isolation Kit - Beef liver and chicken heart samples were ground with a glass Dounce tissue grinder to prepare them for DNA isolation. The PowerLyzer® PowerSoil® DNA isolation kit (MoBio Laboratories) was used to extract bacterial DNA from the tissue samples. Briefly, in this method, the tissue is initially homogenized in a bead beating tube and after a series of washing steps, DNA is bound on a silica membrane and then eluted into a buffer for storage and later evaluation.

Phenol/Chloroform Extraction - Beef liver and chicken heart samples were also extracted using a phenol/chloroform extraction technique as outlined below. This method was found to be more effective than the PowerLyzer® PowerSoil® DNA isolation kit (Mo Bio Laboratories) and was then used for the human cadaver samples as described below. Approximately 10 mg of heart and liver sample was used for bacterial DNA extraction. The tissues were placed into a Lysing Matrix E Tube (MP Biomedicals) containing zirconia and silica beads, 0.5 ml phenol/chloroform/isoamyl alcohol (25:24:1) (TE saturated, pH 8.0) and 0.5 ml of 2X TENS buffer [100 mM Tris–HCl (pH 8.0), 40 mM EDTA, 200 mM NaCl, 2% SDS] (Wan et al., 2011). Tissues were homogenized using a BioSpec Mini-Beadbeater 16 then cooled on ice. Tubes were centrifuged for 6 minutes at 16,000 RPM and supernatant transferred to a 2.0 ml Phase Lock Gel Tube (Invitrogen) containing 0.3 ml of 7.5 M ammonium acetate and 0.5 ml of chloroform. Tubes were centrifuged for 6 minutes at 16,000 RPM and supernatant transferred into new tubes containing 0.3 ml of ice cold isopropanol and 2 µl of GlycoBlue Coprecipitant (Life Technologies). After gently inverting several times, samples were incubated at

−80◦C for 10 min. Following centrifugation at 16,000 rpm for 6 min, isopropanol was decanted and pellets were washed with cold 80% ethanol and allowed to dry for 5 min. Pellets were eluted with 100 µl of TE buffer.

DNA Quality Control - Both types of DNA extracts were checked spectrophotometrically on a Nanodrop 2000® (Thermo Scientific) to assess the purity and concentration measuring the absorbance at 260 nm. The V4 region of the 16S rRNA gene was amplified using PCR with the primers 515F and 806R. The reaction product was run on a 1% agarose gel to check for target sequence amplification.

IV. Illumina MiSeq Sequencing - The initial objectives of the study were to analyze the human cadaver tissues using next generation sequencing (Illumina MiSeq®) to determine if the bacterial taxa that had grown in the tissue since death was related to PMI or the overdose drugs present in the tissues. However, it was determined that the DNA extracts from liver and heart samples were not pure enough for sequencing, so Dr. Javan's laboratory provided DNA samples from liver and spleen tissues from different overdose cases to be sequenced. Microbial diversity and relative abundances of the thanatomicrobiome were examined in these new tissues using next generation sequencing.

V. **Bioinformatic Analysis** - After sequencing, the sequence data were analyzed by Dr. Javan using a microbial diversity analysis pipeline (Javan et al., 2017). Short sequences, singleton sequences, and noisy reads were removed with denoising. Chimera detection was done with the UCHIME software (Edgar et al., 2011). For diversity analysis, each sample was run through the microbial diversity analysis pipeline to group reads into operational taxonomic units using the UPARSE algorithm (Edgar, 2013), and

then aligned using the USEARCH global algorithm (Edgar, 2010) against a database of 16S rRNA gene sequences to determine taxonomic classifications (Javan et al., 2017). Microbial diversity was determined using the Shannon Diversity index of species diversity (McMurdie and Holmes, 2013). Analysis of variance (ANOVA) was used to screen for group (organ) differences in microbial diversity. Overall richness was calculated using the Chao1 richness estimator (McMurdie and Holmes, 2013).

RESULTS

I. Drug Identification: Drugs were found in every liver tissue sample that was analyzed (Table 1). Opioids were found in 70 % of the samples, with methadone being the most common drug found (three samples). The maximum number of drugs found in one sample was nine (G25) and all but one sample contained multiple drugs.

Table 1. Results of Drug Extraction from Liver Tissue Samples.

	C42	G25	G40	G10	G24	C38	C40	G7	G26	C21
Amphetamine					0.26					
Benzodiazepine									+	
Buprenorphine		+								
Bupropion (Breakdown)										+
Citalopram (Breakdown)					+					
Cocaine		+			+					
Codeine			0.55							
Dextromethorphan	+			+						
Diphenhydramine		+						+		
Doxepin							+			
Doxylamine	+									
ECME		+								
Fluoxetine										2.0
Gabapentin (Breakdown)			+							
Hydrocodone		+	+							
Levamisole		+			+					
Methadone			+			+	+			
Methamphetamine		+			1.0					
Metoprolol				+						
Norfluoxetine										+
Nortriptyline									0.13	
Oxycodone								0.15		
Quetiapine		+								
Tramadol										+
Venlafaxine		> 1.0								

II. Illumina MiSeq Sequencing: All samples were successfully sequenced and reached the maximum number of reads (Figure 1). The rarefaction plot shows validation of full coverage to 20,000 reads to observe all taxa by showing that species richness approached an asymptote for all the samples.

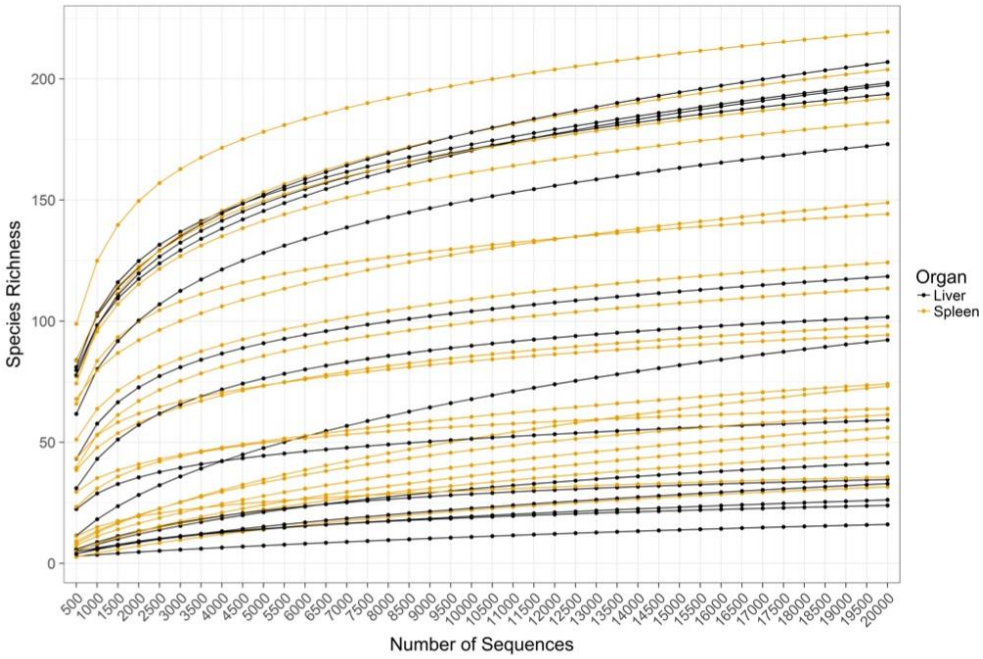

Figure 1. Rarefaction plot of species richness, subsampling from 500 to 20000 reads in increments of 500 reads.

The highest percentage of bacteria on the genus and species levels found in the liver and spleen tissues was *Clostridium* (Figure 2). Over 25% of the bacteria found in the liver and spleen tissues on the genus level was *Clostridium*. Other well -represented genera include *Pseudomonas, Lactobacillus*, and *Bacteroides*.

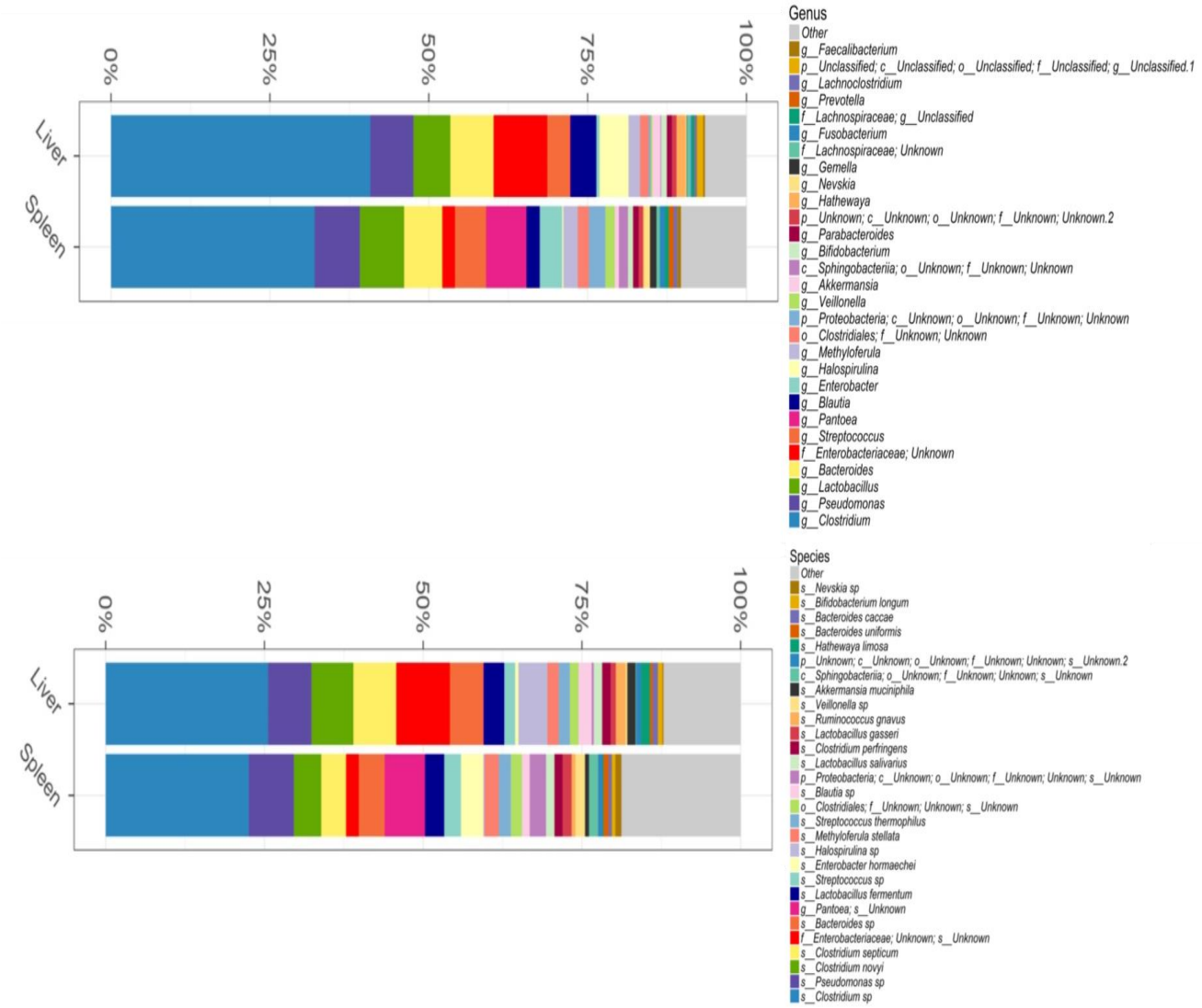

Figure 2. Relative abundances of the top most abundant bacteria in liver and spleen. Both organs are illustrated according to Genus and Species.

The liver and spleen tissues showed similar Shannon Diversity indices (Figure 3). This diversity index accounts for species abundance as well as evenness of species present. There was no significance difference in Shannon Diversity found between the two organs ($F_{(1,31)} = 0.45$, $p = 0.5061$).

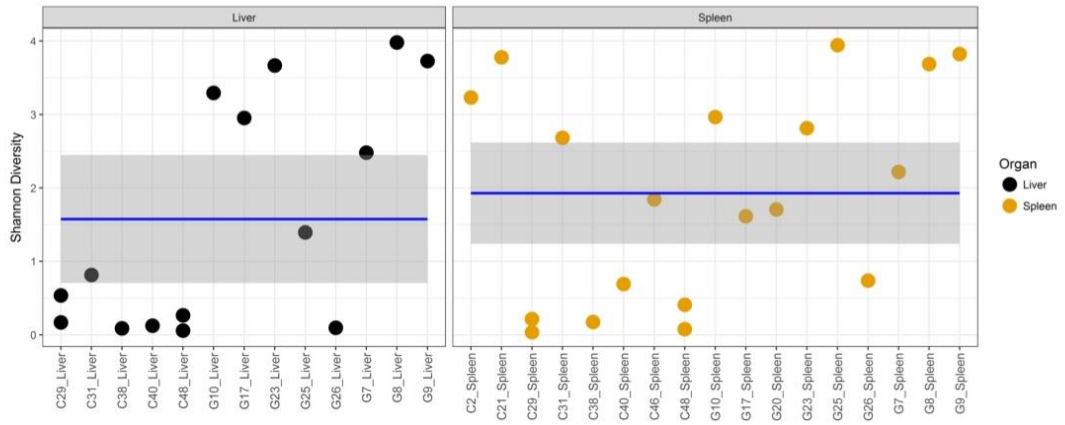

Figure 3. Shannon diversity within the total microbiome data, colored and faceted by organ. The mean value (and confidence interval) in each group also are illustrated.

The predicted number of taxa in the samples is shown in the Chao1 richness index (Figure 4). There was no significant difference in Chao1 richness found between the two organs ($F_{(1,31)} = 0.16$, $p = 0.6893$).

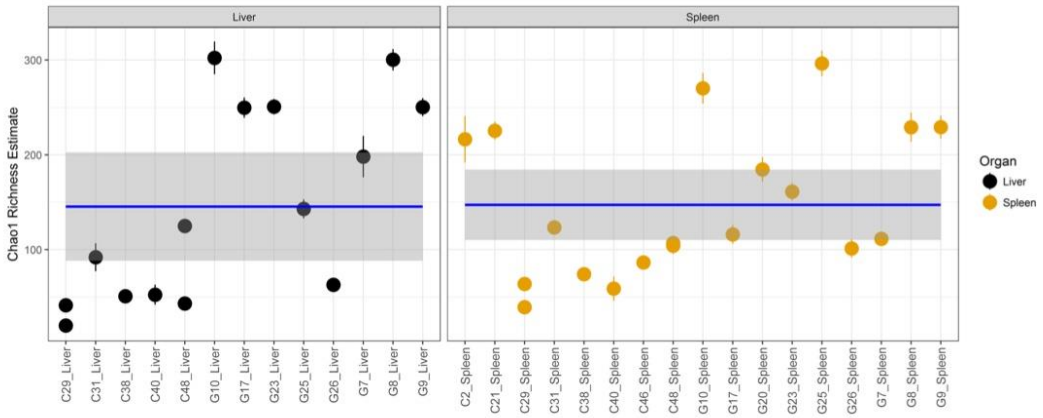

Figure 4. Chao1 richness within the total microbiome data, colored and faceted by organ. The mean value (and confidence interval in gray) in each group also are illustrated.

The heatmap (Figure 5) shows the abundance of bacterial genera by organ, sorted by sample number and bacterial taxon. The most abundant bacterial genus found on the heatmap was *Clostridium* (Figure 5), which was found in 38% of the liver and spleen samples.

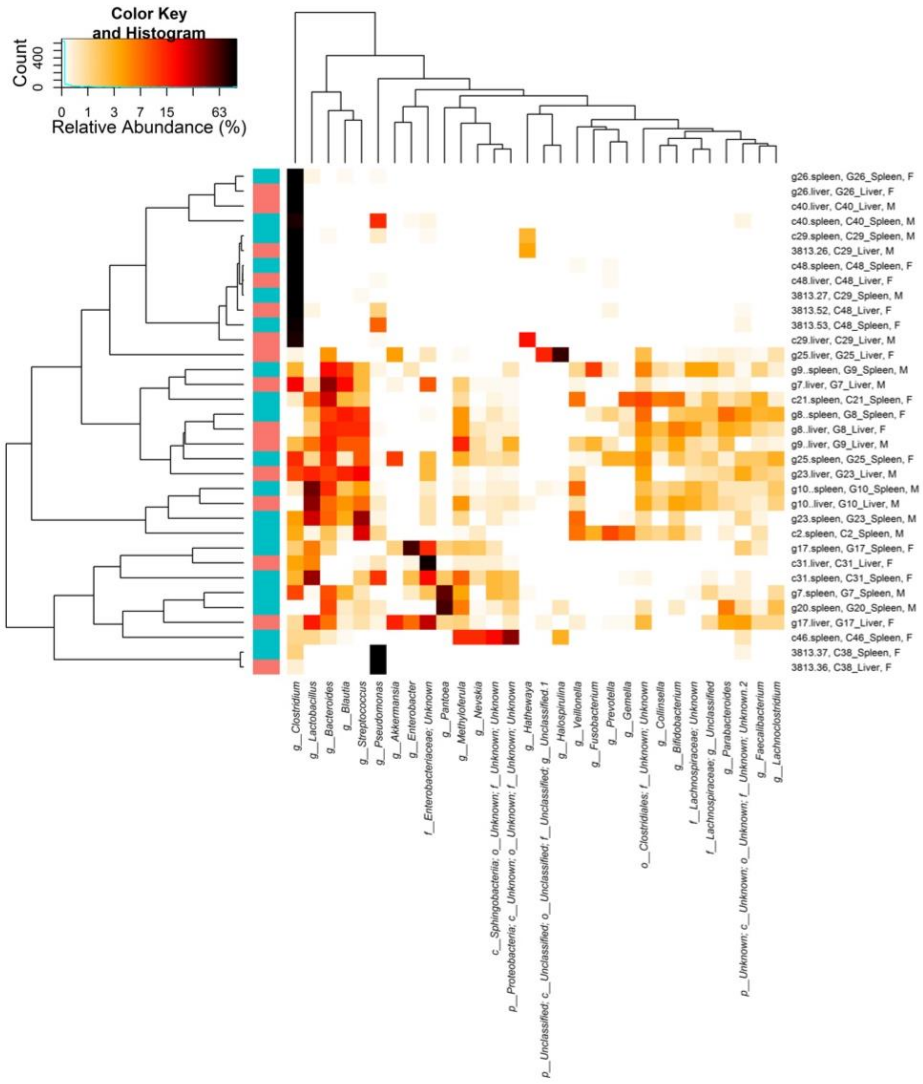

Figure 5. Heatmap of relative abundances of the top 30 bacterial genera by organ. Samples and bacteria are sorted based on weighted UniFrac and Euclidean distances, respectively.

DISCUSSION

The methods of extraction used for the drug identification were successful at extracting drugs out of the postmortem liver tissues. A basic extraction was done, which means that the extraction was only focused on finding basic drugs. The Tennessee Bureau of Investigation (TBI) mainly screens for basic drugs since most of the drugs that are screened for fall under this category (https://www.tn.gov/content/dam/tn/tbi/documents/Toxicology%20Drug%20List.pdf). Other types of drugs might have been present in the liver tissue samples (acidic, neutral), but were not extracted due to the basic extraction method. In future studies, multiple drug extraction methods might be beneficial to find more of the drugs present in the samples. The liver tissue was used for the drug extraction due to the role of the liver in processing toxins, such as drugs, from the blood. The results of the drug extraction were not compared to microbiome data as originally planned due to the microbiome data not being available for the original samples. In future studies, it would be beneficial to do the drug extraction on samples that have microbiome data so that they can be compared to microbiome samples containing no overdose drugs.

There was no significant difference found between the two organs, liver and spleen, regarding Shannon Diversity and Chao1 richness (Figure 3, 4). This indicates that the bacterial communities in these organs are similar in abundance and evenness of species. It may be beneficial to examine the similarity of bacterial communities between other organs in future studies.

The highest percentage of bacteria found in the liver and spleen samples was *Clostridium*, which is consistent with other thanatomicrobiome studies. The abundant

nature of *Clostridium* species in postmortem tissue was established in a recent study looking at 45 different corpses in different stages of decomposition (Javan et al., 2017). In this study, Clostridiales was the most abundant bacteria found on the order level (Javan et al., 2017) and the high relative abundance of *Clostridium* species during human decomposition was termed the "Postmortem Clostridium Effect" (PCE) (Javan et al., 2017). The high abundance of *Clostridium* in decomposition is thought to be due to three main reasons: quick doubling time, proteolytic functions, which allow migration into nearby tissues, and cessation of the heart that results in hypoxia and leads to the flourishing of anaerobic bacteria (Javan et al., 2017). In the future, thanatomicrobiome research hopefully will be used to make predictive models using the Postmortem Clostridium Effect to further recover different bacterial community types related to postmortem microbial communities in internal organs (Javan et al., 2017).

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