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The Gut Microbiome: Is Fecal Matter Microbial Composition a Proxy for Intestinal Microbial Composition in Studies of the Microbiome

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The Gut Microbiome: Is Fecal Matter Microbial Composition a Proxy for Intestinal Microbial Composition in Studies of the Microbiome?

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by

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ABSTRACT

The Gut Microbiome: Is Fecal Matter Microbial Composition a Proxy for Intestinal Microbial Composition in Studies of the Microbiome?

by

Enrique Reyes

As many health phenomena seem to be affected directly and indirectly by the microbiome, gut microbiome research has increased in the last decade. Issues such as allergies, cancer, obesity, and other health complications have been shown to be influenced by the microbiome. Most of gut microbiome research is done by collecting and sequencing the DNA of the microbiome of the fecal matter from model organisms or human subjects. Studies that use this method of sample collection and analysis assume that fecal matter microbiomes are similar to intestinal microbiomes, and that it can be used as a proxy. At present, no published studies exist which directly compare stool microbial composition and intestinal microbial composition. Bacterial composition was found to be different at all levels of taxonomy between stool and the different portions of the intestines tested. Uni-variate analysis shows significance between the two main phyla Firmicutes and Bacteroidetes, as well as main genera like *Odoribacter, Porphyromonas* and *Alistipes.* Relevant species *Desulfovibrio desulfuricans* and *Odoribacter splanchicus* were also found to be significantly different in relative abundance between stool and some parts of the intestines. Alpha-diversity was not significantly different between all parts of the intestine and stool. Beta-diversity was significantly different between the ileum and stool,

with stool having slightly higher diversity. Looking at the bacterial composition of both environments and the relative abundances of the dominant taxa, one can see that that there are key differences between the intestines and stool. The microbial composition at all levels of taxonomy was found to be different, therefore, research that targets the gut microbiome should look closely at the specific taxa being observed. Phyla like *Firmicutes* and *Bacteroidetes* were found to have different abundance within the portions of the intestine and stool, suggesting that phyla level analysis should be performed by observing each community separately. Interestingly, diversity analysis was not found to be significant, suggesting the composition is different, but the number of different taxa is similar across the intestinal tract and stool.

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CHAPTER I

LITERATURE REVIEW

The gut microbiome is currently a very popular field of study. Fifteen years ago, a keyword search using the term “microbiome” on a journal’s search would have resulted in a very limited number of papers. A more recent Google Scholar search using the term “microbiome yielded over 200,000 papers. If the term “gut” is added to the search, 31,100 articles are found. Microbiome scientists have found a field of research that may have an influence in all aspects of biology and human health. Recent studies have shown connections between the composition of the gut microbiome and: the immune system, mental health and brain chemistry, and obesity (1). So far, gut microbiome researchers have only scratched the surface of some of these phenomena, allowing for further research to assess the biological implications of the connections made, adequacy of methodology and medical significance.

Gut Microbial Composition

The intestinal microbiome is a highly diverse system that is mostly composed of commensal organisms. Biodiversity is defined as: “the variety and variability of biological organisms”, Therefore, by these parameters, the microbiome is a highly diverse environment. The gut microbial content varies between portions of the intestinal tract, but generally is made of ~10^3 - 10^{14} microbes per gram of content. The cumulative genomic material of the gut microbiome contains 100 times more genes than our own genome (1). Human gut microflora is dominated by member of two main phyla, the Firmicutes and Bacteroidetes, which form roughly 90% of the gut microbiome. Some of the more prevalent genera are *Clostridium,*

Bifidobacterium, Lactobacillus and *Ruminococcus* (2). Although the microbiome composition varies during early development, in humans, it stabilizes around the age of three (3, 4).

Human Gut Microbial Composition

The area termed "the gut" for humans is shown in Figure 1. The stomach is inhabited predominantly by organisms belonging to the genera *Lactobacillus* and *Streptococcus.* Bacterial biodiversity is very low in this portion of the intestinal tract due to the highly acidic environment of pH 1-2. Bacterial biodiversity in the duodenum and jejunum is higher due to the less acidic environment of 5.7-6.4 pH. Organisms belonging to the genera *Lactobacillus, Escherichia* and *Enterococcus* dominate this section of the intestinal tract. The ileum is a highly diverse environment with many organisms belonging to the genera *Enterobacter, Enterococcus, Bacteroidetes, Clostridium* and *Lactobacillus.* The ileum is a neutral environment with a pH range of 7.3-7.7. In humans, the cecum and colon have similar community structure and diversity. The cecum is the portion of the gut that connects the small intestine and the large intestine. The colon is the portion closest to the rectum in the large intestine. These two sections have neutral environments, and the highest number of species in the intestinal tract. Organisms belonging to the genera: *Bacteroidetes, Eubacterium, Bifidobacterium, Ruminococcus, Propionobacterium,* and others are found in these two sections of the intestinal tract (4; 5).

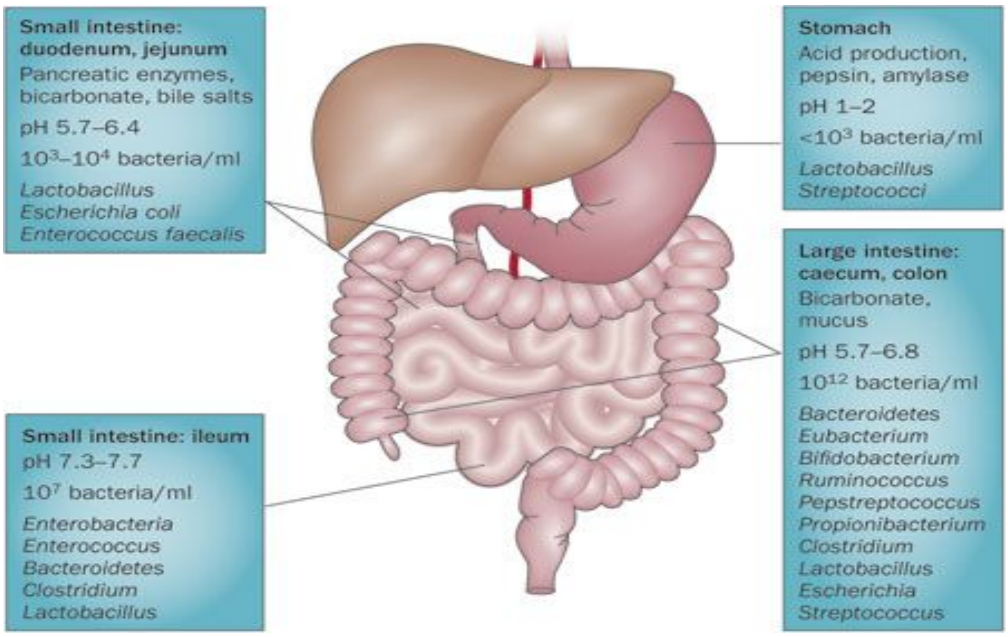

Figure 1. Human Gastro-intestinal Tract microbial composition (4).

Mouse Gut Microbial Composition

Mice are the animal model most commonly used to study gut microbial composition and the effects that changes in diet and exercise have on the gut microbiome. The gastro-intestinal anatomy of the mouse is a similarly complex version of the human intestinal tract, with very comparable structures (Figure 2). The human and mouse stomach are very similar to each other and the human gut microbiome and the mouse gut microbiome are both dominated by the same phyla, Firmicutes and Bacteroidetes. When looking deeper into the species taxonomic level, one can observe differences between the human and mouse microbiomes. According to Ley and colleagues 85% of microorganisms found in the mouse gut microbiome are not found in the human gut microbiome (6). Another study took the task of assessing these finding by using metagenomics techniques in already existing datasets of human fecal matter and mice cecal samples. The researchers recognized that having only fecal matter data for humans (as opposed to intestinal data) is a limiting feature of their study, but they found similar results to other

researchers in the field. The mouse microbiome and the human microbiome share a large number of microorganisms with roughly 79 genera shared by both organisms (7).

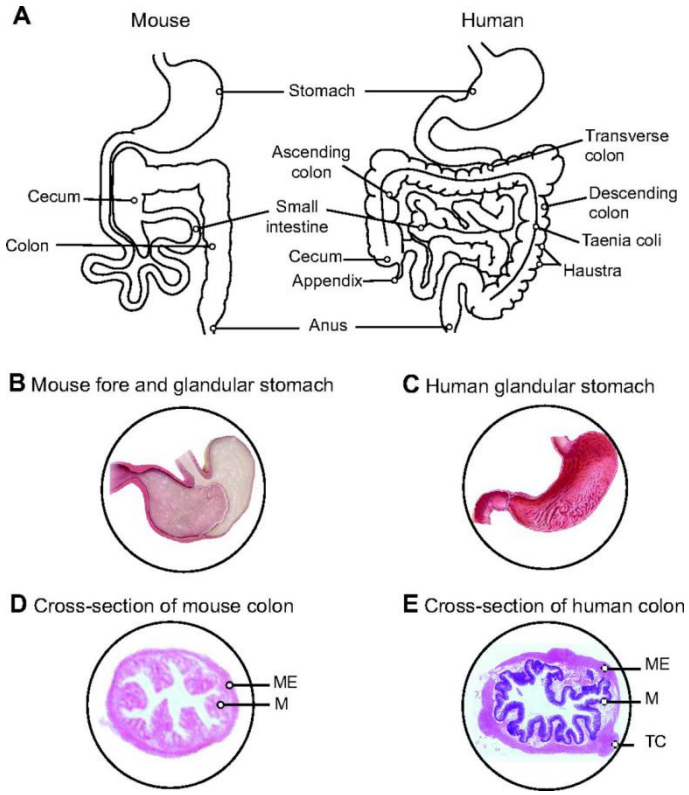

Figure 2. Mouse intestinal tract vs. human intestinal tract (7).

Mouse intestinal microbial communities are composed of species belonging to the genera *Lactobacillus, Alistipes, Roseburia, Bifidobacterium, Clostridium,* and others. This composition is very similar to that of humans, but the content, as in abundance, seems to be different. The relative abundances of these genera differ greatly between mouse and human, but, interestingly, the species richness is similar (Figure 3). Although some differences are observed in abundances of certain taxa, the mouse microbiome is still considered a good model to study microbial communities and the effects of changes to the environment (8). Germ-free mice, for example, are a great way to study the effect of certain microbial communities in the environment. These mice have been bred in completely aseptic conditions; therefore, they do not have any microbiomes.

This allows the researcher to create a gut microbiome specific to individual mice under study. Phenomena like obesity, dieting and probiotics have been studied using germ-free mice (8).

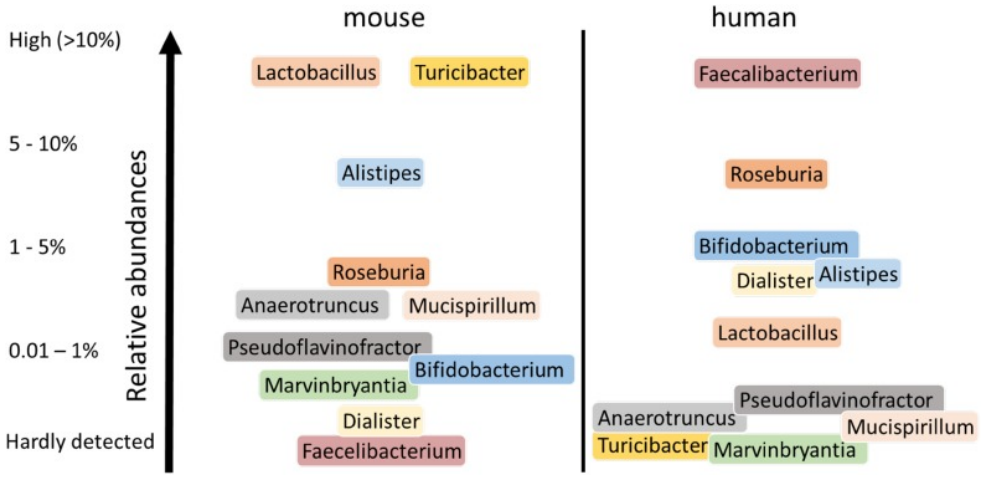

Figure 3. Relative abundances of certain intestinal genera are different between humans and mice, but species richness is very similar (8).

Methodology for Microbial Community Identification and Comparison

Sample source

Recent gut microbiome research uses fecal matter as a source of the gut microbiome, rather than samples taken directly from the intestine, primarily due to the relative ease of collection. In a Google Scholar search for “gut microbiome”, the first twenty-two references that appeared used feces rather than intestinal samples (Table 1). Interestingly, research that specifically compares the composition of fecal microbiota to that obtained from intestinal samples appears to be lacking.

Table 1 Comparison between usage of stool samples as a proxy for gut microbial composition, usage of intestinal samples, and the usage of both sample types.

Used feces as a proxy for intestine microbial composition	Used intestinal samples	Used both fecal and intestinal samples
Turnbaugh *et al..,* 2009	Lagkouvardos *et al..,* 2016	Eckburg *et al..,* 2005
Kalliomaki *et al..,* 2008	Markle, *et al..,* 2013	Kleessen *et al..,* 2001
Mai *et al..,* 2011	Turbaugh *et al..,* 2006	
Gill *et al..,* 2006	Ravussin *et al..,* 2012	
Ley *et al..,* 2006		
Qin *et al..,* 2010		
De Fillipo *et al..,* 2010		
Ley *et al..,* 2005		
Jumpertz *et al..,* 2011		
Routy *et al.,* 2018		
Gopulakrishnan *et al.*, 2018		
Halfuorson *et al.,* 2017		
Xia *et al.,* 2017		
Wu *et al.,* 2017		
InSerra *et al.,* 2019		
Jin *et al.,* 2019		

Nucleic-acid Based Techniques

The gut microbiome is a very challenging system to research and observe. Early techniques used to identify species were culture based and employed biochemical testing. These types of testing are beneficial to observe microorganism in pure cultures, but have proved limiting for capturing the extent of species richness. Because more than 80% of our gut microbiome is of anaerobic nature and fastidious in growth requirements, only about 1% of the microbes in our gut have been cultivated in the laboratory setting with traditional culture-based methods (9). Microbiome research that targets identification, characterization, and multiple community comparisons have moved to use molecular-based techniques that use genetic data to

assess counts and abundance of each species. Nucleic acid-based techniques have been very useful to identify organisms to the species and sub-species level, and have provided a new avenue of research with the different possible questions they can answer. Some of these techniques use bacterial ribosomal RNA and/or DNA, and others use the collective DNA extracted from a particular environment (10) (Figure 4).

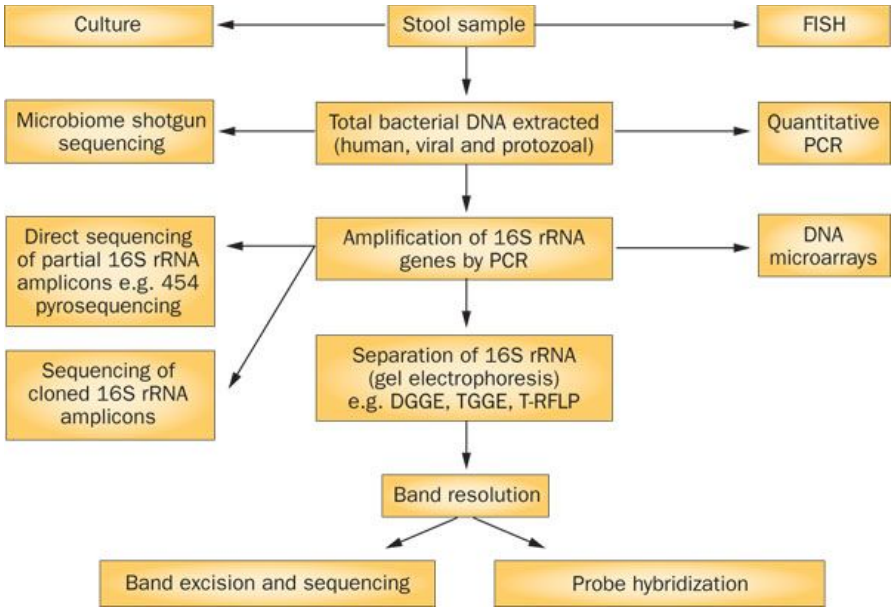

Figure 4. Diagram of microbiome characterization techniques (42).

16S rRNA amplicon sequencing

One technique that has been widely used in the field of gut microbiome research is 16S rRNA amplicon sequencing. This technique uses rRNA sequences to provide a taxonomic profile for microorganisms present in the environment. It is called rDNA amplicon sequencing because it uses ribosomal RNA genes as a basis for taxonomic assignment. The ribosome is a structure that serves as a protein generator (Figure 5). It uses amino acids and mRNA to yield a polypeptide chain that eventually gets folded into a protein. The ribosome consists of several sub-units, which in turn are categorized using a non-SI unit called Svedberg. The “S”, or

Svedberg coefficient, is a non-SI unit that serves as a measure of how big and at what rate a particle moves in an environment subjected to high G-force, such as those incurred in a centrifuge. This unit is based on time, but it is not translated as seconds or minutes, but as a rate of how fast can a molecule move, which correlates to how big it might be as well, therefore the values assigned to each sub-unit are not additive, but only descriptive (11).

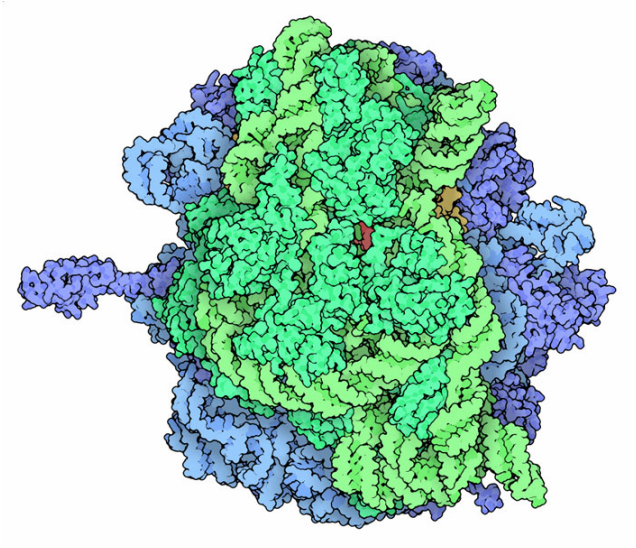

Figure 5. 3D structure of a bacterial ribosome. Green=small sub-unit, Blue=large sub-unit (43).

Prokaryotes (Bacteria and Archaea) have free-floating 70S ribosomes in their cytoplasm. This ribosome is divided into a 30S (small) and a 50S (large) sub-unit, which are each divided each into more sub-units. The 50S sub-unit is divided in two RNA molecules named 23S and 5S. The 30S product contains the sub-unit that is most commonly used in prokaryotic taxonomy, the 16S subunit. Since it is the most conserved sequence and the easiest to track with PCR (Polymerase Chain Reaction), the 16S rRNA gene is the preferred sequence for multiple molecular-based techniques used to identify microorganisms in an environment. Figure 6 shows the process of 16S rRNA amplicon generation. All prokaryotes have portions of the 16S

sequence that is conserved, and multiple variable regions which can be used to differentiate between phyla, genera and species. Some conserved regions are unique to prokaryotes and can therefore be used as sites for primers to bind to amplify prokaryotic DNA. PCR amplification of the variable regions tells the researcher the necessary information to assign taxonomic designations by cross-referencing sample data with a public database that contains many known sequences of bacterial 16S rDNA to pin-point the exact taxonomic designation for all the sequences in the amplified sample (10). 16S rRNA amplicon sequencing has proven better, than other nucleic-acid based techniques, for testing large samples, but it also has lower resolution than other techniques. Other advantages of this technique are: it is able to exclude all non-bacterial organisms, ease of access, it can yield accurate taxonomic designations and is less labor intensive (12).

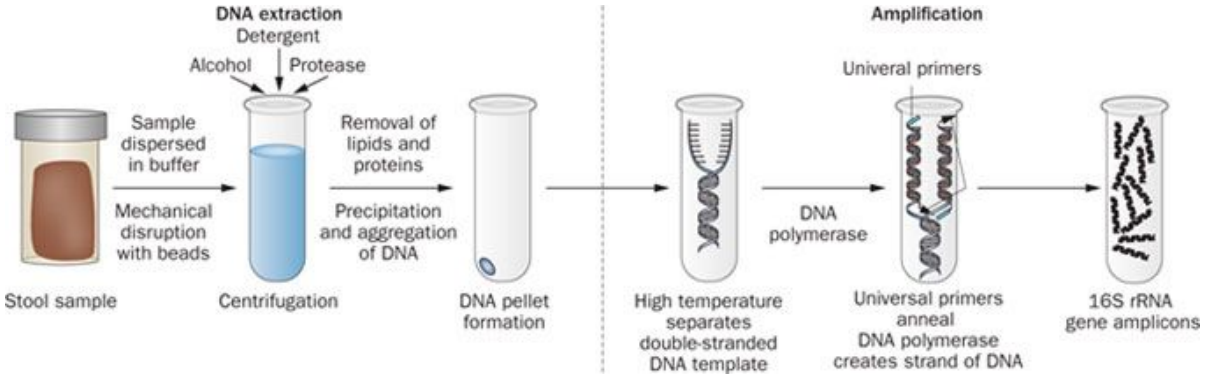

Figure 6. 16S rRNA amplicon production process (42).

Metagenomics

A more sensitive technique that is also highly used in microbiome research is metagenomics. Rather than amplifying and sequencing a single gene, metagenomics involves

extracting and sequencing all the DNA in a sample. This allows the researcher to find important genes (virulence or certain metabolic genes, for example) and observe whether they are present in the environment or not, providing information about metabolic pathways that can be performed by that certain organism (14). Metagenomics not only allows for assignment of taxonomic designations, but also reveals the whole gene repertoire of the system (13). Techniques like "shotgun sequencing" and metagenome analysis can provide higher resolution, allowing the researcher to assign designations as specific as sub-species and strain. These techniques have proven useful for creating a genetic profile of the microbial environment, often identifying specific genes that produce virulence factors or important enzymes more often than other molecular-based technologies (13).

Metatranscriptomics

Metatranscriptomics is a group of techniques that study the transcriptome of a microbial community. The transcriptome is the collective sum of messenger RNA and other RNA products' sequence information. The focus of these techniques is the isolation and sequencing of RNA fragments to assess expression levels to infer metabolic activity and population viability. (15; 16; 17). This technique can be challenging in environments that include both prokaryotic and eukaryotic RNA. Prokaryote RNA does not have a poly-A tail, therefore selection during cDNA synthesis is not possible as it is with eukaryotes. Probes targeting certain sequences of RNA that are bound to magnetic beads to exclude the unwanted eukaryotic and ribosomal RNA is a highly used method. RNA is then reverse-transcribed to cDNA and sequenced to determine which genes were being expressed (15; 16; 17; 18).

Metabolomics

Metabolomics is the branch of genomics that studies the metabolome. The metabolome is the metabolic profile of microbial community in a particular an environment- it measures metabolites such as sugars, proteins, and lipids. Metabolomics is currently used to identify markers that could lead us to the development of diagnostic techniques for multiple diseases, observe biochemical stresses, identify microbial metabolic products, and characterization of disease-related metabolites (19; 20; 21; 22). Techniques like liquid and gas chromatography (LC, GC), mass spectrometry (MS) and nuclear magnetic resonance (NMR) allow for the researcher to develop a chemical profile of the system that can show what type of biochemical pathways are occurring in the environment. These techniques are better employed in research geared towards understanding the effect of metabolites on health and disease (23; 24; 25). Bacteria are responsible for the breakdown and production of some of these metabolites that can be studied through metabolomics. Metabolites like short-chain fatty acids (SCFAs), vitamins, other acids, bile salts, amino acids and other biomolecules can be produced or transformed by bacteria (Figure 7). Metabolomics can be used to create a metabolic profile that includes which organisms are producing which of these metabolites (23)

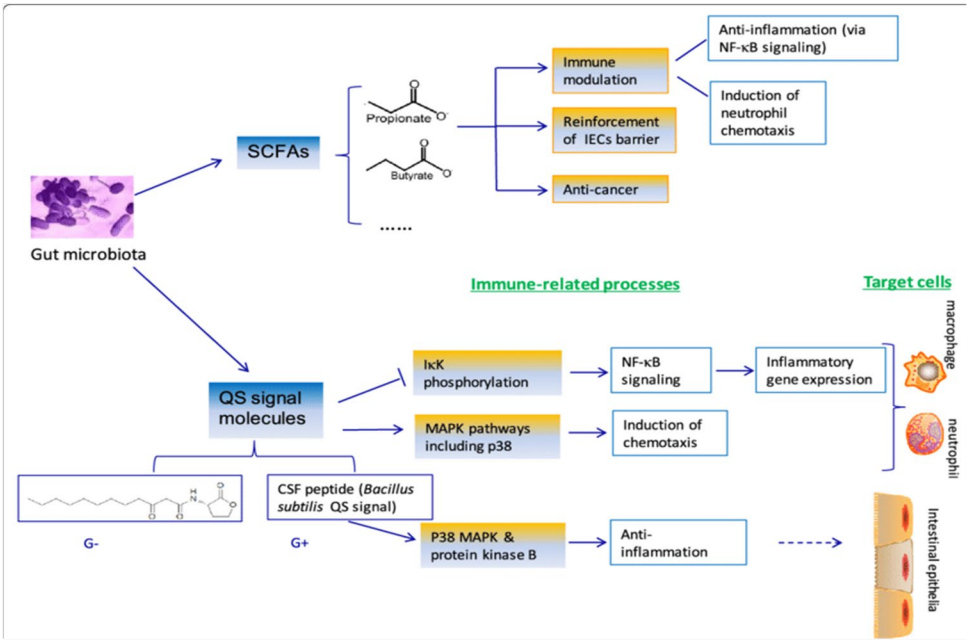

Figure 7. Gut microbial metabolites and signaling molecules (23).

Analysis of 16S rDNA data

Microbial community comparison bioinformatics uses 16S rDNA raw data to assign taxonomic designations and to calculate the genomic relatedness of two or more communities. The data is often extensive and complex. When studying the gut microbiome, each 16S RNA gene amplicon is referred to as an operational taxonomic unit (OTU). Each OTU represents a species of prokaryote. A single gut sample may have over 1000 species, where each species is represented by a population ranging from 10 to several million members. It would take a great amount of time to sort, group, analyze and plot these data. Therefore, multiple software platforms have been developed to accelerate the process and to create informative figures that accurately represent the composition of the microbiome. Software like mcaGUI, PICRUSt and UniFrac, which use the R programing language, are some of the most widely used programs in

microbiome research. Other programs like Qiime and Mothur are also effective, but are Python-based and require a higher skill level in coding and bioinformatics. All of these programs are ultimately used for 4 general categories of tasks. These categories are: (i) taxonomic assignation of individual OTUs, (ii) functional profiling, or assigning genes to metabolic pathways that can show the researcher the overall functional profile of the microbiome, (iii) community comparison, which identifies differences and similarities between the OTU make-up of two or more microbial communities, and (iv) meta-analysis, or the use of previously collected data to draw conclusions about certain phenomena or environments (26).

mcaGUI

This open-source program uses the R language and environments to provide multiple statistical tools and packages that can be used for microbial community comparison. It allows the researcher to create OTU tables with the abundances and counts of organisms obtained from the 16S rDNA raw data. Other analysis can then be performed. Principal component analysis (PCA), richness and diversity estimates, and multiple-community comparisons are the most popular analyses performed (27).

UniFrac

UniFrac is a community comparison measurement that takes into account the genetic relatedness of multiple communities. To assess the genetic distance between two communities it uses phylogenetic trees that are individually formed for each sample (28). A phylogenetic tree is a model used to display the genetic composition and history of an environment. This tree has

branches and nodes, which represent genetic distance and taxa or common ancestors respectively. Branches that are closer together correspond to closely related microorganisms/communities. Branches that are further apart correspond to less related organisms and communities (29). UniFrac uses this model and by quantifying the phylogenetic distance between different sets of taxa in terms of length of the branch, it can provide a coefficient from 0-1 that corresponds to the level of relatedness between two or more communities. Microbial communities can also be clustered in a plot, visually showing similarities or differences between them (28). By using this measure one can observe that if relative abundances of each OTU are not taken into account, then all organisms have the same weight, yielding a UniFrac value that is only based on OTU richness. To reduce the impact that presence has over abundance, a weighted UniFrac can be used. This measure allows the researcher to observe similarities of communities by relative abundances and not just by presence, diminishing the effect of low abundant present organisms. The un-weighted version just looks at the presence of organisms giving equal statistical power to all OTUs even if they are in low abundance in the environment (28;30).

MicrobiomeAnalyst

MicrobiomeAnalyst is a recently-developed web-based software that contains four modules of work, which are the following: Maker Data Profiling, Shotgun Data Profilin, Projection with Public Data and Taxon Set Enrichment Analysis. Maker Data Profiling is a module that takes 16S rRNA sequence data and uses it to calculate measures like α−diversity and β−diversity using a variety of statistical methods (Shannon, Chao-1, ACE, etc) (26). These measurements are important because they measure the level of species richness and evenness in

an environment, and having high biodiversity is a marker of community health. α–diversity is the richness and evenness of the organisms within a single community and β–diversity is the richness and evenness between communities. Shannon's index is a measurement of how diverse a community is, while taking into account the total amount of organisms and total amount of each organism. This index yields a value from 0-1. The closer to 1 it gets the more diverse the environment is. SDP is a module meant to analyze and organize metagenomics and meta-transcriptomics data. PPD allows for the researcher to compare the data obtained with already published and analyzed data. Finally, TSEA is a module designed to assess the biological implications or effects of a certain list of OTUs in the environment. For the purpose of this project, MDP will be used, since it is the most appropriate module for the questions asked (26). Figure 8 represents a flowchart that shows all possible analytic capabilities of MicrobiomeAnalyst, depending on format input.

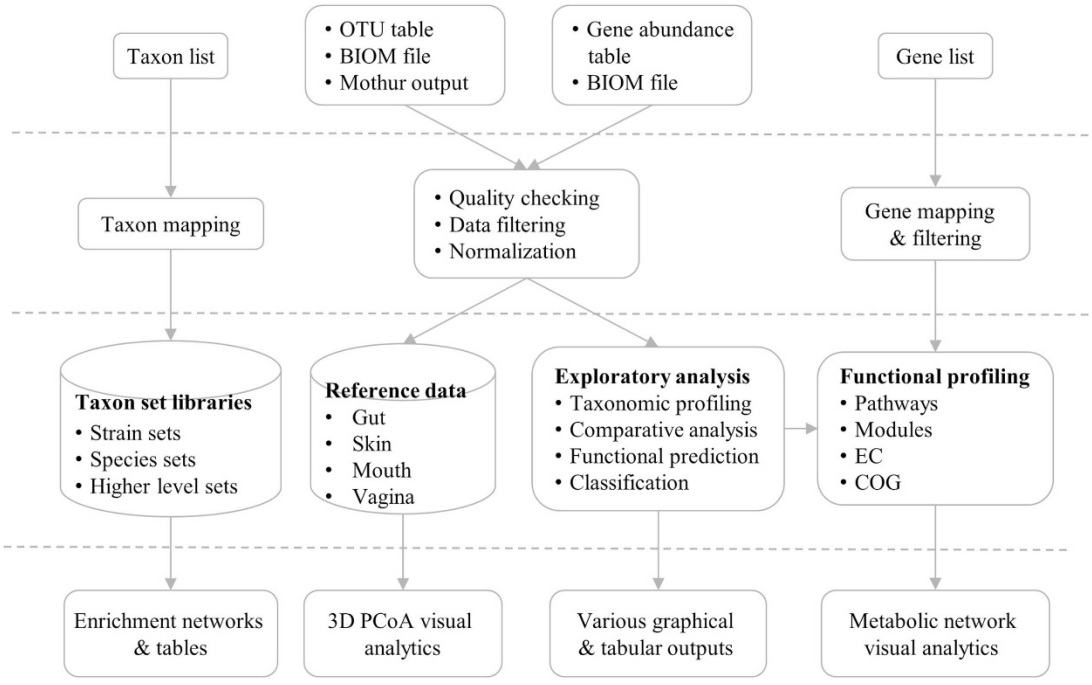

Figure 8. Microbiome Analyst flowchart representing all types of analysis depending on file input and data type (26).

Gut Microbiome in Health and Disease

Multiple health related connections have been made between the gut microbiome and various diseases and conditions. Microbiome researchers have connected diseases and disorders like obesity, atopic diseases, allergies, stress, and others with the microbial composition of the gut (31). Most of these studies have one aspect in common: the source of the samples used to assess the composition of the gut. Evidence supporting connections between conditions and diseases is based on the premise that stool microbial composition is a proxy for intestinal microbial composition; therefore, only fecal matter is used for microbiome samples. Even though no studies have demonstrated that fecal microbiomes are representative of the gut microbiome, many studies have relied on fecal microbiome biome data for their experiments. Table 2 summarizes the relevant findings from select studies that have used mouse and human data from both fecal and cecal microbiomes.

Knowledge gleaned from analysis of fecal samples

In an experiment aimed to investigate if gut microbial composition can precede obesity, the researchers found that the presence of the genus *Bifidobacterium* was significantly decreased in overweight children. *Bifidobacterium* species are responsible for controlling the populations of other microbes during infancy. This suggested to the researchers that there is a link between *Bifidobacterium* and obesity (32). Phyla like Firmicutes and Bacteroidetes have also been

connected to obesity. Obese individuals show an increase in the phylum Firmicutes compared to Bacteroidetes, and lean individuals have a balanced proportion (33).

Germ-Free (GF) mice are a great model for assessment of the implications of certain shifts in microbiome (dysbiosis) or the consequences of growth in a completely sterile environment for gut microbial composition and organismal health. Some immunological diseases and allergies have been correlated to microbial composition irregularities, or mal-developed microbiome (34). Germ-Free mice show mal-developed gut associated lymphoid tissue, lower counts of multiple leukocytes, poorly developed germinal centers, and low immunoglobulin levels, suggesting that not having a microbiome causes an overall detriment to the development of the immune system. (34). Certain microorganisms have been observed to aid in some of these processes and in immunomodulation as well. Organisms like *Lactobacilli* and *Escherichia* have been observed to induce T cell differentiation to a T helper lymphocyte 2 or T regulatory responses. This led researchers to conclude that these organisms promote a tolerogenic environment in the gastrointestinal tract (35). This, in turn, led microbiome researchers to connect allergies and atopic diseases to gut microbial composition and microbiome development.

Disorders and conditions like stress, anxiety and depression, Autism Spectrum Disorder, Parkinson ’s disease (Pd) and others have also been connected to dysbiosis and abnormalities in the gut microbiome (36). In an experiment aimed at understanding the relationship between the microbiome and stress, anxiety and depression, researchers found that GF mice have higher corticosterone when challenged with restraint to promote stress, than Specific Pathogen Free (SPF) mice. GF mice also exhibited reduced neurotrophic factor expression, which is a sign of potential neuronal mal-development. All of these issues were reversed by reconstitution of certain organisms such as *Bifobacterium infantis* (37). Other disorders and neurological diseases

also share connections with the gut microbiome. For example, Pd was found to have a microbial aspect to the underlying cause of the disease. Sampson and colleagues (38) investigated the relationship between the gut microbiome and Pd's pathophysiology. They found that GF mice colonized with Pd microbiota display Pd symptoms and behavior as well as reduces microglia activation. Microglia size and branching was reduced in Pd mice. The same was observed when GF mice were given Pd microbiota. Mice that had Pd symptoms and Human Pd patient samples had a higher UniFrac value than to the other sample groups. This indicates that Pd mice have very similar microbiomes to human Pd patients, alluding to a microbial profile that can be correlated to Pd (38).

Cecal Sample Collection

Another common sample collection method in the study of the effects of the gut microbiome on human health using mice as a model organism is to take tissue sections from the cecum. A few studies have used samples gained from tissue excision from the cecum or other portions of the gut and extract DNA from that to assess the microbial composition of the intestines (39;40;41). The research performed using this method of sample collection is scarce and uses mouse models. From all the research done for this project only 3 relevant articles were found to use only cecum sample collection (39;40;41).

An experiment focused on understanding the microbial composition of obese mice and comparing it to control mice found that diet induced obese mice had higher bacterial diversity that control mice. Also, when these mice were put under a regime to reduce weight, microbiome

diversity increased greatly. The phylum Firmicutes was observed to decrease in this weight reduction process, suggesting that the levels of these organisms are related to the obese phenotype. The phylum Bacteroidetes was observed to decrease in obese mice and to significantly increase during the weight reduction process, which suggests that there is a relation between this phylum and the lean phenotype. All of this evidence showed the researchers that the ratio of these two phyla might be correlated to the degree of obesity (39).

Results from another experiment changed the focus of study from obese phenotype to energy harvest and determined how well the microbiome from an obese animal is able to harvest nutrients and break down foods (40). The researchers found that samples from obese mice where observed to have higher amounts of sequences that code for enzymes responsible for breaking down indigestible elements. Also, GF mice gavaged with the microbiota of obese mice showed a significant increase in fat percentage compared to that of mice gavaged with lean microbiota or control microbiota (40). This evidence suggests that there is a strong bi-directional relationship between metabolic processes of the body and the gut microbiome. This has been observed with other systems as well, for example the immune system and the central nervous system (41;38).

Atopic diseases and autoimmune diseases like diabetes have also been correlated to gut microbial composition. In an experiment geared towards understanding sex-biased microbial composition and the potential of a hormonal induced microbial composition profile found that female mice have a different microbiome than male mice (41). It also suggested, that this can be a reason as to why women are more prone to diabetes. Using non-obese diabetic mice, the researchers assessed the relatedness and the implications of having male and female microbiomes in terms of glucose tolerance and insulin production. They concluded that females gavaged with male microbiome increased glucose tolerance and decreased the degree of

diabetes. This suggests that there is a sex-biased microbial composition that may confer protection or prevention to diabetes. This is still being researched, therefore there are no known mechanisms that underlie this process, but it is hypothesized that testosterone levels might be a factor (41).

Table 2. Relevant finding from select studies that have used fecal and cecal data.

Microbiome Sample Source	Process or Condition	Finding	Reference No.
Feces	Obesity	↓ *Bifidobacterium* sp.	32
Feces	Obesity	↑ *Firmicutes:Bacteriodetes*	33
Feces	Stress	*Bifidobacterium infantis* reduces stress	37
Feces/Intestinal cell imaging	Immunomodulation	*Lactobacillus, Escherichia coli* promote a Th2 or Treg differentiation.	34,35
Feces	α-Synucelopathies	Bacterial gavage of human fecal microbiome into germ free mice causes Parkinson's pathophysiology in mice	38
Cecum	Obesity	*Bacteroidetes* was less abundant in obese mice	39
Cecum	Obesity	Microbial gavage of lean mice into obese mice reduces fat percentage of obese mice	40
Cecum	Diabetes	Non-obese diabetic mice show a sex bias towards diabetes incident, which changes as microbial gavage is exchanged between test groups	41

CHAPTER II

THE GUT MICROBIOME: IS FECAL MATTER MICROBIAL COMPOSITION A PROXY FOR INTESTINAL MICROBIAL COMPOSITION IN STUDIES OF THE MICROBIOME?

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Abstract

Background: As many health phenomena seem to be affected directly and indirectly by the microbiome, gut microbiome research has increased dramatically in the last decade. Issues like allergies, cancer, obesity, and other health phenomena have been found to be influenced by the microbiome. Most of gut microbiome research is done by collecting and sequencing the DNA of the microbiome of the fecal matter from model organisms or human subjects. Studies that use this method of sample collection and analysis assume that fecal matter microbiomes are identical to intestinal microbiomes. At present, no published studies exist which directly compare stool and intestinal microbial composition.

Results: Bacterial composition was found to be different at all levels of taxonomy between stool and the regions of the intestines tested. Univariate analysis shows significance between the two main phyla Firmicutes and Bacteroidetes, as well as main genera like *Odoribacter, Porphyromonas* and *Alistipes.* Relevant species *Desulfovibrio desulfuricans* and *Odoribacter splanchicus* were also found to be significantly different in relative abundance between stool and some parts of the intestines. α-Diversity was not significantly different between all parts of the

intestine and stool. β-diversity was found significant between the ileum and stool, with stool having slightly higher diversity.

Conclusions: Looking at the bacterial composition of both environments and the relative abundances of the dominant taxa, one can see that that there are key differences between the intestines and stool. The microbial composition at all levels of taxonomy was found to be different, therefore, research that targets the gut microbiome should look closely at the specific taxa being observed. Phyla like Firmicutes and Bacteroidetes were found to have different abundance within the portions of the intestine and stool, suggesting that phyla level analysis should be performed by observing each community separately. Interestingly, diversity analysis was not found significant, suggesting the abundance is different, but the number of different taxa is similar across the intestinal tract and stool.

Keywords: gut microbiome, microbial community comparison, 16S rDNA amplicon sequencing

Background

Microbiome research, specifically projects assessing the composition of the gut microbiome, have become a popular avenue of exploration. From the year 2000 to 2014 a pear reviewed article internet search for “Gut Microbiome” yielded ~40,000 articles, and when the word “Ecology” is added to the search it yielded ~13,500 papers. This is a huge increase from what was observed in the 90’s, where it was reported by Sekirov and colleagues that from 1990 to 1995 no more than 500 articles were published on the gut microbiome (1). It would seem that microbiome researchers have successfully found a system that affects all other systems of the body. For example, research suggested that obesity could be explained by the composition of the gut microbiome, as well as a potential mechanism as to why allergies occur and why they are on the rise. Connections were also found between the immune system and the gut microbiome, and many more phenomena were connected to the gut microbiome (2;3;4). Microbial composition has become an important phenomenon to look at. With the help of the murine (mouse) microbiome, interesting research was performed showing, for example, the effects of diet and stress on the microbiome, microbial composition along the intestinal tract, what occurs when the microbiome is removed at an early age, among others (2;3;4).

Mouse are a commonly used animal model used to study gut microbial composition and the effects of changes like diet and exercise have on the gut microbiome. The gastro-intestinal anatomy of the mouse is a similarly complex version of the human intestinal tract, with structures that are alike, although the composition is slightly different. Cross-sections of organs show some differences, but the same structures are present in both organisms and they perform similar functions (7). The human gut microbiome and the mouse gut microbiome are both dominated by the same phyla: Firmicutes and Bacteroidetes. When looking deeper into the

species taxonomic level, one can observe differences between the two microbiomes. According to Ley and colleges 85% of species found in the mouse gut microbiome are not found in the human microbiome, (5). Even though this dissimilarity is observed there are still roughly 79 genera shared by both humans and mice (6). Both of these findings suggest that composition is not necessarily dissimilar, but the lower one gets in taxonomy the more diversification these two organisms have.

Mouse intestinal microbial communities are composed of species belonging to the genera *Lactobacillus, Alistipes, Roseburia, Bifidobacterium, Clostridium,* and others. This composition is similar to that of humans. Relative abundances, on the other hand, are different, as well as certain communities that are not in humans are found in mice (6; 7). Although, some researchers have alluded to the issue that these discrepancies and dissimilarities, between human and mouse, could be better studied if they did not lack comprehensive microbial data due to the inability to perform research in intestinal matter and fecal matter rather than just fecal matter (8). Even though mice and humans have dissimilarities, the mouse is still a good model to study interactions between microbes and the system, changes due to exogenous factors and relative compositions between portions of the intestine (7).

The inability of these researchers to provide complete conclusions due to the lack of intestinal data in humans alludes to the question “is fecal matter an adequate proxy for intestinal microbial composition?”. Many of the conclusions that microbiome researchers have drawn from studies of the gut microbiome are founded in the assumption that fecal matter is enough to describe the entirety of the intestinal tract, in terms of microbial composition (Table 1). Research using mouse models has the ability to extract intestinal matter and study said samples for microbial composition allowing for a better picture of the microbial communities found in the

intestines. No one has taken the task to provide a complete comparison of stool matter to intestinal matter microbial composition by looking at different portions of the intestine and stool to answer the question is fecal matter can be used to assess adequately the composition of the intestines. This experiment will allude to this dilemma by looking at the microbial composition of the intestinal tract in mice as well as stool and performing microbial community comparison analysis. It is of up most importance to answer this question, since conclusions about the intestinal tract have been made based off the assumption that fecal matter microbial composition is an adequate proxy for intestinal microbial composition.

Table 3. Relevant findings from select studies that have used fecal and cecal data.

Microbiome Sample Source	Process or Condition	Finding	Reference No.
Feces	Obesity	↓ *Bifidobacterium* sp.	2
Feces	Obesity	↑ *Firmicutes:Bacteriodetes*	10
Feces	Stress	*Bifidobacterium infantis* reduces stress	30
Feces/Intestinal cell imaging	Immunomodulation	*Lactobacillus, Escherichia coli* promote a Th2 or Treg differentiation.	3,31
Feces	α-Synucelopathies	Gavage of human fecal microbiome into germ free mice causes Parkinson's pathophysiology in mice	32
Cecum	Obesity	*Bacteroidetes* was less abundant in obese mice	33
Cecum	Obesity	Gavage of lean mice into obese mice reduces fat percentage of obese mice	14
Cecum	Diabetes	Non-obese diabetic mice show a sex bias towards diabetes incident, which changes as microbial gavage is exchanged between test groups	34

Results

Microbial composition: Stool vs. Intestinal Tract

Microbial composition at the phylum level was found to be different in the stool than in all other sections of the intestine. The Bacteroidetes to Firmicutes ratio was specifically different in the stool from all intestinal sections, showing an increase in organisms belonging to the Bacteroidetes phylum and a decrease in organisms belonging to the Firmicutes phylum (Figure 9). The colon showed the most resemblance to the composition of the stool, and the cecum (Figure 9). This was the case at all levels of taxonomy, although species level community comparisons were more complex and similar than those for higher taxonomic levels. At the class level, differences between *Bacteroides* and *Clostridia* were observed between stool and all portions of the intestine tested. Other classes like *Bacilli, Deltaproteobacteria,* and *Verrucomicrobiae* were found to be more abundant in the ileum and colon than in the stool and cecum. *Verrucomicrobiae* was also found in small quantity in the cecum, but not as much as in the colon and ileum (Figure 10).

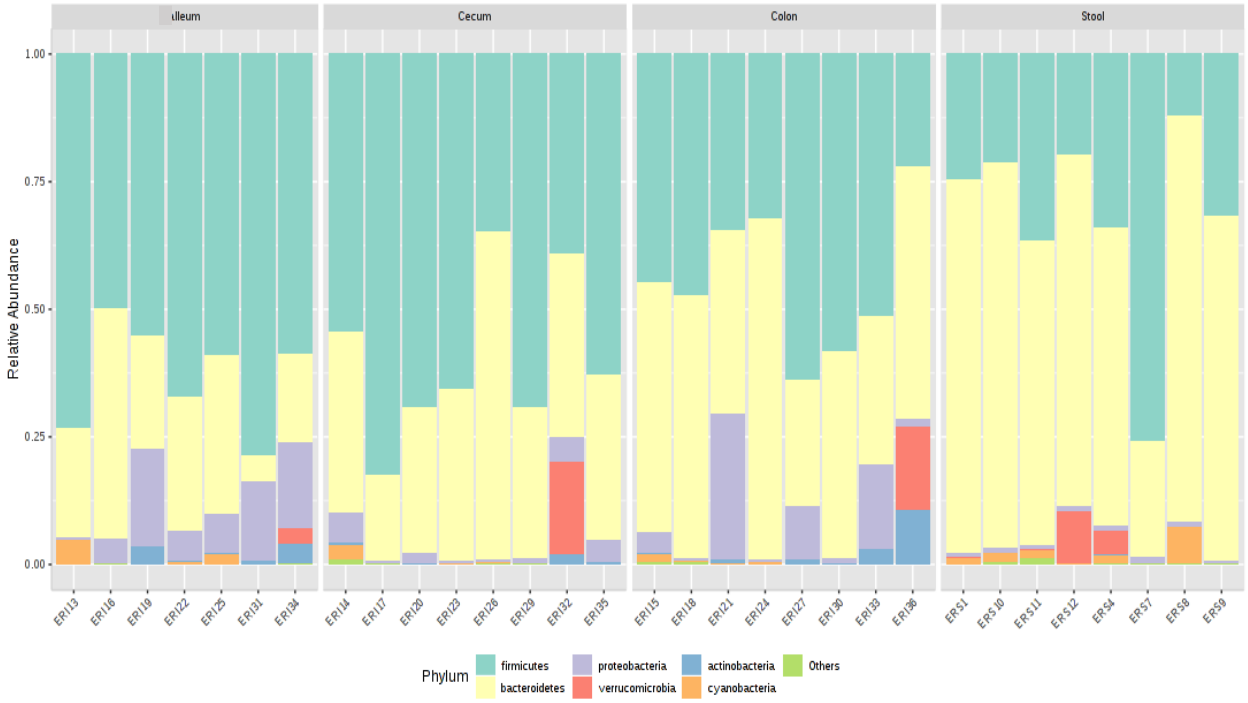

Figure 9. Phylum level microbial composition of stool and intestinal tract (ileum, cecum and colon). Each designation on the X-axis is a mouse sample that corresponded to the specific class assigned.

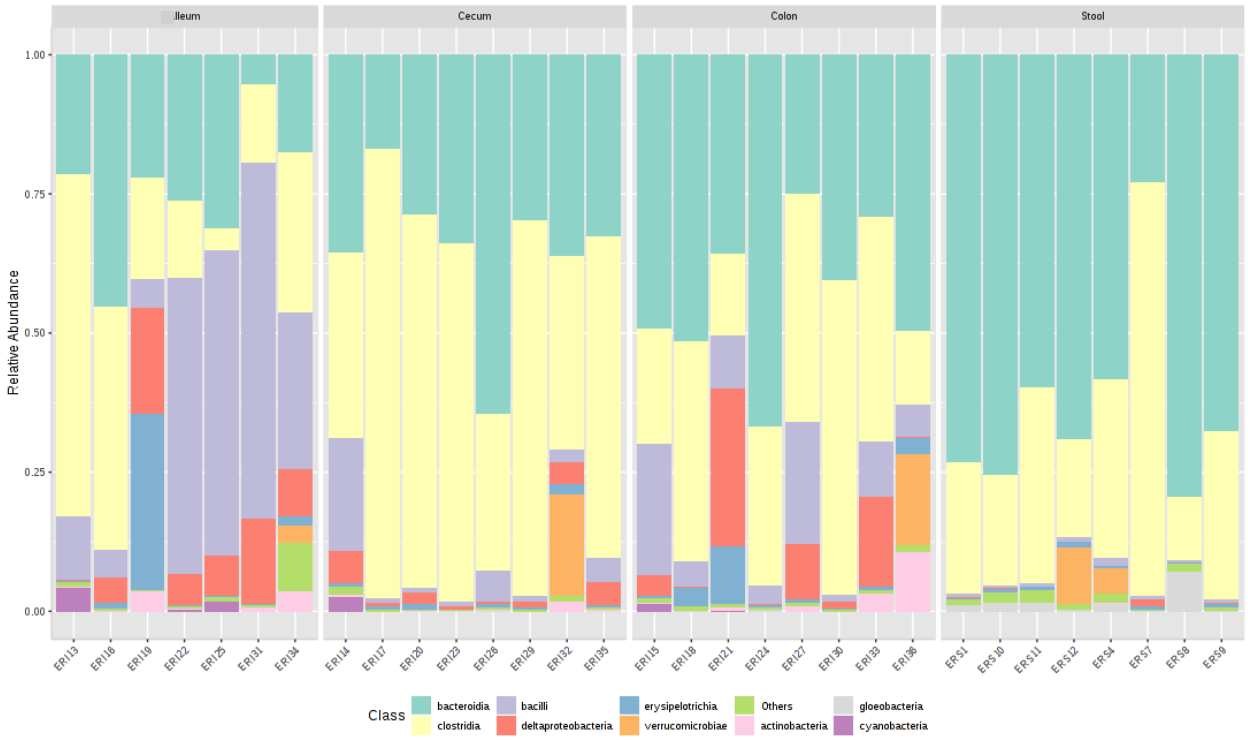

Figure 10. Class level microbial composition of the stool and the intestinal tract (ileum, cecum and colon).

Significant differences among taxa were identified with an ANOVA using MicrobiomeAnalyst software. At the Phylum level, Firmicutes and Bacteroidetes were found to have significant differences between portions of the intestine and stool. Bacteroidetes abundance was found to be significantly different between stool and cecum samples, and stool and ileum samples (Table 2). Although not statistically significant, there were observable trends of differences between the ileum and the colon through less abundance of this phylum (Figure 11A). Firmicutes was also found to have a reversed profile from Bacteroides. Firmicutes was found to be significantly less abundant in the stool than in the cecum and to the ileum (Table 2). Observable trends also show that this phylum is not uniformly abundant across the intestinal tract. Although, no statistical significance was found, the colon also seems to have reduced abundance of Firmicutes compared to the cecum and the ileum, but it is still more abundant than in the stool. The ileum and cecum have very similar abundance of this phylum (Figure 11B).

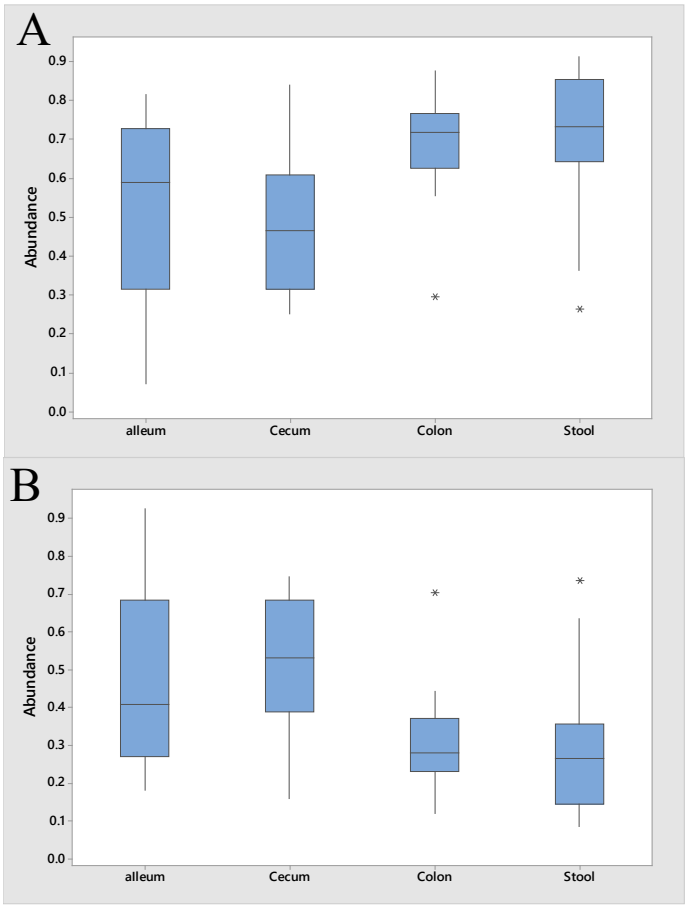

Figure 11. Relative abundance of statistically different phyla. (A) Bacteroidetes relative abundance by section of the intestinal tract ad stool. (B) Firmicutes relative abundance by section of the intestinal tract and stool. The asterisks represent outlier.

Table 4. P-values for statistically significant comparisons at the phylum level. all P-values were calculated using a one-way ANOVA.

Significant Comparison	Phylum	P-value
Stool-Cecum	Bacteroidetes	0.003
Stool-Ileum	Bacteroidetes	>0.001
Stool-Cecum	Firmicutes	0.001
Stool-Ileum	Firmicutes	0.005

At the class level there were two significant classes that had relevant abundance. The class Bacteroidea was found to be significantly more abundant in the stool than in the cecum and in the ileum. Although no significance was found, there is also an observable trend between the colon and stool as well, where this class is slightly more abundant in the stool than the colon. All intestinal portions were found to be very similar (Figure 12B). The class Bacilli was also found to be significantly different between the stool and portions of the intestine. Interestingly, the ileum was found to be significantly different for Bacilli communities than the colon, cecum and stool. The ileum was most different from stool, followed by the colon, and the cecum (Table 3). Differences within the Bacilli class can also be observed between the stool and the colon and cecum, but no statistical significance was found between these three communities (Figure 12A).

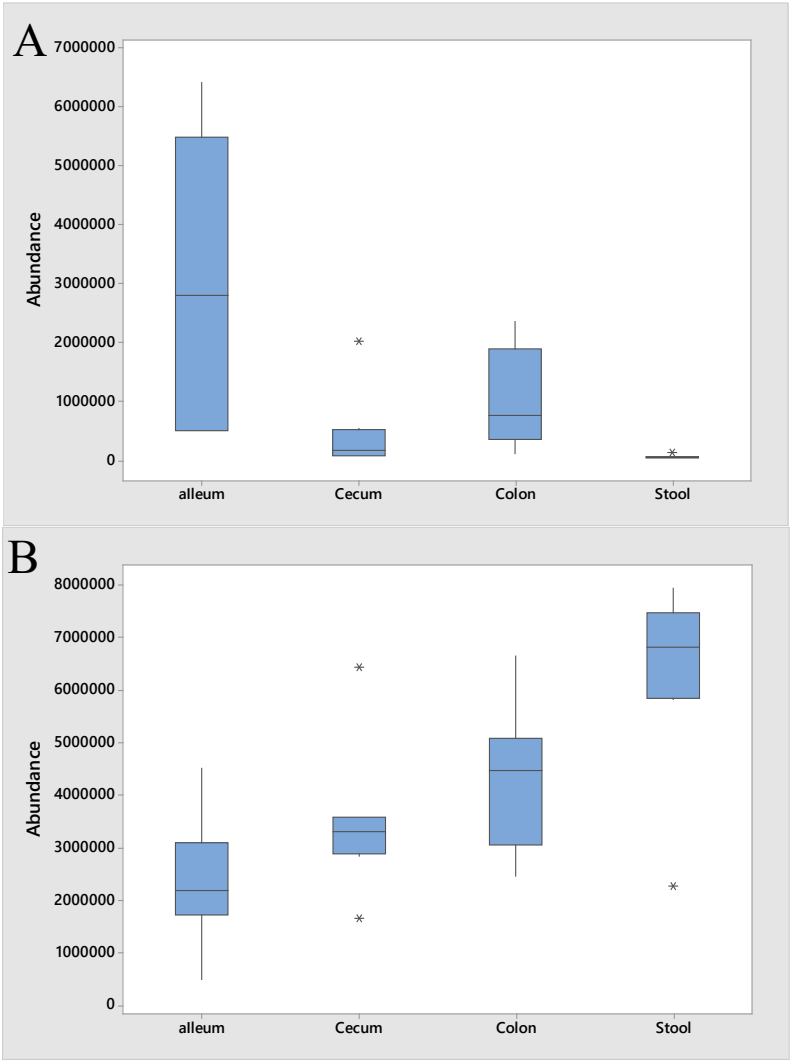

Figure 12. Relative abundance of statistically significant classes. (A) Relative abundance of the class Bacilli by section of the intestinal tract and stool. (B) Relative abundance of the class Bacteroidea by section of the intestinal tract and stool. Asterisks represent outliers.

Table 5. P-values for statistically significant comparisons at the Class level. all P-values were calculated with a one-way ANOVA.

Significant Comparison	Class	P-value

Stool-Cecum	Bacteroidea	0.003
Stool-Ileum	Bacteroidea	>0.001
Ileum-Colon	Bacilli	0.018
Ileum-Cecum	Bacilli	0.002
Ileum-Stool	Bacilli	0.001

At the order level two taxa were found to be significant from all others. The order Bacteroidales was found to be significantly more abundant in the stool. Significance was found between stool and ileum samples and between stool and cecum samples (Table 3). Colon samples were not significantly different than those from the stool, but a trend can be observed, where Bacteroidales was found to be more abundance in stool than in the colon samples (Figure 13A). The order Lactobacillales was found to be significantly less abundant in samples from the stool as compared to the ileum, as well. Lactobacillales was also found to be significantly more abundant in the ileum than in the cecum and colon (Table 4). Trends in differences between the stool and the colon samples were also observed, but none were significantly different. The range of abundances for Lactobacillales samples was found to be very broad between the samples, especially for samples taken from the ileum portion of the intestine (Figure 13B).

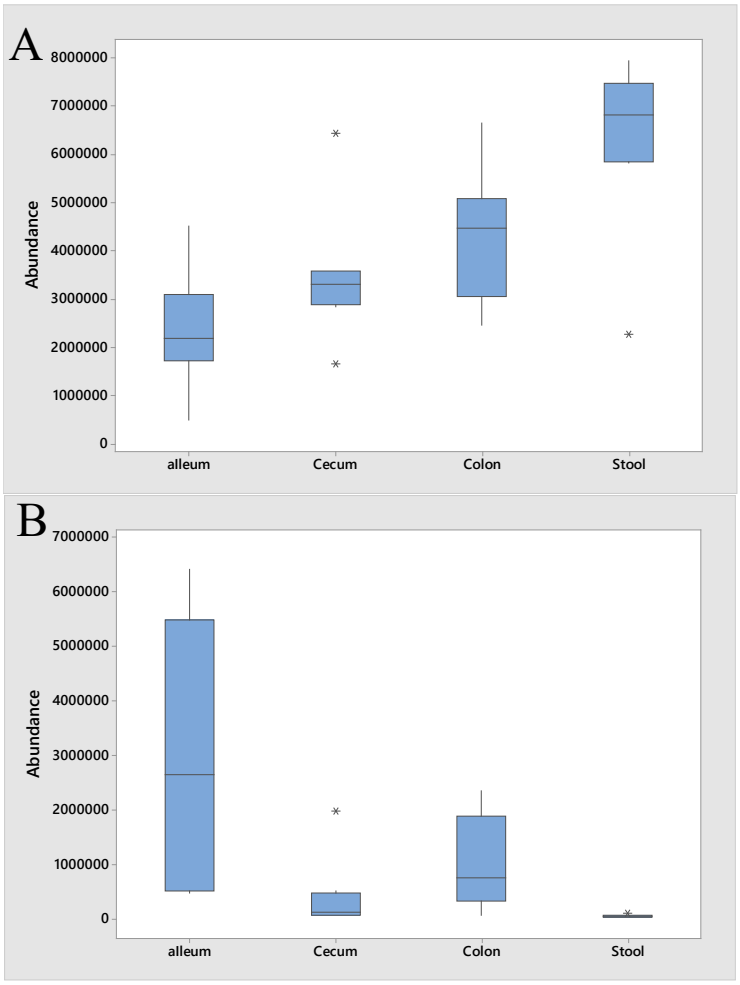

Figure 13. Relative abundance of statistically significant orders. (A) Relative abundance for the order Bacteroidiales by section of the intestinal tract and stool. (B) Relative abundance of the order Lactobacillales by section of intestinal tract and stool. Asterisks represent outliers. Asterisks represent outliers

Table 6. P-values for statistically significant comparisons at the Order level. all P-values were calculated with a one-way ANOVA.

Significant Comparison	Order	P-value
Stool-Ileum	Bacteroidales	<0.001
Stool-Cecum	Bacteroidales	0.003

Stool-Ileum	Lactobacillales	0.001
Ileum-Cecum	Lactobacillales	0.003
Ileum-Colon	Lactobacillales	0.019

At the family level two features were found to be significantly different from the rest. The family *Rikenellaceae* was found to be significantly more abundant in stool samples than in all other intestinal portions. The largest difference was observed between stool and ileum samples, followed by stool and cecum samples, and stool and colon samples (Table 5). There were also two observable trends that showed cecum samples being different than the ileum and a large range of differences among samples taken from the colon (Figure 14). The family Porphyromonadaceae was found to be significantly different between the stool and two portions of the intestine. This family was found to be significantly more abundant in samples from the stool than from the cecum, or from the ileum (Table 5). Observable trends show that Porphyromonadaceae was also more abundant in the stool than in the colon, although no statistical significance was found between these two sample types (Figure 14).

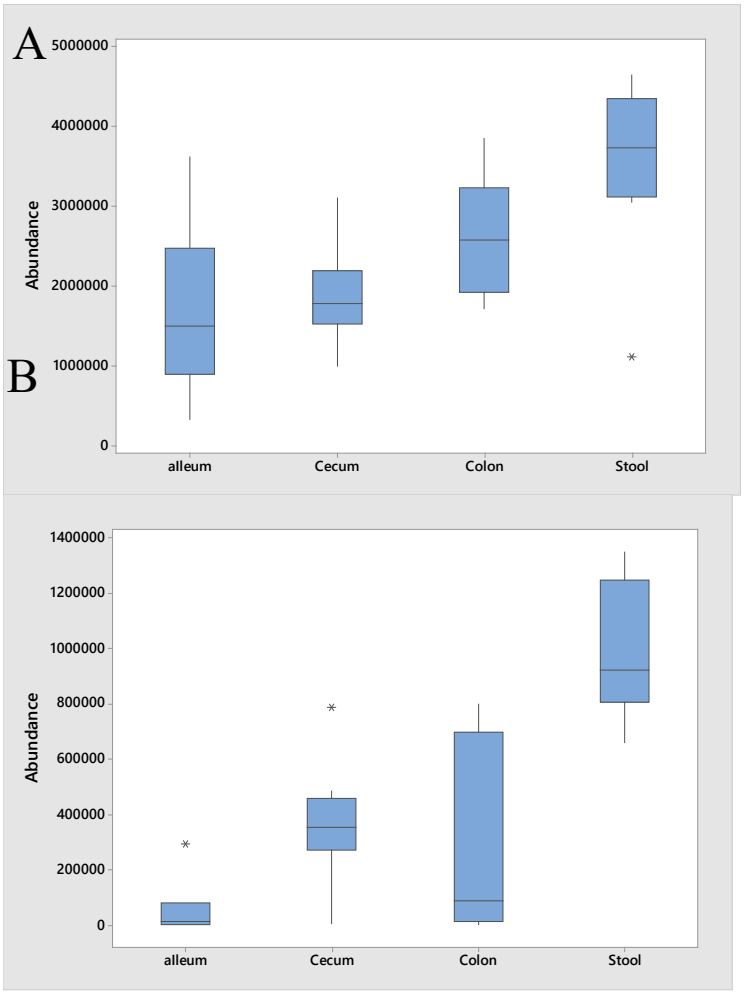

Figure 14. Relative abundance of statistically significant families. (A) Porphyromonadaceae relative abundance across the intestinal tract a stool. (B) Rikenellaceae relative abundance across the intestinal tract and stool. Asterisks represent outliers. Asterisks represent outliers.

Table 7. P-values for statistically significant comparisons at the Family level. all P-values were calculated with a one-way ANOVA.

Significant Comparison	Family	P-value
Stool-Cecum	Porphyromonadaceae	0.006
Stool-Ileum	Porphyromonadaceae	0.003
Stool-Ileum	Rikenellaceae	>0.001
Stool-Cecum	Rikenellaceae	0.001
Stool-Colon	Rikenellaceae	0.001

Three genera were found to differ significantly in types and relevant abundance across the intestinal tract. The genus *Alistipes* was found to be more abundant in the stool samples than in all other intestinal portions tested. The largest difference was found between samples from the stool and the ileum, followed by the cecum and the stool, and the stool and the cecum (Table 6). Interestingly, samples from the cecum and the ileum seem to be very different, although there was no statistical significance found between these two sample types. Samples from the colon showed a high range of data points (Figure 15). The genus *Odoribacter* was also found to differ significantly between samples from the intestine and stool. Bacteria belonging to the genus *Odoribacter* were found to be significantly more abundant in stool samples than in all three portions of the intestine tested. The largest difference was found between samples from the stool and the ileum, followed by the colon, and the cecum (Table 6). All portions of the intestine seem to be very similar although cecum samples showed some difference when compared to the ileum, no statistical significance was found (Figure 15). Lastly, the genus *Porphyromonas* was found to have significantly different abundances between samples from the stool and from portions of the intestine. Stool samples were found to have higher abundance of *Porphyromonas* than samples from the cecum and the ileum (Table 6). There is also a trend that can be observed between colon and ileum samples showing that the colon has higher abundance of *Porphyromonas* species than the ileum, although no statistical significance was found. There was a high variability data points among the colon samples differences between samples (Figure 15). Species level univariate analysis showed many significant features, but they were omitted due to most of them having extremely low abundance.

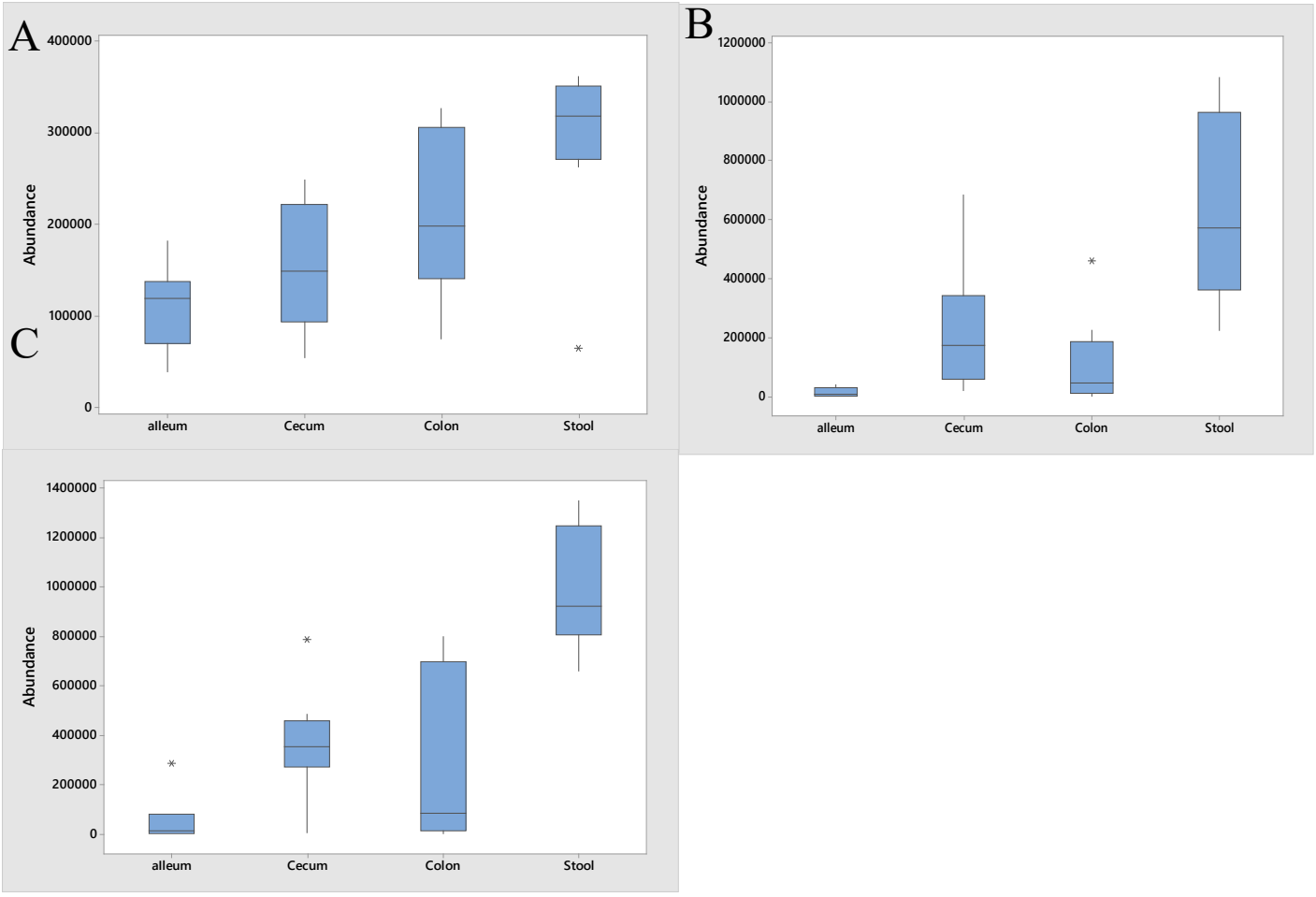

Figure 15. Relative abundance for statistically significant genera. (A) *Porphyromonas* relative abundance across the intestinal tract and stool. (B) *Odoribacter* relative abundance across the intestinal tract and stool. (C) Alistipes relative abundance across the intestinal tract and stool. Asterisks represent outliers.

Table 8. P-values for statistically significant comparisons at the Genus level. all P-values were calculated with a one-way ANOVA in MiniTab.

Significant Comparison	Genus	P-value
Stool-Ileum	*Alistipes*	<0.001
Stool-Cecum	*Alistipes*	<0.001
Stool-Colon	*Alistipes*	<0.001
Stool-Ileum	*Odoribacter*	<0.001
Stool-Cecum	*Odoribacter*	<0.001
Stool-Colon	*Odoribacter*	0.003
Stool-Ileum	*Porphyromonas*	0.008
Stool-Cecum	*Porphyromonas*	0.001

At the species level there were two species that were relevantly abundant and significantly different between samples from the intestine and the stool. The species *Bacteroides acidifaciens* was found to be slightly more abundant in samples from the stool than from the ileum. Although only this comparison was found to be significant, observable trends show more abundance in stool than the cecum (Table 7; Figure 16). The organism was similarly abundant between portions of the intestine. Also, a high amount of variation was found among stool samples (Figure 16). The species *Dorea massiliensis* was more abundant in samples from the stool than in all portions of the intestine. This organism was more abundant in samples from the stool than from the ileum the most, followed by the colon and cecum. All portions of the intestine where similar (Table 7). The range of the colon samples was very high suggesting large differences in *D. massiliensis* abundance from individual to individual. Although, only the stool was significantly different from the intestine, observable trends suggest that there might be a difference between colon and ileum, but due to the large variability within colon samples, significance was not found between these two sample types (Figure 16).

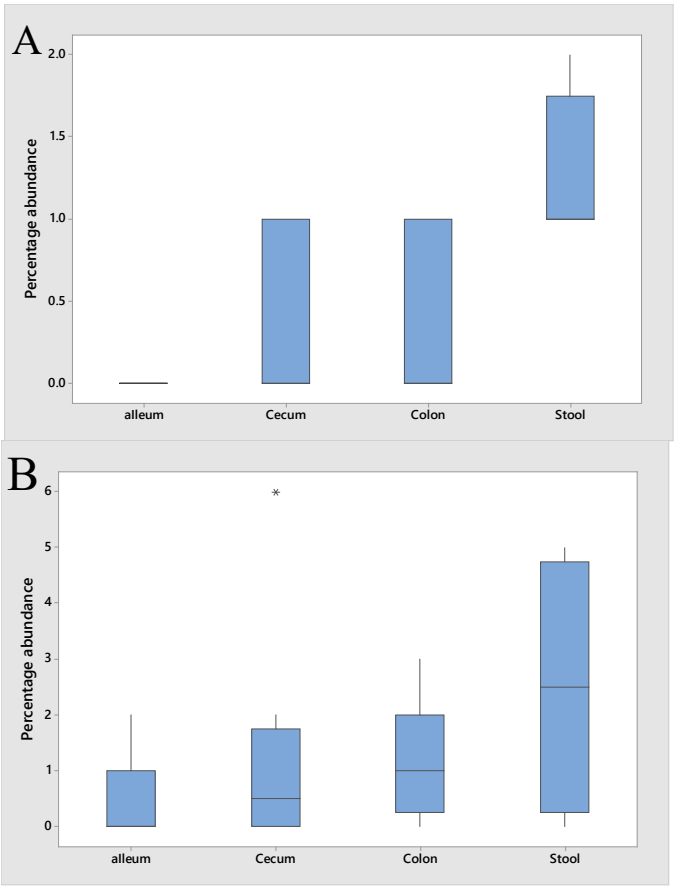

Figure 16. Relative abundance for statistically significant species. (A) *Bacteroides acidifaciens* percentage abundance across the intestinal tract and stool. (B) *Dorea massiliensis* percentage abundance across the intestinal tract and stool. Asterisks represent outliers.

Table 7. P-values for statistically significant comparisons at the Species level. all P-values were calculated with a one-way ANOVA.

Significant Comparison	Species	P-value
Stool-Cecum	*Dorea massiliensis*	0.001
Stool-Ileum	*Dorea massiliensis*	<0.001
Stool-Colon	*Dorea massiliensis*	<0.001
Stool-Ileum	*Bacteroides acidifaciens*	0.004

Diversity Analysis

Alpha-diversity was assessed by Shannon's Diversity Index and β-Diversity was assessed by Bay-Curtis dissimilarity test. At the genus level of taxonomy there were no significant difference between the intestine and the stool when looking at α-diversity (Figure 17). The Bay-Curtis analysis for β-diversity showed significance within the comparisons between the stool and intestine. Stool was found to be more diverse than the ileum (P-value: 0.003). Observable trends show individual clustering between the parts of the intestine and stool, suggesting that there is a difference between all portions of the intestine and stool (Figure 18).

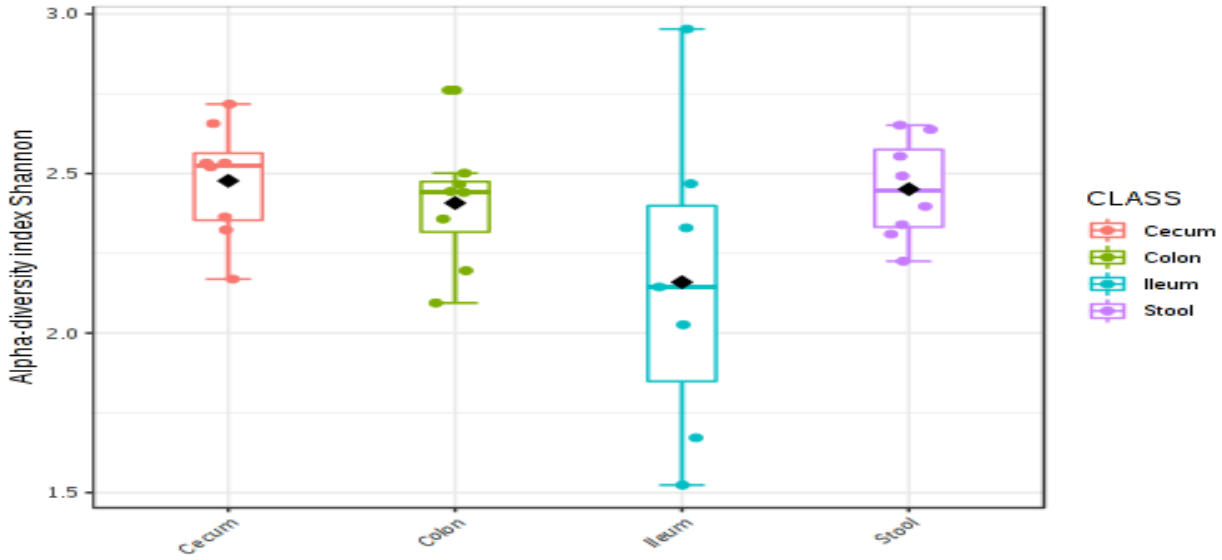

Figure 17. Shannon's diversity index of each intestinal portion and stool. No significance was observed between any of the groups.

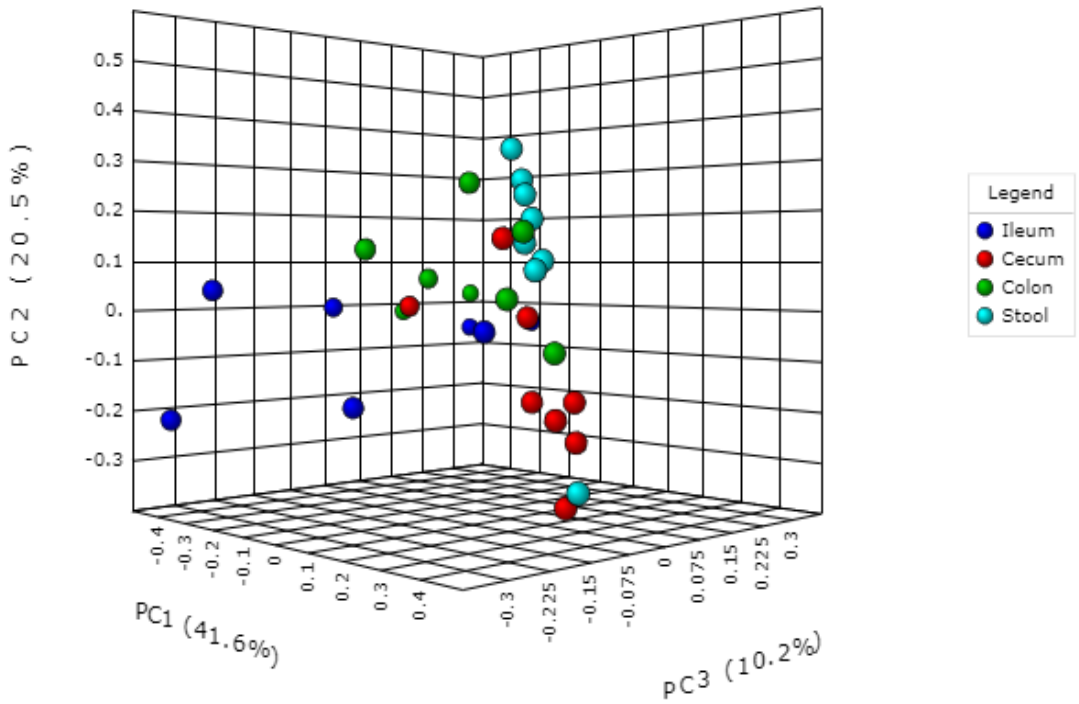

Figure 18. β-diversity analysis. PCOA showing Bay-Curtis Dissimilarity test data. Statistical significance was only found between the Ileum and Stool, but clustering is observed with all sources (P-value: 0.003).

Discussion

Bacterial composition and diversity were compared between fecal samples and intestinal samples in mice. Mouse bacterial composition is similar to human bacterial composition, although abundance seem to be different. For example, mice and humans share some genera including *Alistipes, Odoribacter* and *Turicobacter* but some are in lower abundance in mice than in humans (6). Even so, mice are a good model for intestinal microbial community comparison because the intestinal tract is similar in composition, although the researchers admit that the strength of these findings is lowered due to only observing fecal matter. (7;8).

The intestinal microbial composition of humans and mice a like, has been explored thoroughly, while fecal matter has been used extensively to draw conclusions about the many phenomena that the gut microbiome is connected to, no one has taken on the task of comparing both communities and the communities across the intestinal tract. By comparing the bacterial

composition of the intestinal tract and stool of mice as well as the diversity of the microbiome across the mouse intestinal tract we can shed some light into the adequacy of the assumption that fecal matter bacterial composition is a proper proxy for intestinal microbial composition.

At the phylum level of taxonomy, bacterial composition was found to be different between stool and all portions of the intestine. The phylum Firmicutes was found to be significantly more abundant in stool and the ileum (Figure 15). This profile, then, is very similar to that of the cecum and colon where the Firmicutes to Bacteroidetes ratio is highly dominated by Bacteroidetes. This finding suggests that as the intestinal tract proceeds from proximal to distal end, there is a change in microbial composition observed at the phylum level. Previous studies in the composition of the intestinal tract have found similar observations, by concluding that diversity and complexity of the intestinal tract increases from proximal to distal end (4). This does not only occur at the phylum level, but at all lower levels of taxonomy. The phyla Firmicutes and Bacteroidetes have are highly studied taxa, and have been correlated to obesity. The Firmicutes to Bacteroidetes ratio has been previously used to determine an obese composition. In a study aimed to assess the energy harvest capacity of an obese microbiome, researchers used fecal samples to assess the composition of the intestinal tract and how it is correlated to obesity. They found higher Firmicutes than Bacteroidetes is a characteristic of an obese phenotype (11). Our findings suggest that Bacteroidetes is often lower than Firmicutes in stool (Figure 11B). At the class level of taxonomy, the microbiome for the ileum was very different, not only from the stool, but also from all portions of the intestine sampled. Classes such as Clostridia and Bacteroidea are very similar across the intestinal tract, but are found to be in different proportions in the stool; the class Clostridia is found in low numbers in stool samples whereas Bacteroidea is highly abundant. This is very distinct from all other portions of the

intestines. Other classes are also more abundant in certain portions of the intestinal tract than in the stool, for example the class Bacilli was found to be highly abundant in the ileum, but not in the stool, suggesting that along the intestinal tract, the organisms belonging to this class are not reaching stool. Some of these findings are in agreement with previous research. Sheghuan and colleagues (9) found the class Bacilli to be highly abundant in the ileum, but less abundant in other portions of the intestinal tract. This class is a target of study for the effects of diet in the gut microbiome. A study assessing the effects of diets on the humanized mice microbiome found that the class bacilli is highly abundant in Western diets, but less abundant in other diets, suggesting that western countries have different microbiomes due to the diet that is often consumed (12). This experiment only used fecal matter to draw conclusions about the intestinal tract composition. Our findings suggest that the mouse microbiome has low bacilli in stool. The class Bacteroidea, was found to have a similar abundance across the intestinal tract, although they found it to be high in the Ileum, which is in opposition to our findings (9). This difference might be due to strain and diet differences between the studies.

The genera *Odoribacter, Porphyromonas* and *Alistipes* were found to be more abundant in the stool than in all portions of the intestine. Organisms belonging to the *Alistipes* are very relevant, and have opened an avenue of research concerning the gut-brain axis (27). Previous research, which used only fecal matter for samples, found that this genus is decreased in mice that experience severe stress and when gavaged into germ-free mice yielded a stress phenotype (27). Interestingly it was found high in stool in our study, which might suggest that the depletion of this organism during stress events might occur in the colon, which was found to be similar form stool across all taxa. The genus *Odoribacter* is a known commensal organism in the human and murine gut. Recent studies have found that this organism is highly present in neonatal stages

(14). It was not specified which portion of the intestine this organism was most prevalent in or if it is transient or if it colonizes the intestine since they only used fecal matter at one point in time (14). Our study suggests that it is not highly abundant in the intestinal tract, but highly abundant in stool, which might suggest why the previously mentioned project observed it to be in such high abundance. Another relevant genus that was found to differ in abundance between stool and the intestinal tract is the *Porphyromona* genus, although most of the research around this genus has been focused in a specific organism, *Porphyromonas gingivalis,* which has been foud to have an effect in Alzheimer patients. Periodontal pathogen antibodies have been found in significantly high amounts in Alzheimer patients. The researchers suggest that organisms like *Porphyromonas gingivalis* are connect pathophysiology of the disease (28).

At the species level, organisms known to be important to health, in terms of immune responses, were found to be different. The organism *Bacteroides acidifaciens* was found to be significantly more prevalent in stool. This organism is known for reducing inflammation, improving glucose sensitivity and reducing obesity in mice. These conclusions were drawn from mouse ceca, but also only from human feces suggesting that the results might be skewed due to inaccuracy (10). *Dorea massiliensis* was found to be more abundant in stool than in any other portion of the intestine. This organism has not been highly studied, since it was a candidate organism, or an organism that is in the process of being assigned to that certain taxonomic assignation, until recently (29). Researchers that assigned this taxonomic name found this organism in a single stool sample from an *Anorexia nervosa* patient (29).

When looking at diversity, specifically the Shannon's index, there was no significance found. These findings contradict previous research that puts the diversity in the ileum being lower than other portions of the intestine, in mice (9). The Bay-Curtis dissimilarity test, on the

other hand, showed significance between the stool and ileum. The stool was more diverse and it clustered together apart from the ileum, which clustered together as well (Figure 18). Sheguan and colleagues found similar results, where local diversity was found to be significantly different between the intestinal tract (9). Our findings suggest that just stool is differently diverse, but due to different methodology and diet, conclusions might be different.

There is convincing evidence showing that the microbial composition of the stool and the intestinal tract is different. These findings suggest that fecal matter is not an adequate proxy for intestinal microbial composition. Within the intestine there is also variability in the microbiome. Therefore, individual portions of the intestine should be looked at separately when making conclusions about the microbial composition and abundance of the gut. Similar to our findings, Shenghuan and colleagues also found that each portion of the intestines has its own community, showing multiple differences between the jejunum, ileum, cecum and colon (9). Our findings suggest that microbial composition along the intestinal tract changes, as reported by others (9), and now we add that fecal matter also has different microbial composition from regions of the intestine. Further research needs to be performed on this matter. Research alluding to viability of microbial cells, would answer this question with a more encompassing conclusion. In this experiment we lacked the ability to differentiate between viable and non-viable cells. Most microbes in our gut and mouse gut are obligate anaerobes, therefore it is important to look at viability in stool, specifically. These organisms would have died as soon as contact with oxygen was had and they would not be considered part of the fecal microbiome at said point in time. Although, viability is an issue that needs to be researched more, our findings still suggest that there are differences between the microbial composition of the intestinal tract and stool. Stool bacterial composition and diversity can be a good source of information for studies of the

microbiome, but should not be considered an adequate proxy for intestinal bacterial composition and diversity.

Methods

Tissue and stool collection

Eight approximately six-week-old wild-type C57BL/6 male mice were used in this study. All mice were housed and maintained under Protocol #A101604, which was approved by the Central Washington University Institutional Animal Care and Use Committee. All mice had access to food *ad libitum* and were fed Mazuri Rodent Breeder 6F diet. This diet is composed of 16% crude protein, 6% crude fat, 7% crude fiber, 12% moisture and ~8% mixture of various minerals. Stool samples were collected 30 seconds before euthanasia by placing the mice on a disinfected tray and allowing the mice to produce stool pellets, which were collected aseptically and stored at -80°C until DNA extraction. Following stool collection, mice were euthanized by rendering CO_2 followed by cervical dislocation. Following euthanasia and using aseptic technique, the intestinal tract was removed from each mouse. The intestinal tract was aseptically divided into ileum, cecum and colon, then stored at -80°C until DNA extraction.

DNA extraction and 16S rDNA amplicon sequencing

Intestinal samples were sub-sectioned into three 25mg-35mg tissue sections. DNA from all intestinal samples was extracted using the DNeasy kit (Qiagen, Inc., Germantown, MD) for tissue and blood. DNA was then quantified using absorbance at 260 nm on a Beckman DU 640B UV spectrometer, and quality was assessed using the 260/280 nm ratio. The extracted DNA were then stored at -80ºC. DNA from stool samples was extracted using the QIA AMP Stool DNA

extraction kit (Qiagen, Inc., Germantown, MD). DNA for these samples was also quantified and checked for quality via UV absorbance using the same parameters, and stored at -80ºC. All DNA samples were sent to Mr. DNA Laboratory (Shallowater, TX) for 16S rDNA amplicon sequencing and taxonomic assignment. The 16S rRNA V4 variable region PCR primers 515/806 were selected for use in a one-step 30 cycle PCR. The HotStarTaq Plus Master Mix Kit (Qiagen, USA). The following conditions were used for PCR: 94°C (3 minutes), followed by 30 cycles of 94°C (30 seconds), 53°C (40 seconds) and 72°C (1 minute), after which a final elongation step at 72°C (5 minutes). Sequencing was performed on an Ion Torrent PGM using manufacturer's protocol. Sequence data was processed with proprietary analysis pipeline (MR DNA, Shallowater, TX, USA).

Microbial Community Comparison and Statistical Analysis

Microbial community comparison was assessed using the MicrobiomeAnalyst Web-based software. The Marker Data Profiling (MDP) module was used, which takes 16S rDNA data and yields relative abundance, univariate analysis, α–diversity, β-diversity and significance through statistical tests like Analysis of Variance and Mann-Whitney test. The data set was not normalized, but was subjected to total sum scaling. This allows for more robust statistical analysis. All OTU counts less than 4 and all sequences data representing less than 10% of the whole data set were removed as a filtering method to minimize possible sequencing errors and DNA contaminants. Performing this data filtering removed a total of 408 features from the data set.

Alpha diversity was assessed by Shannon's Diversity Index, and significance was assessed using an ANOVA with a Tukey post-hoc test. Beta Diversity was assessed by Bay-Curtis dissimilarity test, and significance was assessed using a one-way ANOVA followed by a

post hoc Tukey test. All statistical tests were performed by MirobiomeAnalyst or in MiniTab (Ryan *et al.,* University of Pennsylvania, 1972). All statistical tests were performed with a 0.05 P-value parameter in MiniTab.

Univariate analysis was performed using MicrobiomeAnalyst at all levels of taxonomy. Individual significant features that had relevant abundance within the data set were separated and assessed for significance individually. Significance was assessed by a one-way ANOVA followed by a post hoc Tukey test to assess the source of the difference or similarity. All statistical tests were performed with a 0.05 P-value parameter in MiniTab.

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