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Interrogation of the Distal Gut Microbiota of Healthy Adolescents and those with Irritable Bowel Syndrome

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Interrogation of the Distal Gut Microbiota of Healthy Adolescents and those with Irritable Bowel Syndrome

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By

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I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Laura Jean Rigsbee ENTITLED Interrogation of the Distal Gut Microbiota of Healthy Adolescents and those with Irritable Bowel Syndrome BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science

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Abstract

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The human-associated microbiota has been the focus of much current research, with the microbiota inhabiting the gastrointestinal tract of particular interest. These organisms play many roles in human health and well-being. However, shifts in the composition of the intestinal microbiota have been associated with diseases such as irritable bowel syndrome, inflammatory bowel disease, and colon cancer. Several recent studies have reported on the distal gut microbiota composition of healthy adults and those with IBS, while there is a lack of studies devoted to adolescents. This study utilized a custom-designed Affymetrix Microbiota Array capable of detecting 775 phylo-species of intestinal bacteria to determine the composition of the distal gut microbiota of 22 adolescents suffering from IBS-D (diarrhea-predominant) and 22 healthy adolescents. High sample-to-sample variation was observed in both groups at genus level. While some differences were observed in mean relative abundance of several bacterial genera between IBS-D and healthy adolescents, including *Bifidobacterium, Lactobacillus, Veillonella,* and *Prevotella*, these differences were not significant. Sample groups also failed to separate in PCA space. Therefore, we cannot conclude that the distal gut microbiota of adolescents with IBS-D is significantly different than that of healthy adolescents.

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1. INTRODUCTION

Introduction to the Human Intestinal Microbiota

Unique microbial communities are found in many different niches on Earth, including communities within the soil, fresh and saltwater, on the leaves of plants, and those associated with animals. Both vertebrates and invertebrates harbor prokaryotes which play important roles in health and disease. Birds and mammals have high numbers of prokaryotes associated with the skin, and especially the gastrointestinal tract (Whitman, *et al.*, 1998). Virtually every surface on the human body that is exposed to the outside environment is colonized by microbes, including, but not limited to, the skin, oral cavity, respiratory tract, esophagus, and genitourinary tract (Sekirov, *et al.,* 2010). According to Willett *et al*., there are approximately 10^3-10^4 bacterial cells/cm^2 on the human skin, except in the groin and axilla where the density increases approximately 1000-fold to 10^6 cells/cm^2. This pales in comparison to the vast numbers that are found throughout the gastrointestinal tract, which contains up to 100 trillion bacterial cells, with the vast majority located within the colon.

All three domains of life, bacteria, eukarya, and archaea, can be found within the human intestinal tract (Whitman *et al.,* 1998). According to Ley *et al*., 2006, within a given intestinal habitat, some of the microbial members function as "residents," which would generally be always present in fairly stable numbers within the intestinal tract, while others act more like "hitchhikers", resulting from ingested food, water, and the environment, and may not always be present within the gastrointestinal tract of an individual. While the bacterial population density in the gastrointestinal environment is among the highest known, the diversity of the major phyla present is actually quite limited as compared to the diversity

found in other environments, such as the soil and ocean. The human gastrointestinal microbiota is composed of mainly obligate anaerobes, dominated by the phylum Firmicutes, followed by Bacteroidetes and Actinobacteria. Several other phyla can also be found in the gastrointestinal tract, although they are much less abundant. They include Proteobacteria, Spirochaetes, Fusobacteria, Verrucomicrobiae, and Lentisphaerae. Frank *et al*. examined biopsy samples from the small intestine and colon of healthy individuals, and noted that the small intestine samples were composed mainly of Bacilli, Streptococcaceae, Actinobacteria, Actinomycinaceae, and Corynebacteriaceae, while colon samples were dominated by Bacteroidetes and Lachnospiraceae. A few acid-tolerant bacterial genera were detected in the stomach; they included *Lactobacillus*, *Veillonella*, and *Helicobacter*.

The colon is estimated to contain approximately 70% of all microbes associated with the human body (Sekirov *et al.,* 2010). According to Gebbers *et al*., 1989, the surface area of the human gut is similar to that of a tennis court ($200m^2$), which provides a very large area for microbial colonization. The number of microbes in the human gastrointestinal tract increases dramatically moving from the stomach, to the small intestine, and into the colon. Starting at the stomach, there are approximately 10 bacterial cells per gram of contents, increasing to 10^3 cells/gram in the duodenum, 10^4 cells/gram in the jejunum, 10^7 cells/gram in the ileum, and 10^{12} cells/gram in the colon (Sekirov *et al.,* 2010). Collectively, these microorganisms contain 100 times more genes than the human genome (Sartor *et al.,* 2008). Moving from the stomach to the colon, oxygen levels and pH change drastically, therefore; the composition and diversity of the inhabiting microbes vary greatly between the different areas of the gastrointestinal tract. The stomach has a pH of about 2, while the proximal small bowel ranges from 5.5-7.0, and the distal ileum from 6.5-7.5. In the caecum, a decrease in pH is seen, ranging from 5.5-7.5, which then rises again in the distal colon to 6.1-7.5 (Nugent *et al.,* 2001).

The human intestinal microbiota plays many roles in human health and well-being. Among those roles are carbohydrate and fiber degradation, modulation of uptake and deposition of dietary lipids, production of certain vitamins and short-chain fatty acids, development and proper stimulation of the immune system, modulation of gut motility, and protection of the host from intestinal pathogens (Sekirov *et al.,* 2010). Germ-free animals were observed to require higher caloric intake to maintain the same body weight as control animals. A study by Backhed, *et al*., 2005, looked at germ-free versus conventionally-raised mice. They noted that the conventionally-raised mice had 40% more body fat than the germ-free mice, even though the germ-free mice ingested more food. They also experimented with transplantation of the gastrointestinal microbiota from conventionally-raised mice into germ-free mice. This resulted in the body fat levels of the germ-free mice becoming similar to that of conventionally-raised mice. These studies prompted more research into the mechanisms that are employed by the intestinal microbiota to maximize the nutrient availability of ingested food (Sekirov *et al.,* 2010). Since the conventionally-raised mice were ingesting fewer calories, yet still gained more weight than the germ-free mice, it is reasonable to infer that the gastrointestinal microbiota may be degrading certain nutrients into compounds that can be absorbed and utilized by the host. Many non-digestible carbohydrates including polysaccharides, oligosaccharides, fiber, and lignin are optimal energy sources for the colonic microbiota. (Jacobs, *et al.,* 2009) These compounds are broken down into monosaccharides and short chain fatty acids (SCFA). The major SCFA's produced as a result of bacterial fermentation, which mainly takes place in the proximal colon, include acetate, propionate, and butyrate. Butyrate, in addition to being the major energy source for colonocytes, has been examined for its effects on human health. Some studies indicate that butyrate may protect against colon cancer (Hu, *et al.,* 2011, Tang, *et al.,* 2011), intestinal inflammation, and oxidative stress (Hamer, *et al.,* 2008). Levels of different SCFA's can be measured in fecal

extracts through the use of NMR or gas chromatography (Jacobs, *et al.,* 2009, Murphy *et al.,* 2010).

Certain bacterial phyla present in the colon are responsible for breaking down carbohydrates into specific SCFAs. The Firmicutes, which are important starch and fiber degraders, make up the majority of bacteria present in the colon, and include genera such as *Ruminococcus*, *Faecalibacterium, Roseburia, Papillibacter*, and *Clostridium*. The main carbohydrate fermentation products of the Firmicutes include acetate, lactate, formate, butyrate, and succinate (Jacobs, *et al.,* 2009). Bacteroidetes, which include major genera such as *Bacteroides* and *Prevotella*, are responsible for production of acetate, propionate, and succinate. Actinobacteria, represented in the colon mainly by *Bifidobacterium*, produce lactate, acetate, and formate.

Initial colonization of the human intestinal tract

Prior to birth, the intestinal tract of the human fetus is sterile. While the intestinal tract is first colonized within hours after birth, the mode of delivery and feeding is important in determining the types of bacteria that will first colonize the intestinal tract. It is thought that the early intestinal microbial community could affect future fiber fermentation, short chain fatty acid metabolism, and vitamin K synthesis (Biasucci). This initial colonization is also thought to be important in the development of the immune system. Vaginally-delivered infants are colonized with microorganisms from the mother's intestinal and vaginal microbiota, while those delivered by Cesarean section are colonized with microorganisms from the skin and environment (Biasucci, *et al*., 2010). Penders, *et al*., 2006, report that Cesarean-delivered infants had lower numbers of *Bifidobacterium* and *Bacteroides* and were more likely to be colonized with *Clostridium difficile* than vaginally-delivered infants. They also found that infants who were delivered vaginally at home and were breastfed exclusively

seemed to harbor the highest numbers of "beneficial" microbes. These infants had the highest numbers of *Bifidobacterium* and lowest numbers of *E. coli* and *C. difficile*, while formula-fed infants were more often colonized with *E. coli, C. difficile, Bacteroides*, and *Lactobacillus*.

During the first year of life, the composition of the microbial residents of the gastrointestinal tract is much simpler and less diverse than that of an older child or adult, and varies widely between individual infants. Both infants and adults are exposed to microbes by ingesting milk and other foods, however, infants are much more likely to actually be colonized by these organisms rather than healthy adults with established gastrointestinal microbial communities (Mackie, *et al*., 1999). After the first year of life, the intestinal microbiota becomes more stable and starts to resemble that of children and young adults (Mackie*, et al*., 1999). There is also a shift from facultative anaerobes, mainly Enterobacteria, to strict anaerobes (Hopkins, *et al*., 2005). As facultative anaerobes colonize the colon, they consume oxygen, which enables obligate anaerobes, such as Clostridia, Bifidobacteria, and *Bacteroides*, to enumerate (Enck *et al*., 2009). Obligate anaerobic species dominate the colon in children and adults. Enck *et al*., 2009 studied fecal samples by conventional culture techniques from over 12,000 infants and children up to 18 years of age. They found that *Bacteroides spp*. and lactobacilli increased with age, while Enterococci and *E. coli* decreased, and Bifidobacteria numbers remained stable.

Microbiota in Human Disease

While our intestinal microbiota plays several important roles in human health, as discussed previously, many recent studies have suggested that deviations in certain types of bacteria may be linked to several diseases, such as irritable bowel syndrome (IBS) (Matto, *et al*., 2005, Maukonen, *et al*., 2006, Kassinen, *et al*., 2007, Krogius-Kurrika, *et al*., 2009, Tana,

et al., 2010), inflammatory bowel disease (IBD) (Friswell *et al*., 2010), and possibly even colon cancer (Sobhani *et al*., 2011).

Irritable Bowel Syndrome

According to Longstreth *et al*., 2006, IBS is a common disorder worldwide, with approximately 10-20% of adults and adolescents affected. It is a functional bowel disorder that has varied symptoms among those affected, but is associated generally with abdominal pain, bloating, and changes in bowel habit, but without any visible damage or high-level inflammation to the large intestine as seen in patients suffering from Crohn's disease and ulcerative colitis. Although IBS does not cause as severe of an illness as IBD, it still significantly affects the quality of life (Salonen, *et al*., 2010). The Rome III criteria are used to group IBS-affected individuals into three groups based on stool form and frequency: IBS-D (diarrhea-predominant), IBS-C (constipation-predominant), and IBS-M (mixed, or alternating) (Salonen, *et al*., 2010, Longstreth, *et al*., 2006). For a diagnosis of IBS to be made, pain must be experienced at least 3 days per month over 3 consecutive months, and the pain must have two of the following three features: (Karantanos, *et al*., 2010)

1. Relief after defecation
2. Onset of pain associated with change in stool frequency
3. Onset of pain associated with change in stool form

Unfortunately, the actual pathophysiology of IBS is very complex and not yet completely understood. While deviations in the intestinal microbiota have been noted in studies involving healthy subjects vs. IBS-affected subjects, other factors are also thought to be involved, such as visceral hypersensitivity, abnormal gut motility, autonomous nervous system dysfunction, and psychological factors, such as stress and anxiety (Karantanos, *et al*., 2010). Low-level

inflammation of the GI mucosa in IBS patients has also been reported in several studies (Aerssens, *et al*., 2008, Chadwick, *et al*., 2002, Macsharry, *et al*., 2008).

Irritable Bowel Syndrome and the Intestinal Microbiota

According to Salonen, *et al*., 2010, there are three main convincing pieces of evidence that tie the intestinal microbiota to involvement in IBS. The first involves the correlation between gastrointestinal infections and a particular type of IBS, post-infectious irritable bowel syndrome (PI-IBS). The gastrointestinal microbiota has also been shown to be altered in IBS patients in many recently published studies involving subjects with different subtypes of IBS. In addition, other studies have shown that IBS symptoms can be improved with treatments that are meant to alter the composition of the intestinal microbiota, such as probiotics, prebiotics, and antibiotics.

Post-infectious IBS

After a gastrointestinal infection, some patients (7-31%) will go on to develop what is known as PI-IBS. Most studies of PI-IBS have involved adult patients. While IBS affects approximately 14% of high school patients and 6% of middle school patients, a study by Marshall, *et al*., 2006, reported a high incidence of post-infectious IBS symptoms (36%) in 88 children with positive stool culture results. Microorganisms that are most often associated with PI-IBS include *Salmonella* spp., *Campylobacter jejuni*, and *Shigella* spp. The length and severity of the infection are also important factors in the likelihood of developing PI-IBS. According to Neal, *et al*., 1997, an illness that lasts longer than 3 weeks has a relative risk of 11.4 compared to an illness lasting less than 1 week. Antibiotic use also increases the risk of PI-IBS. Maxwell, *et al.,* 2002, state that patients who were given antibiotics were 4 times more likely than controls to develop bowel problems 4 months after treatment.

SIBO (Small Intestinal Bacterial Overgrowth) and IBS

Small intestinal bacterial overgrowth, or SIBO, is a condition that is characterized by abnormally high numbers of bacteria (>10^5 organisms/ml) present in the proximal small intestine, which leads to a competition for nutrients between the host and the bacteria (Rana, *et al*., 2008, Scarpellini, *et al*., 2009). As a result of unusually high bacterial catabolism in this area of the intestine, toxic metabolites are produced, which can cause injury to the intestinal enterocytes (Rana, *et al*., 2008). Symptoms include chronic diarrhea, flatulence, nausea, and abdominal pain (Rana, *et al*., 2008). The proper method for SIBO diagnosis has been debated, but it is generally diagnosed by aspiration and culture of the jejunal contents, or with lactulose breath testing (Posserud, *et al*., 2007, Scarpellini, *et al*., 2009). In a study by Bouhnik, *et al.,* 1999, jejunal samples from patients with suspected SIBO were cultured, and bacterial genera recovered included microaerophiles *Streptococcus, E. coli, Staphylococcus, Micrococcus, Klebsiella*, and *Proteus,* and anaerobes *Lactobacillus, Bacteroides, Clostridium, Veillonella*, *Fusobacterium*, and *Peptostreptococcus*.

SIBO has been shown to be associated with IBS. In a 2009 study by Scarpellini, *et al.,* 2009, children with IBS and healthy children were given lactulose breath tests (LBT), and they found that the LBTs were abnormal in 65% of children with IBS, while only 7% were abnormal in control children. Several adult studies also suggest a correlation between SIBO and IBS (Lupascu, *et al*., 2005, Pimentel, *et al*., 2003, Carrara, *et al*., 2008, Majewski, *et al*., 2007, Posserud*, et al*., 2007). Treatment of SIBO usually involves antibiotics. Broad-spectrum antibiotics have been effective against Gram negative organisms. Other antibiotics, such as norfloxacin, amoxicillin, rifaximin, chlortetracycline, and ciprofloxacin were also used in treatment of SIBO (Rana, *et al*., 2008). Posserud treated seven SIBO patients with

ciprofloxacin, and five showed decreased levels of small-intestinal bacteria after treatment. Three patients reported an improvement of symptoms after treatment.

Recent Studies of Intestinal Microbiota Among Different IBS Subtypes

While there have been a number of recent studies (Lyra, *et al*., 2009, Matto, *et al*., 2005, Maukonen, *et al*., 2006, Kassinen, *et al*., 2007, Krogius-Kurrika, *et al*., 2009, Tana, *et al*., 2010) aimed to determine whether there are significant differences in the intestinal microbiota between healthy subjects and IBS subjects, the results of these studies do not show a particular consensus, which may be due to the varying experimental methods used. Among these methods are conventional culturing, DGGE, qPCR, sequencing, FISH, and microarray technology, or a combination of these methods. In addition, methods such as qPCR only allow for study of specifically selected groups of bacteria. The majority use the Rome II criteria for recruitment of subjects, and all involve adult subjects.

Stability and Variability of IBS Microbiota vs. Healthy Microbiota

A culturing and DGGE-based study by Matto, *et al*., 2005, suggested that IBS subjects showed greater temporal instability (changes over time) of their intestinal microbiota than healthy controls. However, some of the IBS subjects were on antibiotics, which may explain this finding. A slightly higher number of coliforms and a higher proportion of aerobes to anaerobes were also reported. A follow-up study by Maukonen, *et al*., 2006, also studied temporal stability of the microbiota of IBS vs. healthy subjects by DGGE, and also looked at the abundance of certain groups of bacteria by affinity capture, a type of quantitative hybridization-based technique. Similar to the findings by Matto, greater temporal instability of IBS microbiota as compared to healthy controls was reported. They also saw a decrease in the proportion of the *C. coccoides-E. rectale* group in IBS-C subjects compared to healthy

controls. In contrast to the previous two studies, a DGGE-based study by Codling, *et al.,* 2010, reported greater instability and variation in healthy subjects compared to IBS subjects.

Key Differences Observed in the Microbiota of IBS Subtypes

Kassinen, *et al*., 2007, reported a significantly altered fecal microbiota in IBS. After 16S rRNA library sequencing, they noted decreases in *Collinsella aerofaciens, Clostridium cocleatum,* and *Coprococcus eutactus* in IBS subjects, which were verified by qPCR. Another 16S rRNA library sequencing and qPCR-based study by Krogius-Kurrika, *et al*., 2009, found significant differences between IBS-D subjects and healthy controls. They saw increases in Proteobacteria and Firmicutes in IBS-D subjects, with a particular increase in the family *Lachnospiraceae* within Firmicutes. Decreased Actinobacteria and Bacteroidetes were also observed compared to healthy controls. A qPCR-based study by Lyra, *et al*., 2009, measured levels of 14 different bacterial phylotypes in IBS-D, C, M, and healthy controls. After multivariate analysis, they reported that the microbiota of IBS-D subjects differed the most from all other sample groups. Increased *Ruminococcus torques* and *Clostridium thermosuccinogenes* in IBS-D subjects, and increased *Ruminococcus bromii* in IBS-C subjects were observed compared to controls. *C. thermosuccinogenes* was also found to be increased in IBS-M subjects, while *R. torques* was decreased as compared to healthy controls. A second qPCR-based study by Malinen, *et al*., 2005, examined levels of 20 bacterial phylotypes. Decreased Lactobacilli were seen in IBS-D subjects compared to IBS-C. An increase in *Veillonella* was observed for IBS-C subjects compared to healthy controls. Overall, in IBS subjects *Bifidobacterium catenulatum* was decreased, while *Ruminococcus productus* and *C. coccoides* were increased compared to healthy controls. Tana, *et al*., 2010, reported higher levels of *Veillonella* and *Lactobacillus* in IBS patients compared to controls. This study also examined levels of SCFA's, and found that IBS patients had higher levels of

acetic, propionic, and total organic acids, which are known to be produced by a combination of *Lactobacillus* and *Veillonella*. Higher levels of acetic and propionic acids also correlated positively with increasingly worse GI symptoms associated with IBS.

Treatment of IBS

While there is no known cure for IBS at this time, treatments are available that can help manage symptoms of IBS. The main goal of IBS treatment is to improve symptoms (abdominal pain, bloating, constipation, diarrhea); thereby improving the quality of life for the patient. As mentioned previously, SIBO has been treated with antibiotics. Although low-level inflammation has been reported in the GI mucosa of IBS patients, (Aerssens, *et al*., 2008, Chadwick, *et al*., 2002, Macsharry, *et al*., 2008) treatment with anti-inflammatory drugs such as prednisolone have not been very effective in improvement of IBS symptoms (Dunlop, *et al*., 2003). Other drugs, such as smooth muscle relaxants, tricyclic antidepressants, SSRI's, and anticonvulsants have been used in treatment of abdominal pain associated with IBS with some success (Lacy, *et al*., 2009).

Other treatment options that have been widely used and studied are probiotics and prebiotics. Probiotics are live bacteria which are administered to the host, usually through a food product (yogurt, for example) or as a capsule. Generally, they include one or more members of the genera *Bifidobacterium, Lactobacillus*, or *Streptococcus*. Microorganisms given as probiotics are considered to be beneficial in some way to the host. Once in the host, some of the bacteria must survive the acidic environment of the stomach and eventually establish in the colon among the existing colonic microbiota. In order for this to happen, the bacteria must be administered in large doses over a period of time. Possible health benefits of probiotics include competitive interactions with pathogenic bacteria, production of compounds that inhibit the growth of other bacteria, modulation of intestinal inflammatory

response, and enhancement of the mucosal barrier function (Quigley, *et al*., 2010). Prebiotics, on the other hand, are nondigestible compounds, typically galacto- or fructo-oligosaccharides that selectively stimulate the growth of certain beneficial bacteria, such as *Lactobacillus* and *Bifidobacterium* (Quigley, *et al*., 2010).

How do we study the intestinal microbiota?

In order to understand how changes in the composition of the intestinal microbiota relate to certain disease states (irritable bowel syndrome, inflammatory bowel disease) the basic composition of the normal, "healthy" microbiota must be determined. The vast majority of studies use fecal samples or intestinal biopsies as a means of sampling the intestinal microbiota. Of those studies, fecal samples seem to be used most frequently, mainly due to the ease and non-invasive nature of collection. In some cases though, fecal samples may not be ideal. Biopsy specimens may need to be collected for studies focused on the microbiota associated specifically with the colonic mucosa or the small intestine.

The "classic" method of studying intestinal microbiota involved culturing bacteria from fecal or biopsy samples onto differential media to select for certain groups, and then counting the resulting colonies. Culturing was very time-consuming and labor intensive, and identification of bacteria to the species or strain level was impossible. The majority of gut microbiota are strict anaerobes, which also makes sample preservation and culturing difficult. In addition, an estimated 80% of the gut microbiota cannot be cultured due to their unknown nutritional requirements (Eckburg, *et al*., 2005). Later, molecular-based methods were put into practice. Instead of culturing live bacteria from fecal samples, these methods generally involve extracting DNA or RNA from the bacteria and then amplifying a portion or all of the 16S ribosomal RNA gene for study. This gene is very useful for determining the composition of complex microbial communities because it has been highly conserved through evolution.

The beginning and end of the gene are highly conserved; therefore, universal bacterial primers can be designed for these regions and the entire 16S rRNA gene can be amplified by PCR. Primers can also be designed to variable regions of the gene in order to amplify 16S rRNA sequences for only a certain desired subset of bacteria. Some of the common methods used recently in studies of intestinal microbiota composition will be briefly discussed in the following sections.

Molecular Methods Used to Study Intestinal Microbiota

Full-length 16S rRNA sequencing (Sanger sequencing) was used in a study by Eckburg, *et al*., 2005, to determine the diversity and composition of the colonic microbiota from three healthy individuals. Basically, the "pool" of 16S rRNA sequences were compared and then divided into operational taxonomic units (OTU's) based on their percent sequence identity, or similarity. As the number of samples to be analyzed increases, full-length sequencing can be very costly (Sekirov, *et al*., 2010). Pyrosequencing is used to amplify select variable regions of the 16S rRNA gene, and can have 100-fold higher throughput than Sanger sequencing (Sekirov, *et al*., 2010). The most significant advantage of sequencing methods is the ability to detect completely new phylo-species of bacteria. Other methods that can be used to study the composition of the intestinal microbiota include DGGE (denaturing gradient gel electrophoresis), TRFLP (terminal restriction fragment length polymorphisms), FISH (fluorescent in-situ hybridization), and qPCR. For the most part, these methods have the advantage of being less costly than DNA sequencing, but they cannot give nearly as "complete" a picture of the intestinal microbiota as a whole as DNA sequencing can. DNA fingerprinting methods such as DGGE and TRFLP, for example, are based on the ability of different DNA fragments to be resolved on a gel. However, if a particular phylotype is present at a low abundance, it is unlikely to be detected by these methods. FISH can be very

useful under certain circumstances, and low cost is a definite advantage. This method uses 16S rRNA-targeted oligonucleotide probes that are labeled with some type of fluorescent molecule. These probes can be targeted to large groups of bacteria (phylum or class) or much more specific groups. Generally though, only a small number of different probes are used simultaneously for these experiments. qPCR, like FISH, can either be targeted to broad or very specific groups of bacteria. It is often used as a confirmatory method to FISH experiments, or vise versa (Sekirov, *et al*., 2010).

Microarray technology: a new way of studying intestinal microbiota

A more recently introduced, high-throughput method for studying the intestinal microbiota is the DNA microarray. In a general sense, microarrays contain nucleic acid probes that are complementary to nucleic acid sequences present in the community of interest. These probes are affixed at one end to a glass slide or some other surface in a particular order. Microarray technology in the present day has been applied to many areas of biological research, such as gene expression in cancer cells, gene upregulation and downregulation in bacterial and eukaryotic cells under particular conditions, and microbial community composition studies, to name a few examples. Microarrays used for studying microbial communities are mainly 16S rDNA based. Early applications of 16S rDNA microarrays in microbial community analysis were much smaller in scale, containing probes towards a limited number of specific bacterial species, probes for higher phylogenetic groups of bacteria, or both (Wang, *et al.,* 2004).

In recent years, several custom 16S rDNA-based microarrays have been developed to study complex microbial communities, including the HITChip (Human Intestinal Tract Chip), PhyloChip, and the Microbiota Array that was recently developed in the Paliy laboratory. (Paliy, *et al.,* 2009, Brodie, *et al.,* 2006, Rajilic-Stovanovic, *et al.,* 2009) The probes on these

arrays were developed based on bacterial 16S rDNA sequence information, and contained much higher numbers of probes that aimed to interrogate much larger and more diverse bacterial populations. The PhyloChip, which was the earliest of these efforts, contains approximately 500,000 unique probes to bacterial species (Brodie, *et al.,* 2006). The HITChip, developed by Rajilic-Stovanovic, *et al.,* contains probes to 1140 distinct bacterial phylo-species, and was validated by interrogating bacterial 16S rDNA from fecal samples from young adults and elderly adults, and comparing to results obtained by FISH. The authors also noted the better reproducibility and reliability of results of the microarray compared to results obtained by DGGE. Paliy, *et al*., 2009 also recently developed a custom Microbiota Array designed to interrogate the intestinal microbiota, which contains probes to 775 unique bacterial phylo-species. Validation experiments correctly identified genomic DNA from 15 different bacterial species. The sensitivity of the microarray was also tested; 30 cycles of 16S PCR dropped the detection limit from 4ng to 10pg of genomic DNA. The Microbiota Array was also recently employed to demonstrate how the number of PCR cycles affects bacterial detection and also to examine differences between bacterial presence and metabolic activity (Rigsbee, *et al.,* 2011). This study also proposed mathematical algorithms to account for cross-hybridization of 16S rDNA and for the varying numbers of 16S gene copies in different bacterial groups. After these adjustments were taken into account, results were more consistent with FISH data (Rigottier-Gois, *et al.,* 2003, Lay, *et al.,* 2005), suggesting that certain groups of bacteria were previously over-represented and others under-represented. While these microarrays were all designed to study intestinal microbiota, the technology could theoretically be applied to study many types of bacterial communities.

Thesis overview

The work described in this thesis involves a quantitative comparison of the composition of the intestinal microbiota between healthy adolescents (n=22) and adolescents diagnosed with irritable bowel syndrome (n=22) through the use of a custom Microbiota Array, as described and validated by Paliy, *et al.,* 2009. While there have been previous studies of the intestinal microbiota of adult IBS patients, this study is the first to our knowledge to present a comprehensive, high-throughput view of the composition of the distal gut microbiota of healthy and IBS adolescents. In addition, Agans, *et al.*, 2011, recently showed that the distal gut microbiota of adults is different than that of adolescents. Bacterial gDNA was isolated from fecal samples. 16S rDNA was amplified by PCR, fragmented, and loaded onto the microarray. Microarrays were processed in duplicate for each fecal sample. Microarray results were analyzed as described in Materials and Methods. Microarray data was validated via qPCR experiments.

2. MATERIALS AND METHODS

Fecal Sample Collection and Preservation

Fresh fecal samples were collected into sterile containers by healthy adolescent volunteers (n=22) and newly-diagnosed IBS-D (IBS, diarrhea-predominant) adolescent volunteers (n=22) as approved by the Wright State IRB committee. For healthy samples, the age of the donors ranged from 9 to 18 years with a median of 12. For IBS-D samples, the age of the donors ranged from 8 to 18 years with a median of 13.5. For both sample groups, 10 donors were male and 12 were female. Samples were delivered to Children's Medical Center, Dayton, Ohio, and frozen at -80˚C.

Bacterial Genomic DNA Isolation

After homogenization of the fecal samples, bacterial genomic DNA was isolated from each fecal sample with the ZR Fecal DNA Mini Kit (Zymo Research) by Mr. Harshavardhan Kenche at Children's Medical Center. Specifically:

a. 150 mg of homogenized fecal sample and 750 µl of Lysis Solution were added to a Bashing Bead tube.
b. Tubes were processed in Disruptor Genie for 5 minutes.
c. Tubes were centrifuged at 10,000 g in microcentrifuge for 1 minute.
d. 400 µl of supernatant was transferred to Zymo-Spin IV Spin Filter in a collection tube and centrifuged for 1 minute at 7000 g.

e. 1200 µl of Fecal DNA Binding Buffer was added to filtrate and mixed well.
f. 800 µl of mixture from Step 5 was added to Zymo-Spin IIC Column in a collection tube and centrifuged for 1 minute at 10000 g.
g. Flow through was discarded, and previous step was repeated.
h. 200 µl of DNA Pre-Wash Buffer was added to Zymo-Spin IIC Column in a new collection tube and centrifuged for 1 minute at 10000 g.
i. 500 µl of DNA Wash Buffer was added to the Zymo-Spin IIC Column in a collection tube and centrifuged for 1 minute at 10000 g.
j. The Zymo-Spin IIC Column was transferred to 1.5ml Eppendorf tube. 100ul of DNase/RNase free water was added to column matrix. Column was centrifuged for 30 seconds at 10000 g.
k. Eluted DNA was transferred to prepared Zymo-Spin HRC-IV spin column and centrifuged for 1 minute at 8000 g. This column is meant to remove PCR inhibitors that are typically present in fecal samples.

The concentration of isolated genomic DNA was measured on the Nanodrop 1000 spectrophotometer and an aliquot of each sample was also run on an agarose gel to check quality. If quality was not sufficient, samples were reisolated.

Amplification and Purification of 16S rDNA

PCR amplification of 16S rDNA was carried out for fecal genomic DNA samples using universal 16S-rDNA specific bacterial primers Bact_27F_v4 (5'AGRGTTYGATYMTGGCTCAG-3') and Univ_1492R (5'GGYTACCTTGTTACGACTT-3'), with 250ng of starting gDNA in each reaction. 25 cycles of PCR typically produced sufficient product, but occasionally 30 cycles were used.

Table 2.1: PCR Reaction Mix

Component	Amount
Genomic DNA	250 ng
Bact_27F_v4	1 µl
Univ_1492R	1 µl
Nuclease-free Water	Up to 50 µl
Taq 2X Master Mix	25 µl

Table 2.2: Thermocycler Protocol

Step 1	95C for 2:00
Step 2 (Repeat x25)	95C for 0:30
Step 3 (Repeat x25)	55C for 0:30
Step 4 (Repeat x25)	72C for 1:30
Step 5	72C for 10:00

The four PCR reactions for a particular sample were pooled after amplification for purification with the Qiagen Qiaquick PCR Purification Kit. Specifically:

a. Five volumes of Buffer PB were added to one volume of the PCR reaction, transferred to a PCR purification column and centrifuged at 17,900 g for 1 minute.
b. The flow-through was discarded and 750 µL of Buffer PE were added to the column, and column was centrifuged for 1 minute at 17,900 g.
c. Flow-through was discarded, and the column spun down again to ensure removal of buffer.
d. The spin column was transferred to a new collection tube and 30 µL of RNase free water, heated to 50˚C, was added to the center of the column and let to sit for 2 minutes
e. The column was centrifuged at 13,000 g for 1 minute to elute amplified DNA.

The concentration of the purified 16S rDNA was measured on the Nanodrop spectrophotometer and an aliquot of each sample was also run on an agarose gel to check for the expected 1.5kbp band.

Fragmentation of 16S rDNA with DNase I

Ideally, 16S rDNA should be in fragments of 100-300bp for hybridization to the microarray. 1800ng of 16S rDNA was subjected to fragmentation with DNase I (NEB) at a concentration of 0.04U/µg.

Table 2.3: Fragmentation Reaction

Component	Amount
16S rDNA	1800 ng
DNase I (0.04 U/µl)	1.8 µl
10x DNase Buffer	4 µl
Nuclease-free Water	Up to 40 µl
Total Volume	40 µl

Figure 2.1: Polyacrylamide gel showing proper fragmentation

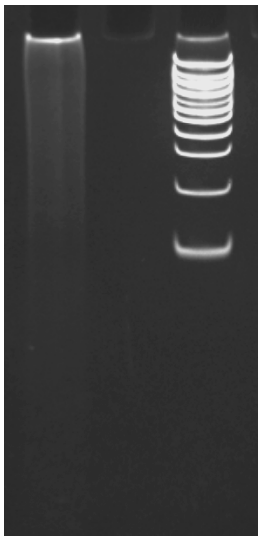


Figure 2.1 shows an ethidium-bromide stained polyacrylamide gel showing an example of properly fragmented 16S rDNA (left) next to a 100-bp ladder (right).

Components were mixed well in a 0.5ml Eppendorf tube and placed in the thermocycler, incubated at 37˚C for 10 minutes, followed by 98˚C for 10 minutes to inactivate the enzyme. 300ng of fragmented 16S rDNA were combined with water and gel loading dye and loaded onto a 10% polyacrylamide gel along with a 100-bp DNA ladder to check for proper fragmentation.

Terminal Labeling with Biotin

The remaining 1500ng of 16S rDNA fragments were end-labeled with biotin. The reaction was mixed well and incubated at 37˚C for 1 hour in the thermocycler. Reactions were stopped with the addition of 2µl of 0.5M EDTA.

Table 2.4: Labeling Reaction

Component	Amount
Fragmented 16S rDNA	1500 ng
$CoCl_2$	5 µl
Terminal Transferase (Tdt)	2 µl
10x Tdt Buffer	5 µl
GeneChip Labeling Reagent	2 µl
Nuclease-free Water	2.7 µl
Total Volume	50 µl

Hybridization to Microarray

Hybridization mixtures were prepared and loaded directly into the Microbiota Array. Microarrays were incubated for 16 hours at 45˚C and 60 rpm in the Affymetrix GeneChip Hybridization Oven.

Table 2.5: Hybridization Mixture

Component	Amount
Fragmented, labeled 16S rDNA	1500 ng
2X Hybridization Buffer	65 µl
Control Oligo B2	2.2 µl
100% DMSO	10.2 µl
10 mg/ml Herring Sperm DNA	1.3 µl
50 mg/ml BSA	1.3 µl
Total Volume	130 µl

Washing, Staining, and Scanning of Affymetrix Microarrays

After 16 hours of hybridization, microarrays were removed from the oven. The hybridization mix was removed from the microarray, frozen at -20˚C and replaced with 160 µl of Wash Buffer A. Staining solutions were prepared according to the Affymetrix protocol. Washing and staining of the microarrays was carried out on the Affymetrix GeneChip Fluidics Station 450, using the "Midi_euk_2v3_450" protocol. Scanning of the microarrays was carried out on the Affymetrix GeneChip Scanner.

Table 2.6: Staining solutions prepared for microarray washing and staining

Streptavidin Vial 1	Amount
2x Stain Buffer	300 µL
50 mg/ml BSA	24 µL
1 mg/ml Streptavidin	6 µL
H2O	270 µL
Total Volume	600 µL

Antibody Soln. Vial 2	Amount
2x MES Stain Buffer	300 µL
50 mg/ml BSA	24 µL
10 mg/ml Goat IgG	6 µL
0.5 mg/ml Anti-strep	6 µL
H2O	270 µL
Total Volume	600 µL

SAPE Soln. Vial 3	Amount
2x MES Stain Buffer	300 µL
50 mg/ml BSA	24 µL
1 mg/ml SAPE	6 µL
H2O	270 µL
Total Volume	600 µL

Quantitative Real-Time PCR Analysis (qPCR)

Two gDNA samples (one from each sample group) were chosen for qPCR analysis based on their contrasting microarray results for relative abundance of selected genera. A standard curve was constructed by combining equal amounts of genomic DNA from each sample chosen for qPCR analysis. Reactions with five gDNA starting amounts (50pg, 200pg, 1ng, 4ng, 10ng) were run in duplicate for each primer pair. The 27F_v4 primer was used as the forward primer for all reactions, and reverse primers were either selected from literature or developed using Ribosomal Database Project (http://rdp.cme.msu.edu/). All qPCR reactions were carried out on the Abi Prism 7000 (Applied Biosystems) using Perfecta SYBR Green Supermix (Quanta Biosciences). One genomic DNA sample from each sample group was chosen based on the contrasting relative abundance values of the genera selected for qPCR analysis (at least a two-fold difference in relative abundance was desired). Reactions were run in triplicate with genomic DNA starting amounts of 500ng and 4ng for each primer pair.

Table 2.7: Reverse primers used for qPCR analysis

Reverse Primer	Sequence (5'→3')	Source
Eub_338R_3W (universal)	GCWGCCWCCCGTAGGWGT	Amann *et al.*, 1990
Bifi_162R (*Bifidobacterium*)	CCGGYATTACCACCCGTTT	Agans *et al.*, (in press)
Osci_236R (*Papillibacter*)	TCAGACGCGAGGCCATCTTTC	Agans *et al.*, (in press)
Prev_496R (*Prevotella*)	CGGAATTAGCCGGTCCTTAT	Matsuda *et al*., 2009
Faec_396R (*Faecalibacterium*)	CCGAAGACCTTCTTCCTCC	Conte *et al.*, 2006

Data Analysis

Data were initially processed in Affymetrix Gene Chip Operating System (GCOS), which provides raw signal values and presence/absence calls for probesets. Normalization of data was carried out with CARMAweb (Rainer) (https://carmaweb.genome.tugraz.at/carma/) using the MAS5 algorithm for background correction, VSN for normalization, MAS5 for PM correction, and Median Polish for expression. After normalization, data were inserted into a custom Microsoft Excel template developed by Dr. Paliy which calculates bacterial abundances at different phylogenetic levels. It also corrects for cross-hybridization and for the varying numbers of 16S gene copies in different bacterial genera. 16S copy numbers were obtained from rrnDB (http://ribosome.mmg.msu.edu/rrndb/index.php) and NCBI Genome Project (http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi). If the average 16S copy number for a particular bacterial genus could not be found, higher phylogenetic levels were examined.

Heatmaps were produced with Genesis (http://genome.tugraz.at/) to show the variation in relative abundance at class and genus levels among individual samples. Principal Components Analysis of experiments was also performed by importing $\log_2$-adjusted signal values into Genesis. Moderated t-tests with BH adjustment were run using CARMAweb to determine if there were significant differences at class and genus level between sample groups.

Core microbiota was assessed by determining the numbers of phylo-species present in all samples for both groups and for each individual sample. The numbers of shared phylo-species (present in at least two samples) and unique phylo-species (present in only one sample) were also calculated.

qPCR data was analyzed using a custom Microsoft Excel template. For standard curves, the log of the DNA starting amount was plotted against Ct values for each primer pair and a slope was calculated. From the slope values, efficiency values were calculated to allow

for unequal amplification rates for different primer pairs. For individual qPCR experiments, average Ct values for each primer pair were assessed against the standard curve using the Excel template. Relative abundance values of each genus being assessed were calculated with the template. Ratios of each genus between tested samples were also calculated and compared to ratios from microarray results.

3. RESULTS

Overview

Fecal samples were collected from twenty-two healthy adolescents and twenty-two adolescents suffering from IBS-D. Bacterial gDNA was extracted from each fecal sample and 16S rDNA was amplified via PCR using universal primers as described. 16S rDNA was fragmented, end labeled with biotin, and hybridized to the Microbiota Array. Microarrays were washed, stained, and scanned according to Affymetrix protocol. Duplicate microarrays were processed for each fecal sample with amplified 16S rDNA from separately run PCR reactions. After analyzing the microarray results in Affymetrix GCOS, raw signal values were normalized in CARMAweb for both kHLT (healthy adolescent) and kIBS (IBS adolescent) fecal samples. Normalized signal values and presence/absence calls for all probesets were copied into the custom Microsoft Excel template to examine the abundance of bacterial groups at different phylogenetic levels.

Correlation of Replicate Microarrays

Pearson correlations of signal values for duplicate microarrays were automatically calculated by the custom Microsoft Excel template. On average, the Pearson correlation for kHLT samples was 0.89±0.05 SD and 0.92±0.03 SD for kIBS samples.

Comparison of Intestinal Microbiota Abundance at the Class Level

At class level, both kHLT and kIBS groups were dominated by Clostridia, at 72.1% and 73.5% mean relative abundance, respectively, followed by Actinobacteria, Bacteroidetes, and Bacilli (Table 3.1). Generally, as mean abundance decreased for a particular class, the coefficient of variation increased. Clostridia, the most abundant class, had the lowest variation from one sample to the next. However, the least abundant classes, such as Alphaproteobacteria, Spirochaetes, and Verrucomicrobiae had much higher variation across samples. In many cases, these low-abundance classes were not present in every fecal sample. Pearson correlations of signal values for replicate microarrays were also determined for low-abundance classes only (Alpha, Beta, Gamma, and Deltaproteobacteria, Mollicutes, Verrucomicrobiae, and Lentisphaerae). For kHLT samples, the average Pearson correlation for low abundance classes was 0.79±0.08, and 0.86±0.07 for kIBS samples. Since these values were lower than those calculated for all classes, it may indicate that the high variation in abundance values for low abundance classes could be due in part to differences in probeset signal values between replicate microarrays. No significant differences in abundance were found at class level between kHLT and kIBS samples ($p<0.05$).

Table 3.1: Comparison of Mean Abundance at Class Level

Class	kHLT Mean Abundance	kHLT Std Error Abundance	kHLT Coefficient of Variation	kIBS Mean Abundance	kIBS Std Error Abundance	kIBS Coefficient of Variation
Alphaproteobacteria	0.1%	0.1%	414.7%	0.0%	0.0%	-
Betaproteobacteria	0.7%	0.1%	92.6%	0.7%	0.1%	91.1%
Gammaproteobacteria	0.5%	0.1%	121.9%	0.7%	0.2%	98.7%
Deltaproteobacteria	0.9%	0.2%	80.3%	0.6%	0.1%	94.0%
Epsilonproteobacteria	0.0%	0.0%	-	0.0%	0.0%	-
Clostridia	72.1%	1.1%	7.1%	73.5%	1.4%	9.0%
Mollicutes	1.3%	0.3%	107.1%	1.2%	0.2%	79.9%
Bacilli	4.3%	0.4%	45.3%	4.8%	0.4%	37.0%
Actinobacteria	10.2%	0.9%	43.8%	8.7%	1.1%	61.9%
Spirochaetes	0.1%	0.0%	218.6%	0.0%	0.0%	154.6%
Bacteroidetes	8.5%	0.6%	31.5%	8.7%	0.7%	39.7%
Fusobacteria	0.0%	0.0%	-	0.0%	0.0%	-
Verrucomicrobiae	1.2%	0.2%	92.2%	0.8%	0.2%	127.3%
Lentisphaerae	0.2%	0.2%	421.1%	0.1%	0.1%	460.5%

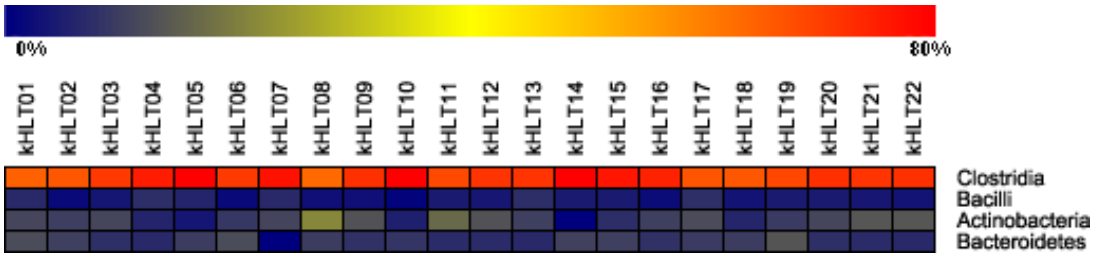
Table 3.1 presents mean abundance, standard deviation, and variation at class level for kHLT and kIBS samples.

Figure 3.1 presents heatmaps showing the variation in relative abundance of the four most abundant bacterial classes among individual kHLT and kIBS fecal samples. The upper maximum (shown in red) was set to 80% relative abundance, and the minimum was set to zero. This was done to ensure that the variations in relative abundance values for all classes could be visualized in the heatmap. Class Clostridia ranged from 63.5% to 83.4% relative abundance in kHLT samples and from 58.9% to 85.1% in kIBS samples, while relative abundance of Bacteroidetes ranged from 0.0% to 13.2% in kHLT samples, and 2.6% to 16.4% in kIBS samples. Actinobacteria also showed wide variation among individual samples, with relative abundance of 0.2% to 21.1% in kHLT samples and 0.1% to 17.5% in

kIBS samples. Actinobacteria are mainly represented in the colon by the genus *Bifidobacterium*, which is frequently included in probiotic supplements and yogurts. Consumption of *Bifidobacterium*-containing products may help to explain the high variability of Actinobacteria levels between samples. Bacilli, the fourth most abundant class, ranged from 1.0% to 7.7% in kHLT samples, and 2.1% to 10.4% in kIBS samples.

Figure 3.1: Relative Abundance at Class Level for kHLT and kIBS samples

A.



B.

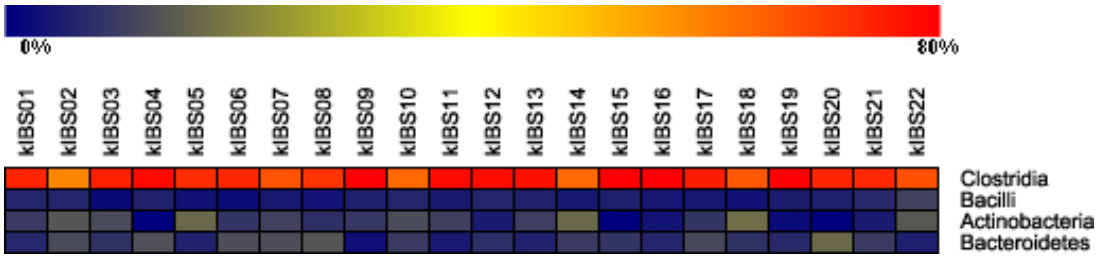


Figure 3.1A shows the variation in relative abundance at class level for kHLT samples and Figure 3.1B shows variation for kIBS samples.

Comparison of Intestinal Microbiota Detection at Class Level

The average number of probesets detected for each class was also assessed for kHLT and kIBS samples, which refers to the number of bacterial phylo-species called present by the Microbiota Array. The array contains probesets for 775 different bacterial phylo-species, and the mean total number of probesets detected was equal for both sample groups at 306, which

corresponds to 39% of all probesets being called present for both sample groups. Bacterial detection varied widely among individual samples in both groups. For kHLT samples, the number of present probesets ranged from a minimum of 207 (27%) to a maximum of 406 (52%), while kIBS samples showed similar results with a minimum of 215 (28%) to a maximum of 399 (51%).

Table 3.2: Comparison of Mean Probeset Detection at Class Level

Class	kHLT Mean Detection	kHLT Std Error	kHLT Coeffcient of Variation	kIBS Mean Detection	kIBS Std Error	kIBS Coeffficient of Variation
Alphaproteobacteria	0.3	0.1	203.1%	0.3	0.1	167.1%
Betaproteobacteria	2.0	0.2	55.6%	2.7	0.3	48.0%
Gammaproteobacteria	2.9	0.3	44.9%	4.4	0.3	34.3%
Deltaproteobacteria	1.2	0.2	81.1%	0.7	0.2	105.5%
Epsilonproteobacteria	0.1	0.1	257.6%	0.2	0.1	188.7%
Clostridia	247.1	10.7	20.4%	241.9	9.2	17.7%
Mollicutes	4.5	0.3	36.1%	4.7	0.3	29.0%
Bacilli	9.9	0.5	24.1%	11.9	0.5	20.1%
Actinobacteria	10.2	0.9	41.8%	11.0	1.0	42.3%
Spirochaetes	0.6	0.2	124.1%	0.8	0.2	104.2%
Bacteroidetes	26.0	2.4	43.3%	26.5	1.9	34.1%
Fusobacteria	0.1	0.1	323.7%	0.1	0.1	257.6%
Verrucomicrobiae	0.7	0.1	62.7%	0.6	0.1	77.4%
Lentisphaerae	0.3	0.1	149.8%	0.1	0.1	257.6%
Total	306.0	13.6	20.9%	306.0	11.6	17.8%

Table 3.2 presents the mean number of present probesets detected for kHLT and kIBS samples, standard error, and coefficients of variation. The mean total number of probesets detected was equal for both groups.

Comparison of Intestinal Microbiota Abundance at Genus Level

While examining the composition of the intestinal microbiota at class level provides a broad picture of present organisms, examining the composition at lower phylogenetic levels, such as genus level, provides a more complete picture of the individual microorganisms that make up the intestinal microbiota across different sample groups, and may help identify key differences between groups. Although the Microbiota Array can detect phylo-species from 115 genera, many are either not detected or detected at very low levels. Genera present at 1.5% relative abundance or higher for at least one sample group are shown in Table 3.3. Twelve genera listed in Table 3.3 belong to the class Clostridia, which made up the majority of relative abundance at class level. *Ruminococcus* was by far the most abundant genus detected in both kHLT and kIBS samples, with mean abundances of 21.5% and 23.0%, respectively. Among all genera listed, *Ruminococcus* also had the lowest coefficient of variation. *Faecalibacterium*, the second most abundant genus, was present at 9.1% mean abundance in kHLT samples and 9.4% in kIBS samples. *Bifidobacterium*, a member of class Actinobacteria, was present at 8.4% relative abundance on average in kHLT samples and 6.5% in kIBS samples. Mean relative abundance of *Prevotella* was 3.5 times higher in kIBS samples, however; this difference can mainly be attributed to two individual kIBS samples (kIBS04 and kIBS17) which had over 7% relative abundance of *Prevotella.* No significant differences were observed between sample groups ($p<0.05$).

Table 3.3: Comparison of Average Abundance of Selected Genera for kHLT and kIBS samples

Genus	Class	kHLT Mean Ab.	kHLT Std Error Ab.	kIBS Mean Ab.	kIBS Std Error Ab.
Clostridium	Clostridia	2.8%	0.2%	2.6%	0.2%
Acetivibrio	Clostridia	2.1%	0.2%	1.7%	0.1%
Anaerotruncus	Clostridia	3.3%	0.3%	2.7%	0.2%
Dorea	Clostridia	2.1%	0.1%	2.5%	0.2%
Faecalibacterium	Clostridia	9.1%	0.6%	9.4%	0.6%
Subdoligranulum	Clostridia	2.8%	0.2%	2.7%	0.2%
Lachnospira	Clostridia	3.3%	0.3%	3.0%	0.2%
Anaerostipes	Clostridia	2.3%	0.1%	2.5%	0.1%
Roseburia	Clostridia	5.3%	0.4%	5.9%	0.4%
Ruminococcus	Clostridia	21.5%	0.9%	23.0%	0.9%
Eubacterium	Clostridia	4.1%	0.2%	4.3%	0.3%
Papillibacter	Clostridia	5.6%	0.4%	5.9%	0.3%
Streptococcus	Bacilli	2.8%	0.4%	3.2%	0.3%
Collinsella	Actinobacteria	1.4%	0.3%	1.8%	0.3%
Bifidobacterium	Actinobacteria	8.4%	0.8%	6.5%	1.0%
Bacteroides	Bacteroidetes	6.0%	0.4%	5.9%	0.6%
Prevotella	Bacteroidetes	0.4%	0.2%	1.4%	0.5%

Table 3.3 shows the average relative abundance and standard error of 17 selected genera for kHLT and kIBS samples.

Figures 3.2 and 3.3 present scatter plots showing the differences in relative abundances of bacterial genera between kHLT and kIBS samples. kHLT mean relative abundance values are plotted on the x-axis, and kIBS mean relative abundance values are plotted on the y-axis. Error bars represent standard error of the mean (SEM). Figure 3.2 shows that the majority of the data points cluster below 1% relative abundance, which represent the genera that were either not present or present at very low levels. Also, it was noted that the vast majority of data points clustered around the diagonal, indicating that mean relative abundances of most genera did not show much variation on average between healthy adolescent samples and IBS samples. Figure 3.3 shows the mean relative abundances of genera present at <2.0% in samples.

Figure 3.2: Mean Abundance at Genus Level for kHLT and kIBS samples

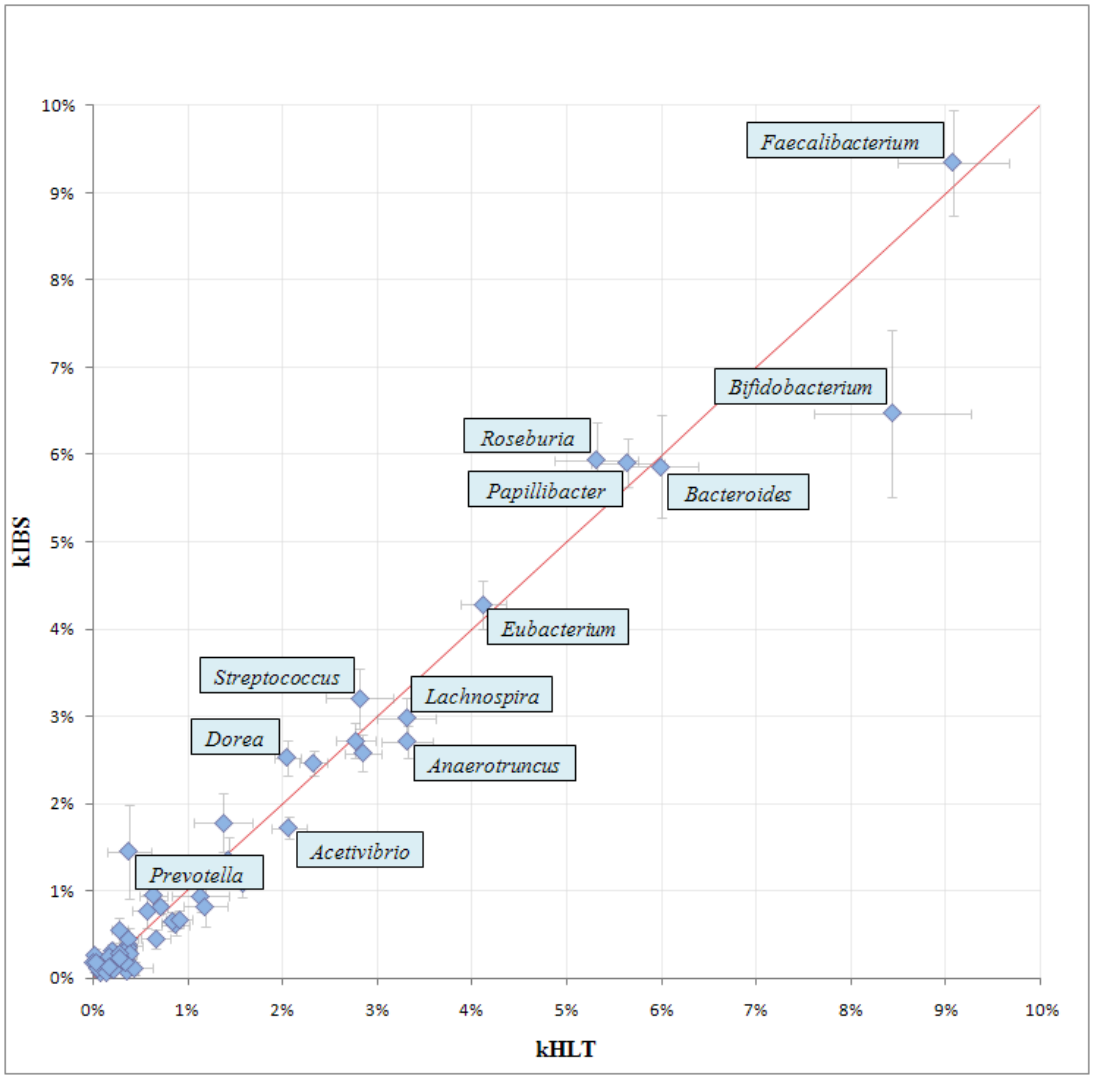


Figure 3.2 presents a scatter plot of the mean relative abundances of all genera for kHLT and kIBS samples. *Ruminococcus*, present at 21.5% abundance in kHLT samples and 23.0% abundance in kIBS samples, is not shown.

Figure 3.3: Mean Abundance at Genus Level for kHLT and kIBS samples for Genera with <2% Relative Abundance

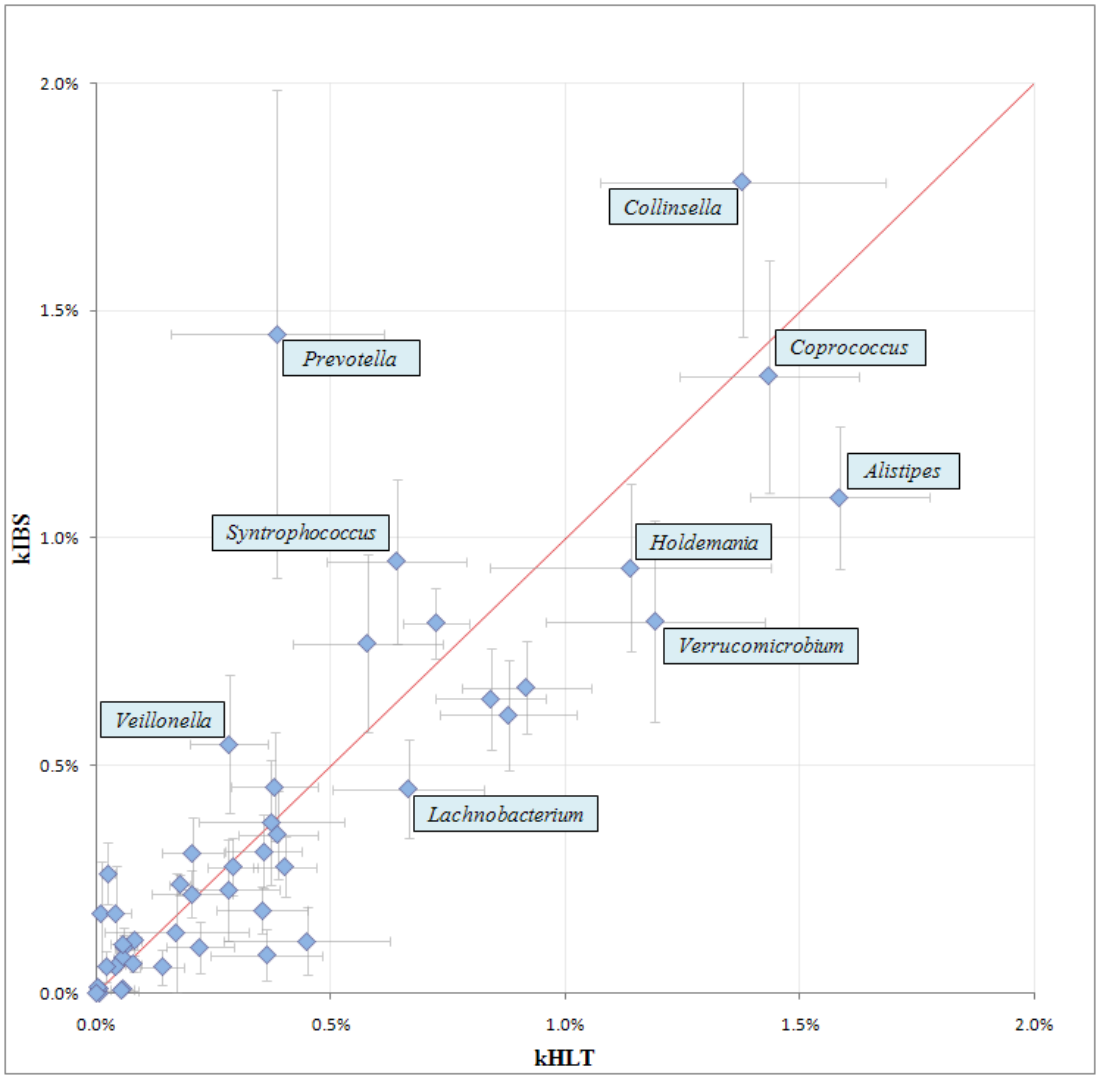
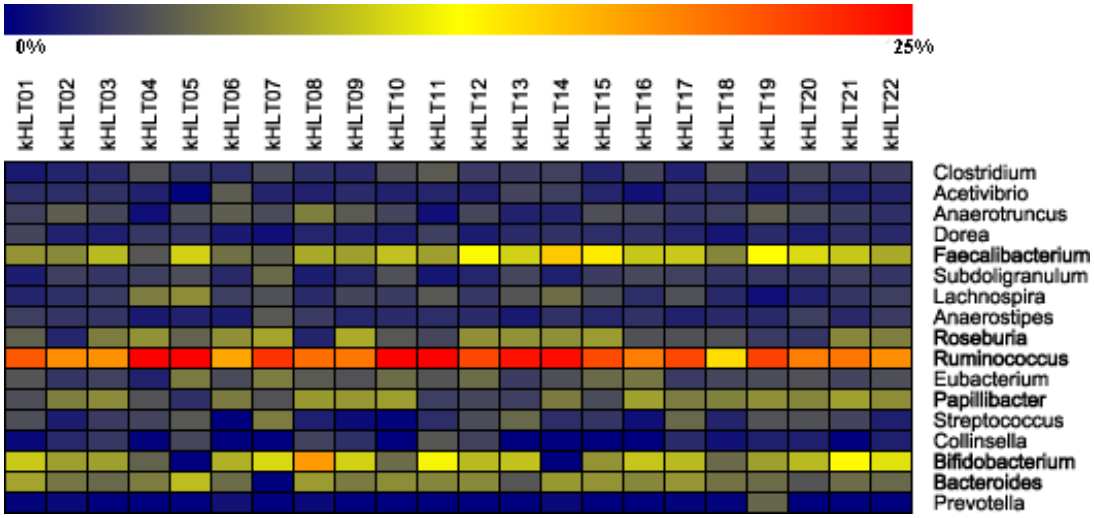


Figure 3.3 presents a scatter plot of the mean relative abundances of genera with <2% abundance for kHLT and kIBS samples.

Figure 3.4 shows variation among individual samples in relative abundance values of the most abundant genera. The upper maximum was set to 25% relative abundance (shown in red), and the minimum was set to zero (shown in dark blue) *Ruminococcus, Faecalibacterium, Bifidobacterium*, and *Bacteroides* were the most abundant genera among individual samples. Relative abundance of genus *Ruminococcus* ranged from 14.2% to 32.9% in kHLT samples and from 15.5% to 31.9% in kIBS samples. *Faecalibacterium* ranged from 4.3% to 15.1% in kHLT samples and from 0.0% (kIBS02) to 13.8% in kIBS samples. A reduction in *Faecalibacterium* has been associated with IBD in some studies (Friswell, *et al.,* 2010, Sokol, *et al.,* 2009); therefore, subject kIBS02 may have been incorrectly diagnosed with IBS rather than IBD. *Bifidobacterium* also varied widely in relative abundance among individual samples, and while a somewhat higher mean abundance was seen in kHLT samples (8.4% vs 6.5%), kIBS samples had more instances in which *Bifidobacterium* was either not present or present at very low abundance. In summary, all genera varied widely in relative abundance among individuals for both kHLT and kIBS samples, and many genera were not present in every sample.

Figure 3.4: Relative Abundance at Genus Level for kHLT and kIBS samples

A.



B.

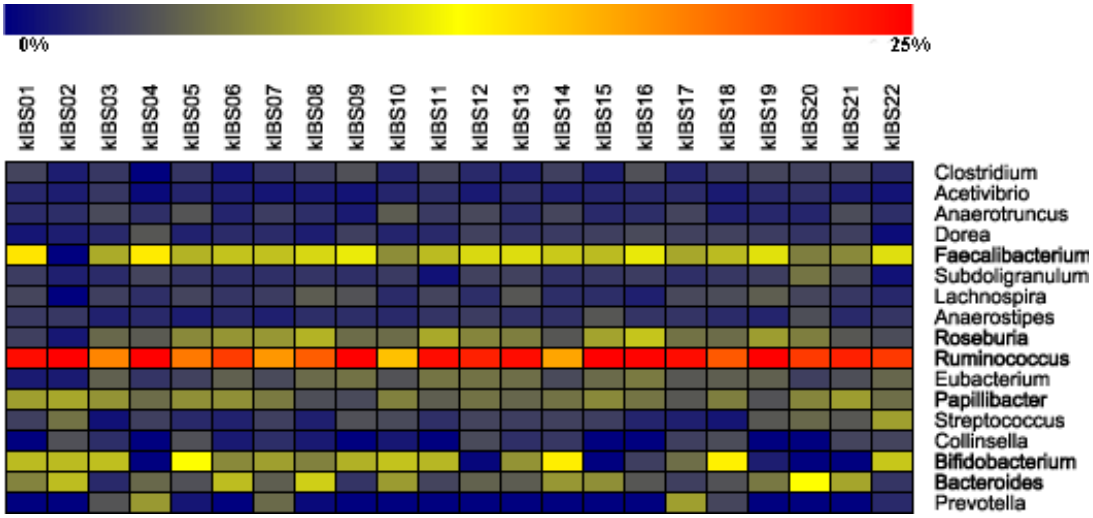


Figure 3.4A shows variation of relative abundances of genera for kHLT samples and Figure 3.4B shows kIBS samples.

Comparison of Intestinal Microbiota Detection at Genus Level

The mean numbers of detected probesets for selected genera are listed in Table 3.4 for kHLT and kIBS samples. Slight differences in the mean number of probesets detected were observed between kHLT and kIBS samples.

Table 3.4: Comparison of Mean Probeset Detection at Genus Level

Genus	Class	kHLT Mean Detection	kHLT Std Error	kIBS Mean Detection	kIBS Std Error
Clostridium	**Clostridia**	**12.0**	**0.5**	**12.1**	**0.6**
Acetivibrio	**Clostridia**	**8.7**	**1.0**	**7.0**	**0.8**
Anaerotruncus	**Clostridia**	**16.4**	**1.2**	**13.4**	**1.0**
Dorea	**Clostridia**	**10.4**	**0.4**	**9.8**	**0.6**
Faecalibacterium	**Clostridia**	**13.2**	**0.4**	**13.6**	**0.7**
Subdoligranulum	**Clostridia**	**7.5**	**0.4**	**8.0**	**0.4**
Lachnospira	**Clostridia**	**6.1**	**0.2**	**5.8**	**0.3**
Anaerostipes	**Clostridia**	**5.6**	**0.3**	**5.5**	**0.3**
Roseburia	**Clostridia**	**23.5**	**0.8**	**24.0**	**1.0**
Ruminococcus	**Clostridia**	**73.2**	**2.0**	**72.8**	**1.6**
Eubacterium	**Clostridia**	**10.6**	**0.6**	**10.3**	**0.5**
Papillibacter	**Clostridia**	**27.3**	**2.1**	**27.0**	**2.0**
Streptococcus	**Bacilli**	**5.7**	**0.3**	**6.8**	**0.2**
Collinsella	**Actinobacteria**	**0.9**	**0.2**	**1.1**	**0.2**
Bifidobacterium	**Actinobacteria**	**6.4**	**0.6**	**5.8**	**0.7**
Bacteroides	**Bacteroidetes**	**17.5**	**1.3**	**18.0**	**1.1**
Prevotella	**Bacteroidetes**	**1.0**	**0.3**	**2.2**	**0.6**

Table 3.4 presents mean number of present probesets detected for kHLT and kIBS samples and standard error.

Microbiota Core

In addition to examining differences in the intestinal microbiota composition between healthy children and children with IBS, the "core" microbiota was also investigated. The core microbiota is essentially bacterial phylo-species that are detected among all samples within a population. This concept of a core microbiota is important because it can identify and separate "indisposable" phylo-species that are present in all samples from those that are detected in only a subset of samples. For example, a phylo-species detected in all 44 samples analyzed may be more likely to play a significant role in intestinal health and digestion than a phylo-species detected only sporadically. The number of phylo-species detected in two or more individual samples (shared phylo-species) and those detected only in one individual sample (unique phylo-species) were also determined for each sample group. The shared species represent the "disposable" core, which also may have important roles in digestion and the breakdown of dietary nutrients, although they are not detected in all samples. Those species detected in only a single sample may not play a major role in the digestive process overall, but could possibly be related to the diet of the individual donor. It is also possible that the core microbiota is closely related to the general tolerance of the immune system of healthy humans towards particular commensal phylo-species of intestinal microbiota. The immune systems of individual human subjects may vary with regards to tolerance of different bacterial species within the GI tract, which may also affect the numbers of shared phylo-species.

Analysis of each sample group individually revealed that there were 56 core phylo-species detected in kHLT samples and 46 core phylo-species detected in kIBS samples (Figure 3.5). Of these, 34 were detected in every sample for both groups, while 22 phylo-species were detected consistently across all kHLT samples and 12 across all kIBS samples (Figure 3.6). Figure 3.5 also shows the numbers of phylo-species detected in at least two

individual kHLT or kIBS samples (shared), and the number of phylo-species present only in one sample for a group (unique). Figure 3.7 shows the genus composition of the 34 probesets called present in every sample across both groups. Twenty-five probesets, or 73%, belonged to *Ruminococcus*, while three probesets belonged to *Roseburia*, and two to *Streptococcus*. One phylo-species from *Clostridium*, *Lachnospira*, *Anaerostipes*, and *Papillibacter* contributed the remaining probesets. Table 3.5 also shows the composition at genus level of the probesets detected across all samples and for each sample group individually. *Ruminococcus* contributed the greatest number of core probesets in all sample groups, while *Faecalibacterium* probesets only contributed to the core phylo-species of kHLT samples. This may be due to the lack of *Faecalibacterium* in sample kIBS02 (discussed previously).

Figure 3.5: Core, shared, and unique phylo-species detected for each sample group or individual sample

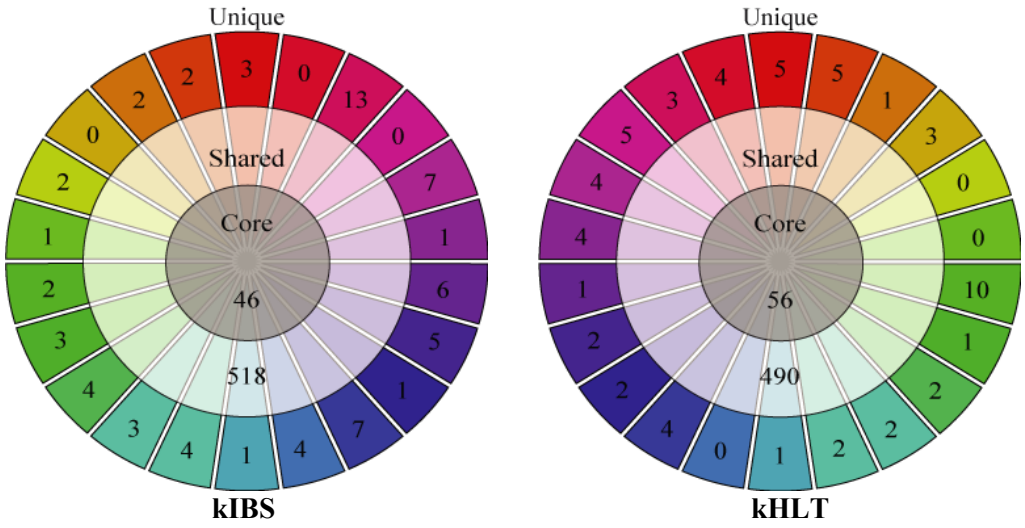


Figure 3.5 shows the number of phylo-species detected among all individual samples (core) for each sample group, the number of phylo-species shared by at least two individual samples (shared), and the number of unique phylo-species in each sample (unique).

Figure 3.6: Core phylo-species detected in kHLT and kIBS groups

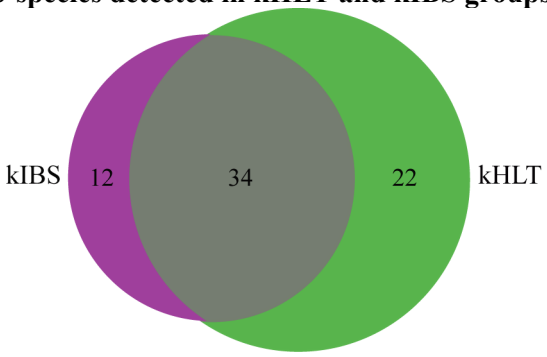


Figure 3.6 shows the number of phylo-species detected consistently across both sample groups and across each group individually.

Note: Special thanks go to Richard Agans for creating the illustrations used in Figures 3.5 and 3.6 and for developing the template used for calculation of core phylo-species values.

Figure 3.7: Composition of core phylo-species shared across both sample groups

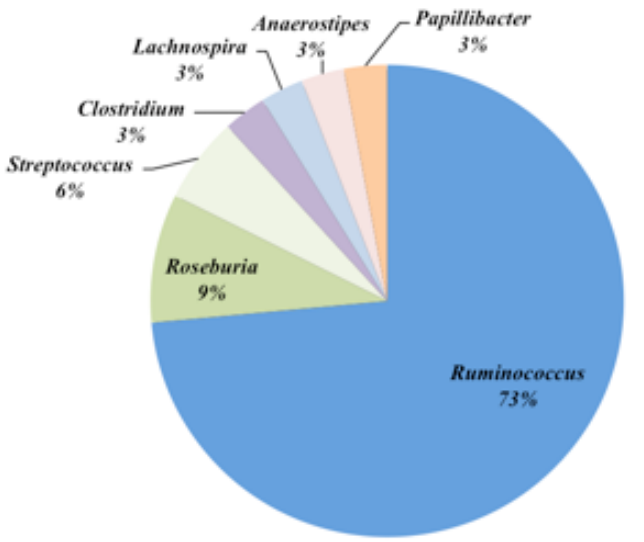


Figure 3.7 shows the composition of the 34 probesets called present in all kHLT and kIBS samples.

Table 3.5: Genus composition of core phylo-species

Genus	kHLT and kIBS	kHLT only	kIBS only
Ruminococcus	25	6	4
Faecalibacterium	-	5	-
Roseburia	3	4	1
Papillibacter	1	1	1
Streptococcus	2	-	1
Clostridium	1	3	1
Lachnospira	1	-	-
Anaerostipes	1	-	1
Turicibacter	-	1	-
Anaerotruncus	-	1	-
Dorea	-	1	-
Acetivibrio	-	-	1
Bacteroides	-	-	1
Subdoligranulum	-	-	1
Total probesets	**34**	**22**	**12**

Table 3.5 presents number of probesets detected for each genus across all samples (kHLT and kIBS) and across all samples for one sample group (kHLT only or kIBS only).

Principal Components Analysis

Principle Components Analysis of experiments was carried out using Genesis. The two sample groups did not separate in PCA space. kHLT samples are shown in blue and kIBS samples in red (Figure 3.8).

Figure 3.8: Principle Components Analysis

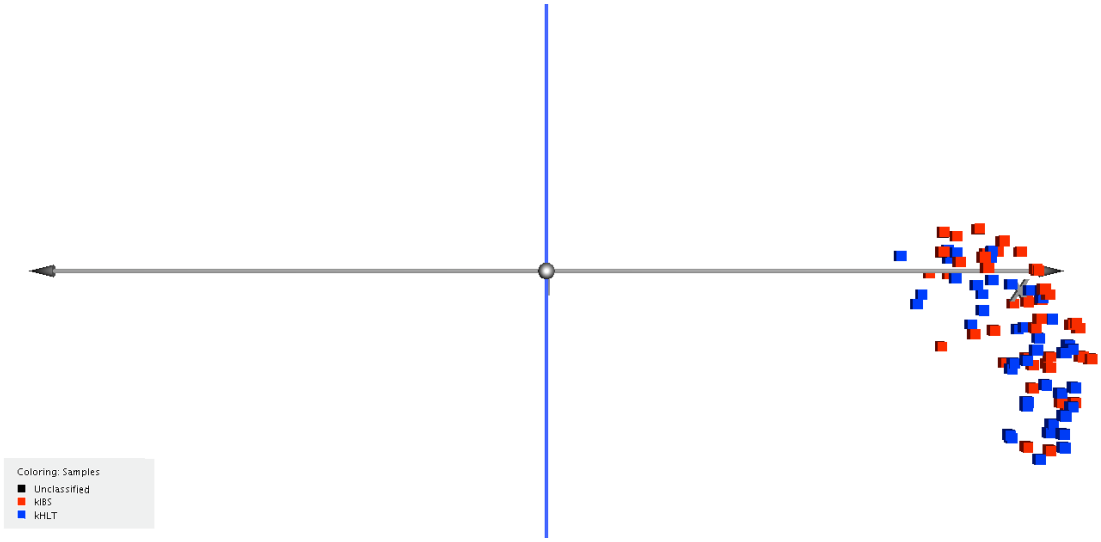


Figure 3.8 shows results of Principle Components Analysis of experiments.

qPCR Validation of Microbiota Array Results

Quantitative real-time PCR was carried out to validate results obtained by microarray. Reverse primers for *Bifidobacterium, Papillibacter, Prevotella*, and *Faecalibacterium* were either selected from literature or developed in our lab. These primers were tested first with qualitative PCR, and agarose gel electrophoresis was carried out to ensure that binding of the primers occurred only at the intended positions and that the amplicon sizes were correct. The 27F_v4 universal primer was used as the forward primer. One sample from each group (kIBS17 and kHLT12) was selected for qPCR analysis based on contrasting microarray results. qPCR reactions were carried out for 40 cycles. Melting curves

were also constructed to ensure that binding of primers only occurred in one position. The results showed that the ratios of relative abundance were in good concordance, although the ratio of Prevotella was higher for microarray results compared to qPCR results. This may be due to the highly sensitive and variable nature of qPCR reactions.

Table 3.6: Results of qPCR Validation Experiments

Genus	Microarray Results	qPCR results*
Bifidobacterium	0.3	0.1±0.1
Papillibacter	0.6	0.4±0.1
Prevotella	15.6	3.8±0.4
Faecalibacterium	1.0	0.8±0.5

Table 3.6 shows the ratio of relative abundance (kIBS to kHLT) obtained by microarray and by qPCR. Only the results from the 500pg starting gDNA reactions were used in calculation of qPCR results.
* Standard error

4. DISCUSSION

This study aimed to examine differences in the intestinal microbiota between adolescents newly-diagnosed with IBS-D and healthy adolescents through the use of a custom Microbiota Array. Although there have been previous publications which investigated the differences in the intestinal microbiota between healthy adults and those with IBS, there has been a lack of studies involving adolescent patients. In addition, those studies did not quantitatively interrogate the entire intestinal microbiome; they only measured levels of certain selected phylo-species through qPCR and sequencing methods. In contrast, the utilization of a microarray in this study gave a much more comprehensive picture of the composition of the intestinal microbiota of healthy and IBS patients.

Twenty-two fecal samples were collected from individuals in each group. Bacterial gDNA was extracted from each sample and 16S rDNA was amplified with PCR, which was fragmented, end-labeled with biotin, and hybridized to the Microbiota Array. On average, 306 probesets were detected for each sample group. Much variation in relative abundance among individual samples was observed at both class and genus levels for healthy and IBS-D groups. No significant differences in mean relative abundance were seen at class or genus level, which differs from previous studies involving healthy and IBS adults (Kerckhoffs, *et al*., 2009, Lyra, *et al*., 2009, Matto, *et al*., 2005, Maukonen, *et al*., 2006, Kassinen, *et al*., 2007, Krogius-Kurrika, *et al*., 2009, Tana, *et al*., 2010). Sample groups also failed to separate in PCA space. Overall, the results suggest that while a few consistencies with previous studies were seen at genus level, no statistically significant differences were observed between sample groups. Lower amounts of *Bifidobacterium* (6.5% vs 8.4%) and higher

amounts of *Veillonella* (0.5% vs 0.3%) and *Lactobacillus* (0.2% vs 0.0%) were observed in kIBS samples, which are consistent with the findings of Kerckhoffs, *et al.,* 2009 and Tana, *et al.,* 2010.

There are several possible explanations for these findings. Currently, the exact role of the intestinal microbiota in IBS is unknown. Some studies indicate that IBS may result from previous gastrointestinal infection (post-infectious IBS), while others suggest that host immune response may be an important factor in development of IBS (Quigley, 2011). Several studies have reported associations between levels of certain bacterial phylo-species and IBS (Lyra, Matto, Maukonen, Kassinen, Krogius-Kurrika, Tana), however; it cannot be said at this time whether or not these phylo-species have a role in the actual development or morbidity of IBS. It is also possible that shifts in the microbiota are seen as a result of the symptoms of the disease itself after a period of time. Also, while somewhat controversial, some studies have also suggested that SIBO (small intestinal bacterial overgrowth) may play a role in the symptoms of IBS (Rana, Scarpellini, Karantanos, Lin, Bouhnik). According to Lin, *et al.,* 2004, in the healthy individual, easily digestible starches, such as rice, undergo complete digestion and absorption in the proximal small intestine, and are not available for fermentation in the distal gut. Fermentation of poorly digestible starches, such as beans, takes place in the distal gut. However, in SIBO, fermentation of poorly digestible starches takes place in the ileum, leading to gas production in the small intestine. With more fermentation occurring in the ileum and fewer nutrients reaching the distal gut, they theorize that the distal gut bacteria move proximally into the small intestine. If the symptoms of IBS are related to bacterial overgrowth in the small intestine, it may be unlikely that differences would be detected in the fecal microbiota between IBS subjects and healthy subjects. In addition, while a jejunal aspiration and culturing study by Bouhnik, *et al.,* 1999 reported the presence of *Veillonella* and *Lactobacillus* in SIBO patients, many of the genera detected are also found in

the fecal samples of healthy subjects. In regards to the wide sample-to-sample variation observed, diet could be an influencing factor. There were no dietary guidelines or restrictions for the sample donors, except that they were not to have been taking antibiotics within the previous three months or consuming probiotic supplements. Certain bacterial species, such as *Ruminococcus flavefaciens*, are responsible for the enzymatic breakdown of plant cell wall polysaccharides, while *Bacteroides thetaiotamicron* is a known starch degrader (Louis). Also, fructo- and galacto-oligosaccharides have been shown to selectively stimulate the growth of *Bifidobacterium* and *Lactobacillus* species (Louis). Different diets could lead to variations in the types and amounts of dietary compounds available for microbial breakdown, so it is plausible that growth of some groups of bacteria may be stimulated or inhibited depending on their particular nutrient requirements and ability to break down certain dietary compounds.

In addition to the primary breakdown of nutrients, other studies have also shown that there is metabolic "cross-feeding" between different groups of bacteria. This cross-feeding could also play an important role in the composition of the intestinal microbiota as it relates to diet. A recent review by De Vuyst and Leroy discusses the breakdown of inulin-type fructans (found naturally in bananas, onions, garlic, leeks, and chicory) by the Bifidobacteria, which results in increased production of lactate, acetate, and butyrate. While Bifidobacteria can produce lactate and acetate, they do not possess a pathway for butyrate production in their genomes. However, several other studies have shown that other members of the gut microbiota can utilize acetate and lactate to produce butyrate. Belenguer, *et al*., showed that lactate-utilizing bacteria *Eubacterium hallii* and *Anaerostipes caccae* produce butyrate when grown in a co-culture with *Bifidobacterium adolescentis*. They also proposed that *Roseburia spp*., while not able to utilize lactate, can consume oligosaccharides produced by *B. adolescentis* to synthesize butyrate. Another recent study by Chalmers, *et al.,* found that *Streptococcus* and *Veillonella spp.* co-aggregate in dental plaques. *Veillonella spp.* are non-

saccharolytic; therefore, they are metabolically dependent on other species to provide SCFAs. In this case, lactic acid produced by *Streptococcus* is utilized as a carbon source by *Veillonella*. While this co-aggregation has not been directly observed in fecal samples, it is plausible that intestinal species could have a similar metabolic relationship. Unfortunately, there is insufficient information available about the roles of many of the members of the distal gut microbiota at lower phylogenetic levels in the breakdown of nutrients, which could be an interesting area for future work. Greater understanding of the metabolic capabilities of the intestinal microbiota at low phylogenetic levels and the metabolic relationships between them, combined with analysis of the diets of the fecal sample donors may shed light on the variations in the microbiota composition between individuals.

The microbiota core was also determined for both sample groups and for each sample group individually. Thirty-four bacterial phylo-species were detected consistently across all fecal samples. Twenty-two phylo-species were detected across all healthy adolescent samples, and twelve were detected across all IBS adolescent samples. The "shared" microbiota core, or phylo-species detected among two or more samples for a group, and the phylo-species detected in only one sample for a group (unique) were also determined. Analysis of the core microbiota may help to show which species are necessary and "indisposable" in the distal gut. A phylo-species found to be present across all samples may play a more important role in the digestive process than a species present in only a subset of samples. *Ruminococcus* species comprised 73% of the core microbiota across all samples. As primary plant cell wall degraders, *Ruminococcus* species not only made up the majority of the core microbiota, but also composed the most abundant genus overall at approximately 22% relative abundance. It also may be important to note that many of the core microbiota genera were also represented by at least two percent relative abundance.

Overall, we were able to examine the composition of distal gut microbiota of healthy adolescents and adolescents suffering from IBS-D and gain a better understanding of the relationship of the microbiota to disease. In contrast to the findings of several adult studies, no significant differences were found in microbiota composition at class or genus level between healthy and IBS fecal samples, but a few consistencies with previous studies were observed. Future efforts may focus on deeper understanding of the nutritional requirements and capabilities of lesser known intestinal bacterial genera such as *Papillibacter*, *Roseburia, Dorea, Anaerotruncus*, *Lachnospira*, and *Eubacterium*. These genera, while not nearly as abundant as *Ruminococcus* and *Faecalibacterium,* are generally present in most (if not all) samples, and usually represent at least two percent of the relative abundance. Understanding the metabolic capabilities of individual genera may also aid in determining the metabolic relationship of one genus to another. While this study focused on newly-diagnosed IBS-D patients, it might also be interesting to focus on the changes in microbiota of IBS and healthy patients over time by collecting fecal samples from the same patients at regular intervals. This may also help to answer the question of whether IBS is actually caused by some form of dysbiosis, or if the reported differences between IBS and healthy may be a result of the disease development itself.

Appendix A

Additional Publications by the Author

Rigsbee L, Agans R, Foy BD, Paliy O. *Optimizing the analysis of human intestinal microbiota with phylogenetic microarray.* FEMS Microbiol Ecol, 2011. **75**(2): p. 332-42.

Agans, R, Rigsbee, L, et al, *Distal gut microbiota of adolescent children is different from that of adults*. FEMS Microbiology Ecology, 2011. **77**(2): p. 404-412.

Rigsbee L, Agans R, et al, *Quantitative profiling of the distal gut microbiota of adolescents with IBS by microbiota array*. *(submitted)*

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