GUT MICROBIOTA AND POST-TRANSPLANT COMPLICATIONS IN KIDNEY TRANSPLANT RECIPIENTS

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By

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ABSTRACT[1]

The gut microbiota is now considered to have a role in regulating the immune system and in drug metabolism. Little, however, is known about its role in infectious and immunological complications and in metabolism of immnosuppressive medications in kidney transplantation. In this pilot study, we prospectively collected fecal specimens in 26 kidney transplant recipients and characterized the changes in the gut microbiota during the first 3 months after kidney transplantation. We utilized 16S rRNA deep sequencing of the V4-V5 hypervariable region to comprehensively characterize the gut microbiota in the fecal specimens. We characterized the changes in the gut microbiota from pre-transplantation to post-transplantation and we evaluated whether the gut microbiota was associated with post-transplant diarrhea, urinary tract infections, acute rejection, and tacrolimus dosing requirements. We report a significant increase in the phylum, Proteobacteria, from pre-transplantation to post-transplantation (0.9% vs. 4.1%, respectively, P=0.04, Wilcoxon signed-rank test) in the 5 kidney transplant recipients who had available pre-transplant fecal specimens. Recipients with post-transplant diarrhea had a lower microbial diversity as measured by the Shannon diversity index than those who did not develop post-transplant diarrhea (2.5±0.3 vs. 3.4±0.8, respectively, P = 0.02, Wilcoxon rank-sum test). Using linear discriminant analysis effect size (LEfSe)

method, we determined that post-transplant diarrhea fecal specimens were associated with a significantly lower abundance of *Bacteroides*, *Ruminococcus*, *Coprococcus*, and *Dorea.* 3 kidney transplant recipients developed *Enterococcus* urinary tract infections (UTI) and the 3 fecal specimens associated with *Enterococcus* UTI had a significantly higher median *Enterococcus* fecal abundance than the 23 time-matched fecal specimens from the kidney transplant recipients who did not develop *Enterococcus* UTI (24% vs. 0%, respectively, P=0.005, Wilcoxon rank-sum test). The same 3 kidney transplant recipients developed acute rejection and the fecal specimens associated with acute rejection had a lower abundance of Bacteroidetes (P=0.02, Wilcoxon rank-sum test), Clostridiales (P=0.01), and Bacteroidales (P=0.03) and a higher abundance of Lactobacillales (P=0.04) than the 23 time-matched fecal specimens from the kidney transplant recipients who did not develop acute rejection. In a subset of 19 kidney transplant recipients who were maintained on tacrolimus and who had available fecal specimens in the first week of transplantation, we evaluated whether the gut microbiota in the first week of transplantation was associated with a dose escalation of tacrolimus at 1 month (Dose Escalation Group, ≥50% increase in initial tacrolimus dosing by 1 month, > 6 mg/day) or with no dose escalation (Dose Stable Group, <50% increase in initial tacrolimus dosing by 1 month, ≤ 6 mg/day). The fecal abundance of *Faecalibacterium prausnitzii* was significantly higher in the Dose Escalation Group (N=5) than in the Dose Stable Group (N=14) (11.8% vs. 0.8%, P=0.002, Wilcoxon rank-sum test). There was a positive linear correlation between the fecal abundance of *Faecalibacerium*

prausnitzii at week 1 post-transplantation and future 1 month tacrolimus dosing (R=0.57, P=0.01). In this pilot study, we report one of the first characterizations of the gut microbiota after kidney transplantation and we report novel associations with post-transplant diarrhea, *Enterococcus* UTI, acute rejection, and tacrolimus dosing requirements in kidney transplantation.

BIOGRAPHICAL SKETCH

Dr. Lee is a transplant nephrologist board certified in nephrology and internal medicine by the American Board of Internal Medicine. He completed his A.B. *magna cum laude* in Biochemical Sciences in 2003 at Harvard College and his M.D. at Weill Cornell Medical College in 2007. He completed his internal medicine residency at New York Presbyterian Hospital – Weill Cornell Medical Center in 2009 and his nephrology fellowship at New York Presbyterian Hospital – Weill Cornell Medical Center in 2013. He is currently on the faculty in the Division of Nephrology and Hypertension at Weill Cornell Medical College.

Dr. Lee's research focus is on the development of biomarkers for the non-invasive diagnosis and prognostication of kidney transplant related complications. His particular research niche focuses on characterizing the gut microbiota after kidney transplantation and its relationship to post-transplant complications and drug metabolism. In the current thesis, he conducts one of the first study describing the changes in the gut microbiota after kidney transplantation and its relationship to post-transplant complications and tacrolimus dosing requirements.

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CHAPTER 1

Introduction[1]

The human gut harbors trillions of bacteria and identification of these bacteria has recently been enabled by high-throughput sequencing technologies. In particular, 16S rRNA deep sequencing of bacterial DNA allows for a non-invasive comprehensive identification of the bacteria in fecal specimens [1]. Based on advances in sequencing technologies, the NIH sponsored the Human Microbiome Project to better characterize the microbiome in adults lacking evidence of disease [2]. In this study, 242 participants provided specimens from over 15 different sites on the body including fecal specimens. With regards to the fecal microbiota, there was high variation in terms of community membership between individual participants and the most common bacteria phyla included Bacteroidetes and Firmicutes [2]. The Human Microbiome Project's main focus was to characterize the microbiota in "healthy" individuals lacking disease. Recent studies have now indicated a role of the gut microbiota in several disease states [3].

The gut microbiome has been implicated in having a role in infectious complications. *Clostridium difficile* is a recognized well bacterium that causes severe diarrhea with the major risk factor for its development being prior

[1] Adapted with permission from 1) Lee et al., Gut microbial community structure and complications after kidney transplantation: a pilot study. Transplantation 98(7): 697-705. Lippincott Williams & Wilkins. Copyright © 2014 and 2) Lee et al., Gut Microbiota and Tacrolimus Dosing in Kidney Transplantation. PLOS ONE 10(3):e0122399. Copyright © 2015.

antibiotic use [4]. Antibiotics have the ability to disrupt the microbiota [5] and allow for colonization by opportunistic organisms like *C. difficile.* The importance of commensal bacteria in *C. difficile* infections is demonstrated in a recent study utilizing fecal transplantation as a potential treatment for *Clostridium difficile* [6]. In a randomized controlled trial for treating recurrent *C. difficile*, treatment with duodenal infection of donor feces was found to be superior to treatment with antibiotics [6]. While the importance of commensal bacteria in the development of *C. difficile* infections is well known, the link between the gut microbiota and the risk for development of other pathogenic infections is not well established. In an allogeneic bone marrow transplant population, *Enterococcus* abundance in fecal specimens as measured by 16S rRNA deep sequencing preceded development of *Enterococcus* bacteremia in 2 recipients [7]. A follow up study of 94 allogeneic bone marrow transplant recipients revealed that *Enterococcus* domination in the stool (defined by ≥30 percent relative abundance) was associated with a 9 fold increased risk for *Enterococcus* bacteremia and that Proteobacteria domination in the stool was associated with a 5 fold increased risk for Proteobacteria bacteremia [8].

In addition to the gut microbiota's association with pathogenic infectious complications, the gut microbiota has also been implicated in having a role in regulating the immune system. Several studies have indicated that certain gut bacteria can induce certain lineages of T lymphocytes [9]. Colonization of the small intestine of mice with segmented filamentous bacterium has been shown to

lead to the induction of Th17 cells [9]. A further study revealed that introduction of segmented filamentous bacterium can induce Th17 cells and production of auto-antibodies, leading to exacerbation of autoimmune arthritis in a mouse model [10]. In terms of transplant immunology, little is known about the effects of the gut microbiota on allograft rejection. One study of 19 small bowel transplant recipients reported an association between a specific gut microbial profile and acute rejection of small bowel transplant recipients [11]. Another study reported the association between gut microbiota and graft-versus-host disease, a form of rejection in allogeneic bone marrow transplantation, specifically that microbial chaos measured in fecal specimens was a potential risk factor for development of graft-versus-host disease [12].

The gut microbiota's potential role in health goes beyond immunological and infectious complications and is also implicated in direct and indirect drug metabolism. Several drugs are well known to be activated in the intestines to produce therapeutic effects such as sulfasalazine, a drug that requires microbial azoreductases to transform itself into 5-aminosalicyclic acid and sulfapyridine [13]. Recently, digoxin, a common anti-arrythymic drug, has been associated with direction inactivation by a common bacteria called *Eggerthella lenta* [14]. Indirect influence on drug metabolism has also been attributed to a gut microbial product called p-cresol and acetaminophen. In this study, levels of urinary p-cresol was associated with low urinary ratios of acetaminophen sulfate to acetaminophen

glucuronide, suggesting competition of O-sulfonation of p-cresol and reduced ability to sulfonate acetaminophen [15].

In kidney transplantation, the role that the gut microbiota plays in infectious and immunological complications and in drug metabolism has not been well defined. This is particularly important since kidney transplant recipients have an increased risk for infectious complications given that they are immunocompromised patients. Furthermore, immunosuppression is the staple for suppressing allograft rejection. Despite mainstay immunosuppressive therapies, allograft rejections still occur and the potential contribution by gut microbiota in this process has not been well characterized. Potentially influencing allograft rejection is also adequate doses of immunosuppressive medications like tacrolimus. Although no direct evidence on the gut microbiota's role in tacrolimus metabolism has been established, indirect evidence includes fluctuations in levels of tacrolimus trough levels in the settting of diarrhea [16-18] and antibiotics [19, 20]. While some studies have proposed downregulation of CYP3A4 and P-glycoprotein in the intestinal cells in the setting of diarrhea [16], the role that the gut microbiota may have on this process has not been explored.

Little is known about the changes in the gut microbiota after kidney transplantation and its relationship to post-transplant complications. One study by Fricke et al. investigated the rectal microbiota after kidney transplantation [21]. In this study of 60 kidney transplant recipients, urine, blood, and rectal swabs were

taken pre-transplantation, 1 month, and 6 months and 16S rRNA deep sequencing of each specimen allowed for microbial characterization in each specimen. The authors reported significant changes in the rectal microbiota after kidney transplantation but did not relate them to specific post-transplant complications [21].

In the current study, we conducted a pilot study of characterizing the serial gut microbiota in 26 kidney transplant recipients during the first 3 months of kidney transplantation. We report the changes in the gut microbiota after kidney transplantation and its relationship to post-transplant diarrhea, *Enterococcus* urinary tract infections (UTI), and acute rejection (AR) [22]. In a subset of 19 kidney transplant recipients, we further report a relationship between a bacterium called *Faecalibacterium prausnitzii* and tacrolimus dosing requirements in kidney transplantation [23].

CHAPTER 2

Methods[2]

Introduction

In this pilot study, we prospectively recruited kidney transplant recipients at the New York Presbyterian Hospital – Weill Cornell Medical Center into serial fecal specimen collections and we characterized the microbiota in the fecal specimens using 16S rRNA deep sequencing. From the medical records, we collected post-transplant complications and tacrolimus dosing and trough levels and we tested whether the gut microbiota are associated with post-transplant diarrhea, *Enterococcus* UTI, acute rejection, and tacrolimus dosing requirements.

Study Cohort and Recruitment

The Institutional Review Board at Weill Cornell Medical College approved the study protocol (IRB: 1206012506; "Characterization of Intestinal Microbiota to Evaluate Kidney Allograft Status"). All subjects provided written informed consent prior to enrolling into the protocol and providing serial fecal specimens. Between August 2012 and January 2013, we recruited 26 kidney transplant recipients (24 kidney transplant recipients and 2 kidney-pancreas transplant recipients) into serial fecal specimen collections. Each transplant recipient provided at least two fecal specimens within the first 3 months of transplantation.

[2] Adapted with permission from 1) Lee et al., Gut microbial community structure and complications after kidney transplantation: a pilot study. Transplantation 98(7): 697-705. Lippincott Williams & Wilkins. Copyright © 2014 and 2) Lee et al., Gut Microbiota and Tacrolimus Dosing in Kidney Transplantation. PLOS ONE 10(3):e0122399. Copyright © 2015.

Kidney Transplant Protocol

Subjects in the protocol received either a kidney alone transplantation or a combined kidney pancreas transplantation. Induction immunosuppressive therapy consisted of either anti-thymocyte globulin therapy or basiliximab therapy with maintenance oral immunosuppressive therapy consisting of tacrolimus and mycophenolate with or without prednisone. Cefazolin, vancomycin, ampicillin/sulbactam, or ampicillin/sulbactam/cefoxitin was given as pre-operative surgical antibiotic prophylaxis while trimethoprim/sulfamethoxazole, dapsone, or atovaquone was given as *Pneumocystis jiroveci* prophylaxis. The transplant recipients also received acyclovir or valgancyclovir for 6 months for cytomegalovirus prophlaxis and clotrimazole for 3 months for thrush prophylaxis. Demographics and pre-transplant, peri-transplant, and post-transplant clinical information were obtained from the electronic medical records of all of the subjects.

Collection of Fecal Specimens and DNA Isolation

Subjects were instructed to collect fecal specimens every 2 weeks after transplantation during the first month and once a month in the second and third post-transplantation months. The subjects self-collected fecal specimens and the fecal specimens were stored at -80°C. DNA from each fecal specimen was extracted using a phenol-chloroform bead-beater disruption method as described in Ubeda et al. [7].

16S rRNA Variable Region PCR Amplification

In each specimen, the V4-V5 variable region of the 16S rRNA gene was amplified using duplicate 50-μL PCR reactions: 50 ng of purified DNA, 0.2 mM dNTPs, 2.5μL of 10X PCR buffer, 1.5 mM MgCl2, 1.25 U Platinum Taq DNA polymerase, and 0.2 μM of forward primer (563F [5'-nnnnnnnn-NNNNNNNNNNNN-AYTGGGYDTAAAGNG-3'] and reverse primer (926R [5'-nnnnnnnn-NNNNNNNNNNNN-CCGTCAATTYHTTTRAGT-3']). The primers possess a 12 base barcode for sample identification and 1-8 additional nucleotides preceding the barcode which offset the sequencing of the primers [24]. The PCR reaction was run with the following cycling conditions: 1) 94°C for 3 minutes; 2) 27 cycles of 94°C for 50 seconds, 51°C for 30 seconds, and 72°C for 1 minute; 3) 72°C for 5 min. The PCR products were purified using the Qiagen PCR purification kit.

16S rRNA Deep Sequencing

Quantity measurements of the DNA were performed using Agilent Bioanalyzer. Illumina adaptors and barcodes were added using the Illumina TruSeq Sample Preparation Kit. The resulting PCR products were sequenced on an Illumina Miseq Instrument (250 by 250 base pair).

Bioinformatics Analysis

Sequences were processed using mothur version 1.31.1 [25]. Sequences were excluded if they were longer than 400 base pairs, had no exact match to the

primer with up to 3 mismatches, had homopolymer stretches longer than 8 base pairs, had sequences containing undetermined bases, or did not align to the V4-V5 variable region of the 16S rRNA variable region. The sequences were aligned to the V4-V5 region (SILVA reference) [26] using mothur through the Needleman-Wunsch algorithm and the sequences were screened for chimeras using Uchime and were removed if deemed chimeras [27]. The sequences were clustered using the function, pre.cluster, which reduces the effects of sequencing errors in overestimating microbial diversity [28]. Sequences were grouped into operational taxonomic units (average neighbor algorithm) and were further arranged on the basis of 97 percent or greater similarity. Phylogenetic classification (to determine the bacteria at the species level) was done with the Bayesian classifier algorithm using a bootstrap cutoff of 60% [29].

Post-Transplant Complications

Post-transplant diarrhea was characterized by a subjective complaint of diarrhea and 3 or more bowel movements for 2 or more consecutive days. Urinary tract infection was characterized as a positive urine culture (≥50,000 colony forming units/mL). Acute rejection was characterized by a biopsy proven allograft biopsy that was classified by the Banff criteria [30].

Dose Escalation and Dose Stable Tacrolimus Group Definitions

At our kidney transplant center, kidney transplant recipients are routinely given 4 mg/day of oral tacrolimus in two divided doses unless clinically relevant drug

interactions were present. Tacrolimus dosing in each transplant recipient was adjusted during the first month of transplantation to maintain a target level of 8 to 10 ng/mL. These changes were based on tacrolimus trough levels which were measured approximately twice a week during the first month of transplantation. Tacrolimus levels were measured at New York Presbyterian Hospital – Weill Cornell Medical Center's clinical laboratory services using the platform of a liquid chromatography tandem mass spectrometry.

Subjects were characterized into tacrolimus groups based on post-transplant day 28: Dose Escalation Group (subjects requiring a 50% increase from the standard initial tacrolimus dosing) (day 28 tacrolimus dosage > 6 mg/day) and Dose Stable Group (subjects not requiring the 50% increase from the standard initial tacrolimus dosing) (day 28 tacrolimus dosage ≤ 6 mg/day).

Statistical Analyses

Alpha diversity (Shannon Diversity Index) was measured using the mothur program [25]. A phylogenetic tree was constructed based on the 16S rRNA sequence alignment with the program, clearcut, in mothur [25, 31]. Unweighted unifrac was calculated using the constructed tree and principal coordinate analysis was calculated using the resulting distance matrix [32].

Fisher's exact test was used to compare groups with dichotomous variables. Wilcoxon signed rank test was used to compare paired groups with continuous

variables and Wilcoxon rank sum test was used to compare unpaired groups with continuous variables. Values that were measured in two groups over time were compared using a two-way between-group ANOVA using contrasts. The linear discriminant analysis effect size (LEfSe) method was utilized to compare significant differences at different taxonomic levels between selected groups [33]. Correlations between two continuous variables were evaluated using a Pearson's correlation and univariate linear regressions were calculated between the two continuous variables. For any log transformed values, a 0 value was assigned the value of half of the lowest value in the series. Variables that were significantly correlated ($P<0.10$) were computed in a multivariable linear regression model. Statistical analyses were performed using R 3.1.1 or STAT 12.1 I/C (Statacorp, College Station, TX).

CHAPTER 3

Gut Microbiota and Post-Transplant Complications[3]

Kidney Transplant Cohort Characteristics

From August 2012 to January 2013, we recruited 26 kidney transplant recipients to provide serial fecal specimens. Table 1 provides a summary of the pre-transplant and peri-transplant characteristics of the patients. We obtained 85 fecal specimens from the 26 subjects.

Fecal Microbiota Characterization

DNA was isolated from each of the 85 fecal specimens and was amplified at the 16S rRNA variable region and sequenced on an Illumina MiSeq as described in Chapter 2. We obtained a total of 1,946,273 high quality 16S rRNA sequences. For subsequent analysis, a maximum of 5000 reads per specimen were randomly selected and the results are based upon 4764±777 reads per fecal specimen.

[3] Adapted with permission from 1) Lee et al., Gut microbial community structure and complications after kidney transplantation: a pilot study. Transplantation 98(7): 697-705. Lippincott Williams & Wilkins. Copyright © 2014 and 2) Lee et al., Gut Microbiota and Tacrolimus Dosing in Kidney Transplantation. PLOS ONE 10(3):e0122399. Copyright © 2015.

Table 1. Clinical Characteristics of the Study Cohort

Transplant Recipients, N (%)	26 (100)
General Characteristics	
Age (Median, Interquartile Range)	56, 46-63
Female, N (%)	13 (50)
Race	
Caucasian, N (%)	16 (61.5)
Hispanic, N (%)	6 (23.1)
African American, N (%)	4 (15.4)
Organ Type	
Kidney, N (%)	24 (92)
Simultaneous Pancreas & Kidney, N (%)	2 (8)
Type of Transplantation	
Living Donor Transplantation, N (%)	14 (53.8)
Deceased Donor Transplantation, N (%)	12 (46.2)
Immunosuppressive Therapy	
Induction Antibody Therapy	
Anti-thymocyte globulin, N (%)	20 (77)
Basiliximab, N (%)	6 (23)
Maintenance Immunosuppressive Drugs	
Tacrolimus and Mycophenolate Acid, N (%)	25 (96)
Tacrolimus and Mycophenolate Mofetil, N (%)	1 (4)
Steroid Protocol	
Steroid Maintenance, N (%)	10 (38)
Steroid Free, N (%)	16 (62)
Perioperative Antibiotics	
Preoperative Surgical Prophylaxis	
Cefazolin, N (%)	21 (81)
Vancomycin, N (%)	3 (11)
Ampicillin/Sulbactam/Cefoxitin, N (%)	1 (4)
Ampicillin/Sulbactam, N (%)	1 (4)
Pneumocystic Jiroveci Prophylaxis	
Trimethoprim/Sulfamethoxazole, N (%)	23 (88)
Dapsone, N (%)	2 (8)
Atovaquone, N (%)	1 (4)

From Lee et al., Gut microbial community structure and complications after kidney transplantation: a pilot study. Transplantation 98(7): 697-705. Lippincott Williams & Wilkins.

Changes in Gut Microbiota After Kidney Transplantation

5 of the 26 kidney transplant recipients provided fecal specimens prior to kidney transplantation. These 5 recipients all received the same induction therapy (anti-thymocyte globulin), *Pneumocystis jiroveci* pneumonia prophylaxis (trimethoprim/sulfamethoxazole), and preoperative surgical antibiotic prophylaxis (cefazolin). All of the kidney transplant recipients had not initiated induction therapy or preoperative antibiotics prophylaxis prior to providing the pre-transplant fecal specimens and all provided a two week post-transplant fecal specimens.

Figure 1 shows the changes in the gut microbiota in the 5 patients after kidney transplantation (Figure 1A – genus level; Figure 1B – phylum level; Figure 1C – order level). At the phylum level, the fecal abundance of Proteobacteria increased from 0.9% (pre-transplant) to 4.1% (2 week post-transplant) (P=0.04, Wilcoxon signed-rank test) (Figure 1B). At the order level, the fecal abundance of Enterobacteriales increased from 0.4% (pre-transplant) to 3.9% (2 week post-transplant) (P=0.04) and the fecal abundance of Erysipelotrichales increased from 5.6% to 10.2% (P=0.04) (Figure 1C) (Table 2).

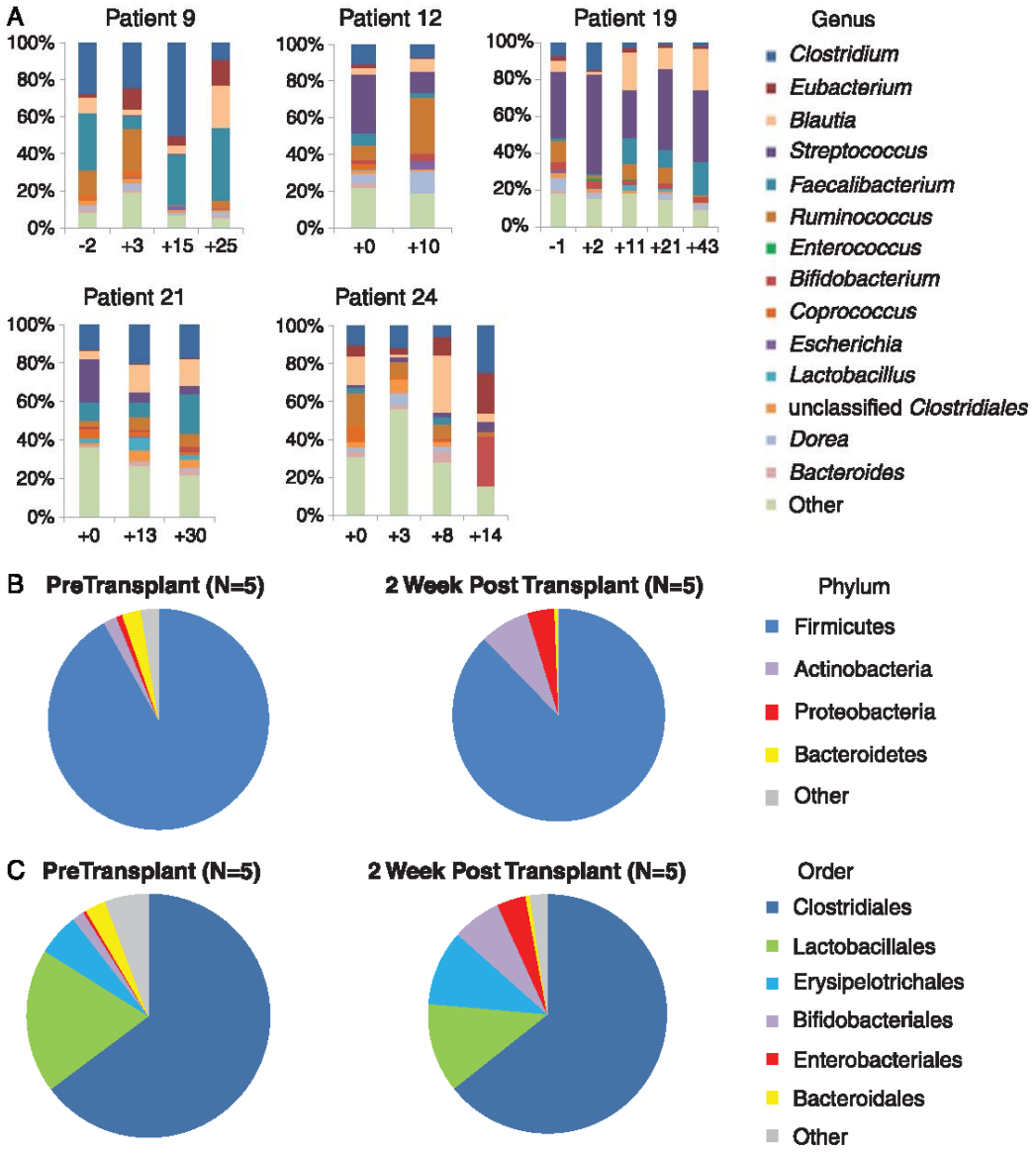

Figure 1: Alterations in the Gut Microbiota Following Kidney Transplantation. Each of the 5 kidney transplant recipients provided a fecal specimen prior to transplantation and prior to receiving any induction immunosuppression therapy or antibiotic prophylaxis and a second fecal specimen approximately 2 weeks after transplantation. All 5 recipients received similar induction therapy, preoperative antibiotics, and PCP prophylaxis therapy. The 5 sets of bar graphs show the gut microbiota of the 5 kidney transplant

recipients at the genus level over time (Panel A). Each bar represents the relative composition of bacteria in the stool sample from each patient. The x-axis indicates the day of specimen collection from the transplantation event as the reference day (Day 0); the y-axis indicates the relative bacterial percentage corresponding to each taxon. Each taxon is labeled by color as defined in the legend. Panels B and C show the differences in gut microbial composition between the pre-transplantation specimens and the 2 week post-transplantation specimens by phylum and order levels, respectively. Each color in the pie chart represents the corresponding taxon group in the legend. At the phylum level, the relative abundance of Proteobacteria (red) was higher in the post-transplantation specimens compared to pre-transplantation specimen in all 5 patients (P=0.04, Wilcoxon signed-rank test). At the order level, the relative abundance of Erysipelotrichales (light blue, P=0.04) and Enterobacteriales (red, P=0.04) were higher in the post-transplantation specimen compared to pre-transplantation specimen in all 5 patients. From Lee et al., Gut microbial community structure and complications after kidney transplantation: a pilot study. Transplantation 98(7): 697-705. Lippincott Williams & Wilkins.

Table 2. Alternations in the Gut Microbiota Following Kidney Transplantation[1]

[1] Pre-transplantation fecal specimens (PreTx, N=5 specimens from 5 patients) were collected a median of 0 days prior to transplantation and the post transplantation samples (PostTx, N=5 specimens) were collected from the same 5 patient a median of 13 days post transplantation. The relative mean bacterial abundance in the PreTx samples and the PostTx samples are shown. P values were calculated using a Wilcoxon signed-rank test.

Phylum	PreTx Cohort N=5	Post Tx Cohort N=5	P value
Firmicutes	91.8%	87.7%	0.22
Actinobacteria	2.0%	7.6%	0.50
Proteobacteria	0.9%	4.1%	0.04
Bacteroidetes	2.8%	0.6%	0.08

Order	PreTx Cohort N=5	Post Tx Cohort N=5	P value
Clostridiales	64.8%	64.3%	0.69
Lactobacillales	19.1%	12.0%	0.22
Erysipelotrichales	5.6%	10.2%	0.04
Bifidobacteriales	1.6%	6.6%	0.89
Enterobacteriales	0.4%	3.9%	0.04
Bacteroidales	2.8%	0.6%	0.08

From Lee et al., Gut microbial community structure and complications after kidney transplantation: a pilot study. Transplantation 98(7): 697-705. Lippincott Williams & Wilkins.

Shannon diversity index is a measure of alpha diversity in microbial ecology and reflects richness (number of different species in an environment) and evenness (relative abundance of each species in the environment) [34]. The Shannon diversity index was 3.7±0.3 (mean±SD) in the pre-transplantation specimens compared to 3.1±0.8 (mean±SD) in the post-transplantation specimens (P=0.22, Wilcoxon signed-rank test).

Gut Microbiota and Post-Transplant Diarrhea

In order to decrease confounding by induction therapy or antibiotic use, we evaluated the 15 kidney transplant recipients who underwent the same induction therapy (anti-thymocyte globulin therapy), the same preoperative surgical prophylaxis (cefazolin), and the same *Pneumocystis jirovecii* prophylaxis (trimethoprim-sulfamethoxazole). 6 of the 15 kidney transplant recipients developed diarrhea with a median duration of diarrhea of 4.5 days and a median number of bowel movements of 4 while 9 of the 15 kidney transplant recipients did not develop post-transplant diarrhea within the first 3 months of transplantation.

We compared the gut microbial composition in the 6 fecal specimens of the 6 kidney transplant recipients at the time of diarrhea to the gut microbial composition in 9 time-matched fecal specimens from the 9 kidney transplant

recipients who did not develop diarrhea. Diversity in the diarrhea fecal specimens was lower than that in the time-matched no-diarrhea fecal specimens (2.5±0.3 vs. 3.4±0.8, respectively, P=0.02, Wilcoxon rank-sum test). Principal coordinate analysis also showed separation between the diarrhea cohort and no diarrhea cohort (Figure 2A). Principal coordinate analysis is based on the dissimilarity between two groups using a distant matrix and allows for a representation in two-dimension space [32].

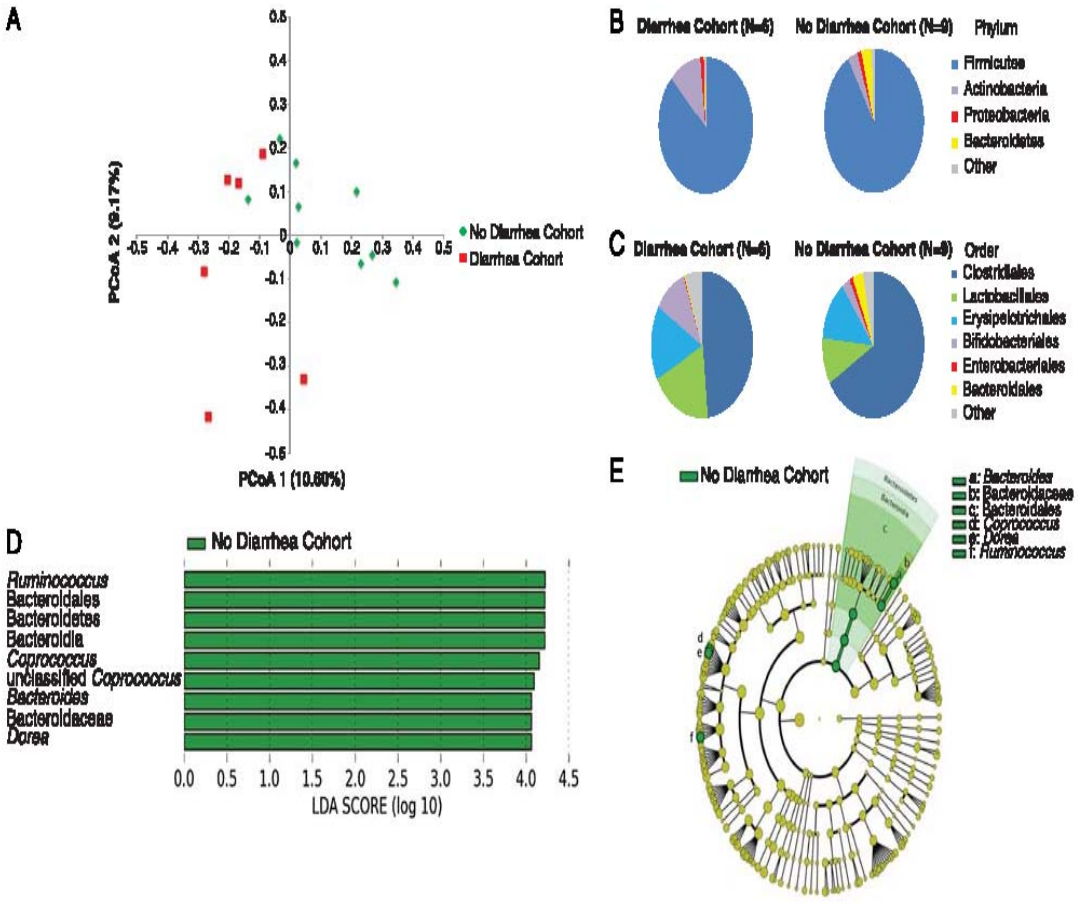

Figure 2. Differential Gut Microbial Composition in Patients with or without Post-Transplant Diarrhea. Panel A shows the principal coordinate analyses of

the 6 patients with diarrhea and the 9 patients without diarrhea. The first two axes of the principal coordinate analysis are represented with principal coordinate axis 1 on the y-axis (10.60% variability) and principal coordinate axis 2 on the x-axis (9.17% variability). The individual red points represent the 6 fecal specimens from the 6 patients with diarrhea and the individual green points represent the 9 fecal specimens from the 9 patients without diarrhea. Panels B and C represent the differences in gut microbiota between the diarrhea cohort and no diarrhea cohort by phylum and order levels, respectively. Each color in the pie chart represents the corresponding taxon group in the legend. Bacteroidetes and Bacteroidales are represented in yellow and were significantly decreased in the diarrhea cohort (P=0.007, P=0.007, respectively). LEfSe method was performed to determine individual taxons that were significantly associated with the diarrhea cohort (red) and the no diarrhea cohort (green) (Panel D). A cladogram based on the LEfSe method is shown in Panel E and highlights the taxonomic groups in the diarrhea cohort (red) and in the no diarrhea cohort (green). From Lee et al., Gut microbial community structure and complications after kidney transplantation: a pilot study. Transplantation 98(7): 697-705. Lippincott Williams & Wilkins. Copyright © 2014. Reprinted with permission.

We also evaluated the differences in gut microbiota between diarrhea-associated fecal specimens and the no diarrhea time-matched fecal specimens at the phylum and order level. At the phylum level, fecal abundance of Bacteroidetes

was significantly lower in the diarrhea cohort than in the no diarrhea cohort (P=0.007) (Figure 2B). At the order level, fecal abundance of Bacteroidales was significantly lower in the diarrhea cohort than in the no diarrhea cohort (P=0.007) (Figure 2C) (Table 3).

Table 3. Microbial Composition of Fecal Specimens From the Patients with or without Diarrhea, by Phylum and Order[1]

[1] The relative mean bacterial abundance in the fecal specimens from the 15 patients who either developed post-transplant diarrhea (Diarrhea Cohort, N = 6 patients) or did not develop post-transplant diarrhea (No Diarrhea Cohort, N = 9). The timing of collection of specimens from the no diarrhea group was matched closely to the day of collection following transplantation in the diarrhea group. P values were calculated using the Wilcoxon rank-sum test.

Phylum	Diarrhea Cohort N=6	No Diarrhea Cohort N=9	P value
Firmicutes	87.2%	91.5%	0.56
Actinobacteria	11.1%	3.2%	0.60
Proteobacteria	1.2%	1.3%	0.44
Bacteroidetes	0.3%	3.4%	0.007

Order	Diarrhea Cohort N=6	No Diarrhea Cohort N=9	P value
Clostridiales	48.4%	66.5%	0.24
Lactobacillales	19.0%	10.1%	0.64
Erysipelotrichales	16.4%	13.2%	0.56
Bifidobacteriales	10.4%	2.6%	0.77
Enterobacteriales	0.2%	1.1%	0.12
Bacteroidales	0.3%	3.4%	0.007

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Linear discriminant analysis effect size (LEfSe) method showed significant differences between taxa in the diarrhea cohort and taxa in the no diarrhea cohort. The LEfSe method allows for biomarker discovery in gut microbiota and uses Kruskal-Wallis statistical testing to compare all taxa at different taxonomic levels [33]. The LEfSe analysis showed that Bacteroidetes, *Bacteroides, Ruminococcus, Coprococcus,* and *Dorea* were significantly lower in the diarrhea cohort (Figure 2D). A cladogram of the LEfSe analysis is represented in Figure 2E.

Gut Microbiota and *Enterococcus* Urinary Tract Infections

In the series of 26 kidney transplant recipients, 3 recipients developed *Enterococcus* urinary tract infections and 23 did not within the first 3 months of transplantation. Patient 2 developed detectable fecal *Enterococcus* 16 days prior to diagnosis of *Enterococcus* UTI; patient 18 had detectable fecal *Enterococcus* a day after the diagnosis of *Enterococcus* UTI; patient 26 had detectable *Enterococcus* 39 days after the diagnosis of *Enterococcus* UTI and 26 days prior to the diagnosis of a recurrent *Enterococcus* UTI. Figure 3 shows the fecal

microbiota over time in the 3 transplant recipients with *Enterococcus* UTI and 3 representative transplant recipients without *Enterococcus* UTI and shows that fecal *Enterococcus* abundance precedes and/or coincides with development of *Enterococcus* UTI.

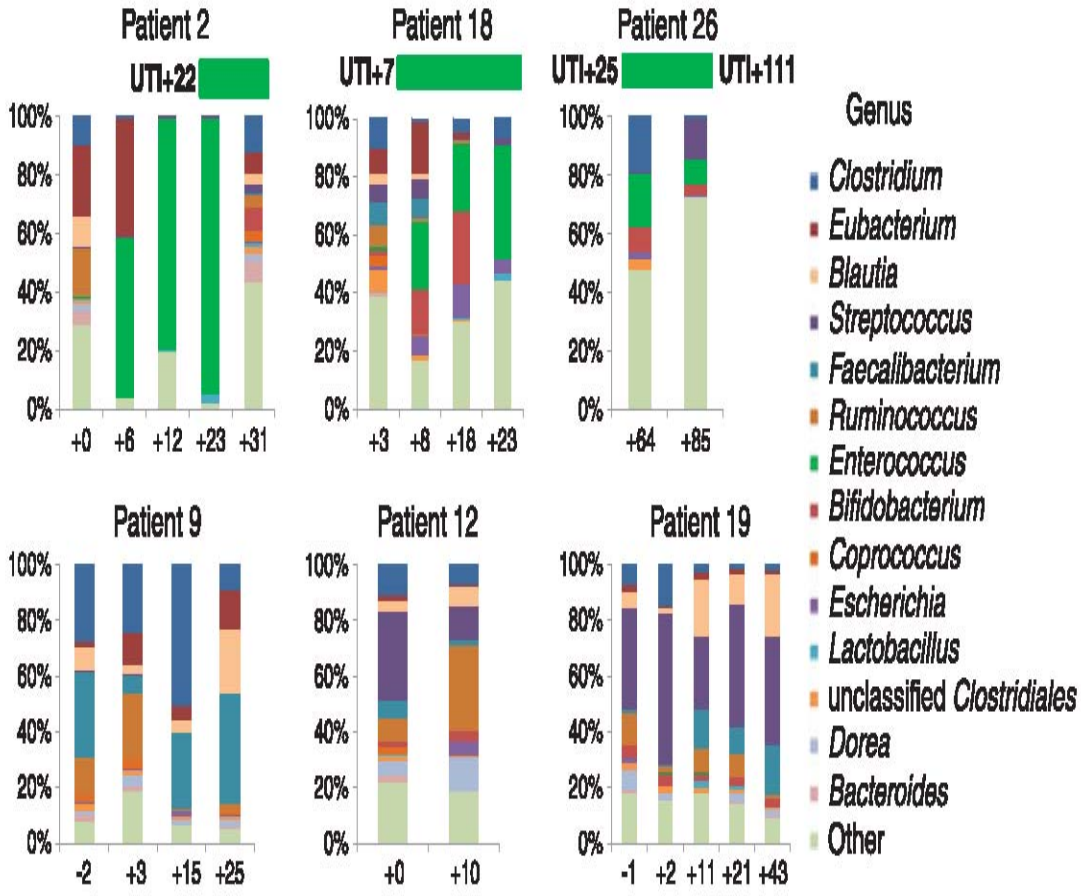

Figure 3. *Enterococcus* Fecal Abundance and *Enterococcus* Urinary Tract Infections in Allograft Recipients. The 6 sets of bar graphs represent 6 of the 26 kidney transplant recipients studied; patients 2, 18, and 26 developed *Enterococcus* UTI and patients 9, 12, and 19 are 3 of the 23 patients who did not develop *Enterococcus* UTI. Each bar represents the relative composition of bacteria in the stool sample from each patient. The x-axis indicates the day of

specimen collection from the transplantation event as the day of reference (day 0); the y-axis indicates the relative bacterial percentage corresponding to each taxon. Each taxon is labeled by color as defined in the legend. *Enterococcus* relative abundance is represented in green and is present in patients 2, 18, and 26 whereas absent in the patients without *Enterococcus* UTI. The timing/day of the *Enterococcus* UTI is indicated by the horizontal bar in green above the bar graphs. From Lee et al., Gut microbial community structure and complications after kidney transplantation: a pilot study. Transplantation 98(7): 697-705. Lippincott Williams & Wilkins. Copyright © 2014. Reprinted with permission.

We also compared the *Enterococcus* abundance in the fecal specimens from the 3 kidney transplant recipients who developed *Enterococcus* UTI (*Enterococcus* UTI group) with that in the time-matched fecal specimens from the 23 kidney transplant recipients who did not develop *Enterococcus* UTI (No *Enterococcus* UTI group). The median *Enterococcus* abundance was 24% in the *Enterococcus* UTI group and 0% in the no *Enterococcus* UTI group (P=0.005, Wilcoxon rank-sum test).

Gut Microbiota and Acute Rejection

The same 3 transplant recipients who developed *Enterococcus* UTI also developed acute rejection while the 23 other transplant recipients did not develop acute rejection within the first 3 months of transplantation. Patient 2

(simultaneous kidney and pancreas transplant recipient) had a biopsy proven acute cellular and antibody mediated rejection on post-transplant day 23; patient 18 (kidney alone transplant recipient) had a biopsy proven antibody mediated rejection on post-transplant day 7; and patient 26 (kidney alone transplant recipients) had a biopsy proven acute cellular rejection on post-transplant day 64.

The gut microbial composition in the 3 fecal specimens associated with the 3 AR events was compared to that in 23 time-matched fecal specimens from the 23 recipients who did not develop AR. Principal coordinate analysis revealed a separation between the AR cohort and no AR cohort (Figure 4A). We also compared the gut microbial composition at the phylum level and at the order level. At the phylum level, the AR cohort had a lower fecal abundance of Bacteroidetes than the no AR cohort (0.02% vs. 3.1%, respectively, P=0.03, Wilcoxon rank-sum test). At the order level, the AR cohort had a lower fecal abundance of Clostridiales and Bacteroidales than the no AR cohort (Clostridiales: 16.9% vs. 63.1%, respectively, P=0.01) (Bacteroidales: 0.02% vs. 3.1%, P=0.03) (Figure 4B) while AR cohort had a higher fecal abundance of Lactobacillales than in the no AR Cohort (49.9% vs. 12.7%, P=0.04) (Figure 4C) (Table 4).

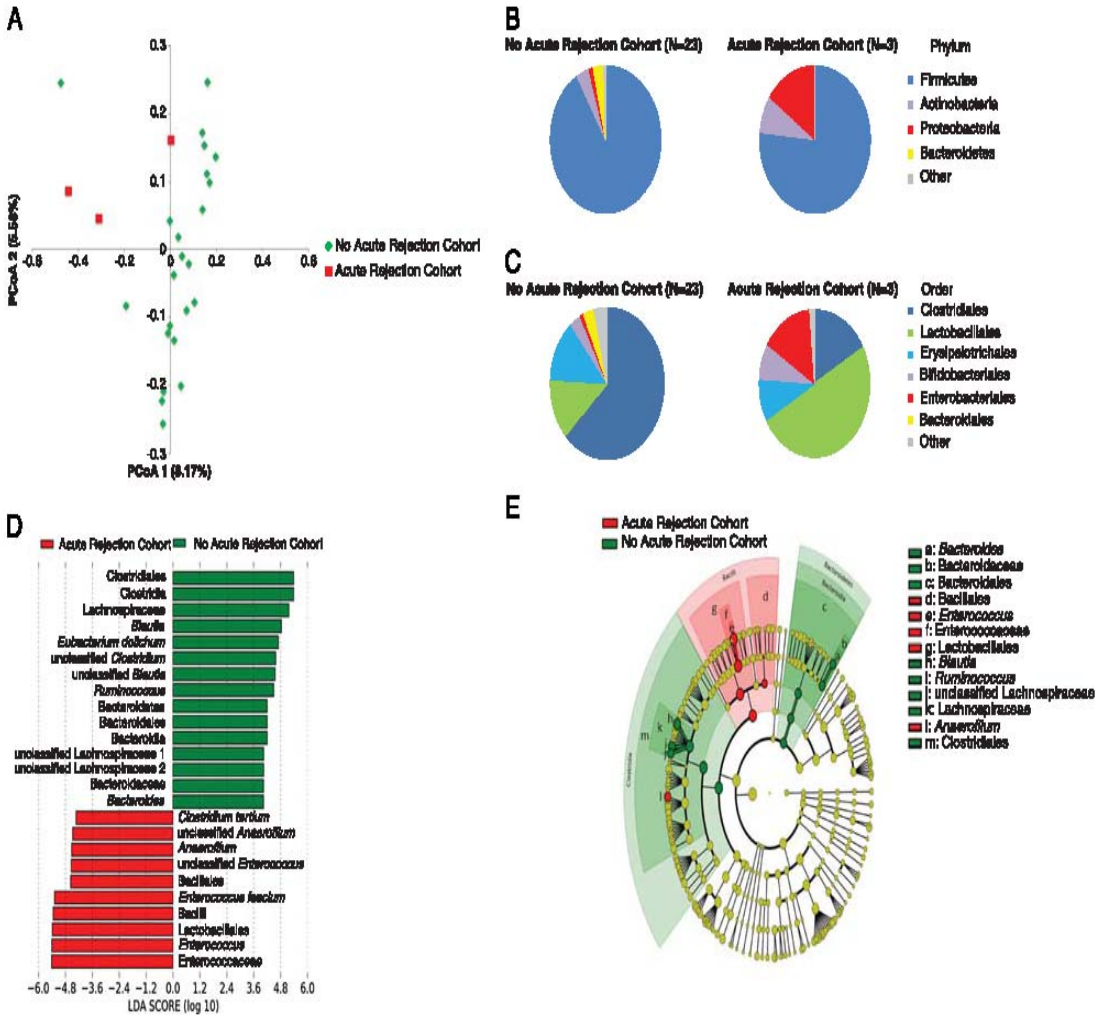

Figure 4. Differential Gut Microbial Composition in Patients with or without Acute Rejection. Panel A represents the principal coordinate analyses of the individual patients with or without biopsy confirmed acute rejection. The first two axes of the principal coordinate analysis are represented with principal coordinate axis 1 on the y-axis (8.17% variability) and principal coordinate axis 2 on the x-axis (5.58% variability). The individual red points represent the 3 fecal specimens from the 3 patients with biopsy confirmed AR and the individual green points represent the 23 time matched fecal specimens from the 23 patients who

did not develop AR. Panels B and C represent the differences in fecal microbiota between the two groups by phylum and order levels, respectively. Each color in the pie chart represents the corresponding taxon in the legend. At the phylum level, Bacteroidetes was lower in the AR cohort than in the no AR cohort (P=0.03). At the order level, Lactobacillales was higher in the AR cohort (P=0.04) and Clostridiales and Bacteroidales was lower in the AR cohort (P=0.01, P=0.03, respectively) when compared to the no AR cohort. LEfSe method was performed to determine individual taxons that were significantly associated in the AR cohort (red) and in the no AR cohort (green) (Panel D). A cladogram based on the LEfSe method is shown on Panel E and highlights the taxonomic groups associated with AR (red) and no AR (green). From Lee et al., Gut microbial community structure and complications after kidney transplantation: a pilot study. Transplantation 98(7): 697-705. Lippincott Williams & Wilkins. Copyright © 2014. Reprinted with permission

Table 4. Microbial Composition of Fecal Specimens From the Patients With or Without Acute Rejection, by Phylum and Order[1]

[1] The relative mean bacterial abundance in the fecal specimens from the 26 patients who either developed biopsy confirmed acute rejection (AR Cohort, N=3 patients) or did not develop acute rejection (No AR Cohort, N=23). The timing of collection of specimens from the AR group was matched closely to the day of collection following transplantation in the no AR group. P values were calculated using the Wilcoxon rank-sum test.

Phylum	No AR Cohort	AR Cohort	P value
	N=23	N=3	
Firmicutes	91.4%	76.6%	0.40
Actinobacteria	3.7%	8.2%	0.60
Proteobacteria	1.3%	15.2%	0.33
Bacteroidetes	3.1%	.02%	0.03

Order	No AR Cohort N=23	AR Cohort N=3	P value
Clostridiales	63.1%	16.9%	0.01
Lactobacillales	12.7%	49.9%	0.04
Erysipelotrichales	13.3%	9.2%	0.32
Bifidobacteriales	3.1%	7.9%	0.44
Enterobacteriales	1.0%	14.7%	0.17
Bacteroidales	3.1%	.02%	0.03

From Lee et al., Gut microbial community structure and complications after kidney transplantation: a pilot study. Transplantation 98(7): 697-705. Lippincott Williams & Wilkins.

Using the LEfSe method, we found several significantly different taxa between the AR cohort and the no AR cohort. The AR cohort had a higher fecal abundance of Lactobacillales, *Anaerofilum, Enterococcus,* and *Clostridium tertium* and had a lower fecal abundance of Bacteroidales, Clostridiales, Lachnospiraceae, *Blautia, Ruminococcus, Bacteroides,* and *Eubacterium dolichum* (Figure 4D). The relationships between the significantly different taxa is represented in a cladogram in Figure 4E.

In terms of antibiotic use in the AR Cohort, it is important to note that the three patients who developed AR had received several multiple antibiotics prior to the occurrence of AR. Patient 2 received metronidazole, ceftriaxone, piperacillin/tazobactam, and trimethoprim/sulfamethoxazole prior to the diagnosis of AR; Patient 18 received flagyl prior to the diagnosis of AR; Patient 26 received piperacillin/tazobactam, cefazolin, cephalexin, vancomycin, daptomycin, and linezolid prior to the diagnosis of AR.

CHAPTER 4

Gut Microbiota and Tacrolimus Dosing Requirements[4]

Study Cohort

We analyzed 19 of the 26 kidney transplant recipients in our pilot study. We evaluated this subset of the patients because all 19 patients had the following criteria: 1) were kidney alone transplant recipients 2) were maintained on tacrolimus maintenance therapy 3) had a fecal specimen within the first week post transplantation 4) did not develop acute rejection within the first month of transplantation. In terms of interactions with tacrolimus, all 19 kidney transplant recipients received clotrimazole twice daily during the first month of transplantation; 2 of the 19 transplant recipients were on diltiazem. No other patient received strong CYP3A4 inhibitors during the first month of transplantation.

Tacrolimus Dosing Groups

Among the 19 recipients, 5 patients required a 50% increase from standard initial tacrolimus dosing (Dose Escalation Group) (tacrolimus dosing > 6 mg/day by the end of the first post-transplant month) and 14 patients did not require a 50% increase from initial standard tacrolimus dosing (Dose Stable Group) (tacrolimus dosing ≤6 mg/day by the end of the first post-transplant month). We compared

[4] Adapted with permission from 1) Lee et al., Gut microbial community structure and complications after kidney transplantation: a pilot study. Transplantation 98(7): 697-705. Lippincott Williams & Wilkins. Copyright © 2014 and 2) Lee et al., Gut Microbiota and Tacrolimus Dosing in Kidney Transplantation. PLOS ONE 10(3):e0122399. Copyright © 2015.

clinical and transplant characteristics between the Dose Escalation Group and the Dose Stable Group and we did not find significantly different characteristics that could affect tacrolimus dosing such as age, gender, weight, race, type of transplantation, and steroid maintenance therapy (Table 5).

Table 5. Clinical Characteristics of the Transplant Cohort.

[1] Categorical variables were compared using Fisher's exact test and continuous variables were compared using the Wilcoxon Rank-Sum test.

Characteristics	Dose Escalation Group (N=5)	Dose Stable Group (N=14)	P value[1]
Age (Mean±SD)	43.4±19.0	58.8±7.8	0.16
Weight (Mean±SD)	81.2±13.6	74.1±14.1	0.23
Male Gender	4 (80.0%)	4 (28.6%)	0.11
African American Race	0 (0.0%)	3 (21.4%)	0.53
Deceased Donor Transplantation	2 (40.0%)	6 (42.9%)	0.99
Induction Therapy			
Anti-thymocyte globulin	5 (100.0%)	11 (78.6%)	0.53
Basiliximab	0 (0.0%)	3 (21.4%)	0.53
Maintenance Therapy			
Tacrolimus and Mycophenolate	5 (100.0%)	14 (100.0%)	0.99
Steroid Maintenance Protocol	1 (20.0%)	6 (42.9%)	0.60
Prophylaxis Therapy			
Preoperative Antibiotic Prophylaxis			
Cefazolin	5 (100.0%)	11 (78.6%)	0.53
Vancomycin	0 (0.0%)	3 (21.4%)	0.53
Clotrimazole	5 (100.0%)	14 (100.0%)	0.99
CMV Prophylaxis			
Valgancyclovir	4 (80.0%)	13 (92.9%)	0.47
Acyclovir	1 (20.0%)	1 (7.1%)	0.47
PCP Prophylaxis			

Trimethoprim/Sulfamethoxazole	5 (100.0%)	11 (78.6%)	0.53
Dapsone or Atovaquone	0 (0.0%)	3 (21.4%)	0.53

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Tacrolimus dosing increased in the Dose Escalation Group when compared to the Dose Stable Group during the first month of transplantation, which is in response to lower tacrolimus troughs in the Dose Escalation Group than in the Dose Stable Group. Figure 5 shows the tacrolimus trough levels and tacrolimus dosing in intervals of 7 days during the first month of transplantation, stratified by the Dose Escalation Group and in the Dose Stable Group. The Dose Escalation Group had initially lower tacrolimus trough levels than the Dose Stable Group (4.4±2.6 ng/mL vs. 9.7±3.2 ng/mL, respectively) ($P<0.001$ at day 7, two-way between-group ANOVA using contrasts) but by post-transplant day 28, the Dose Escalation Group and the Dose Stable Group had similar tacrolimus trough levels) (7.3±2.2 ng/mL and 9.0±2.1 ng/mL, respectively) ($P=0.22$) (Figure 5A). In terms of tacrolimus dosing, the Dose Escalation Group had similar initial tacrolimus dosing to the Dose Stable Group (4.2±1.1 mg/day vs. 3.8±0.8 mg/day, respectively) ($P=0.61$, two-way between-group ANOVA using contrasts), but by post-transplant day 28, the Dose Escalation Group had a higher tacrolimus

dosing than the Dose Stable Group (9.6±2.4 mg/day vs. 3.3±1.5 mg/day, respectively) (P<0.001) (Figure 5B).

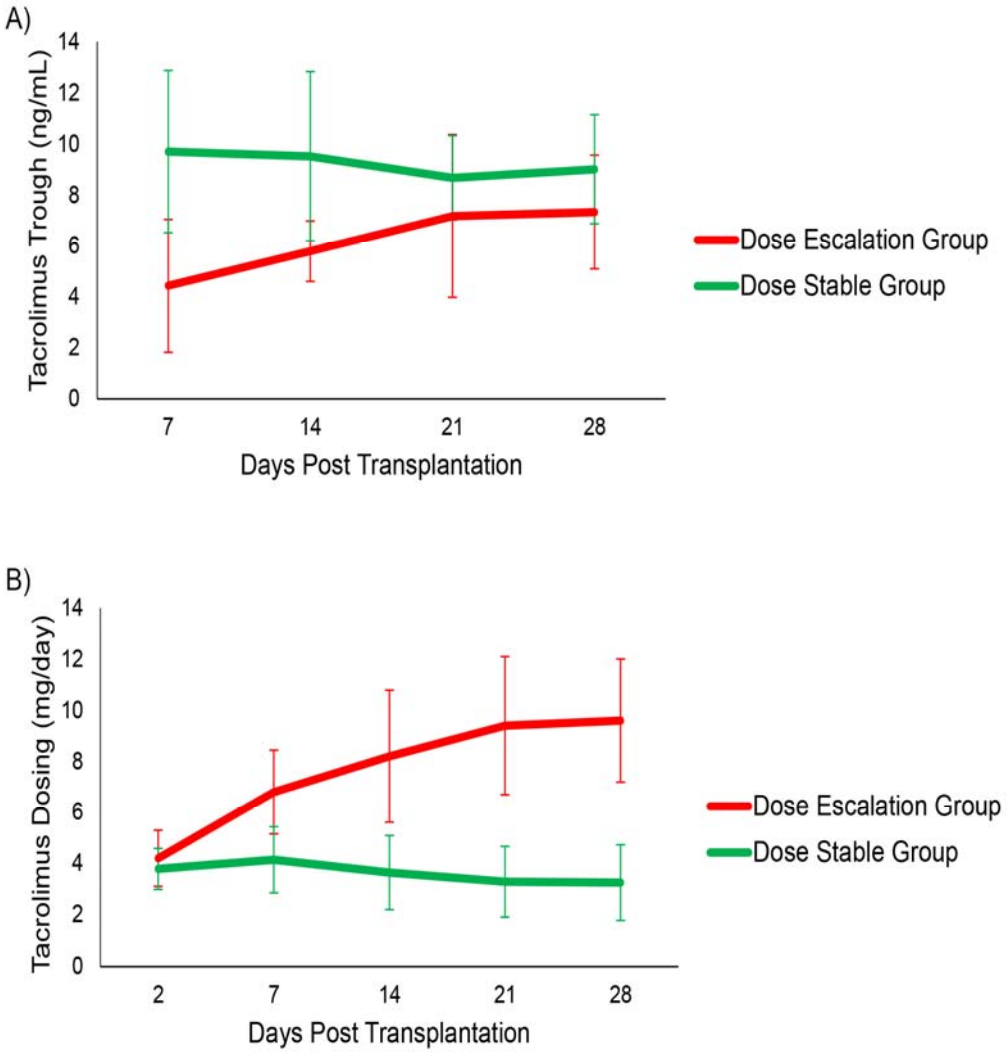

Figure 5. Tacrolimus Trough Levels and Tacrolimus Dosing in the Kidney Transplant Cohort. A) Tacrolimus troughs are shown over the course of the first month. On the x-axis is the day after kidney transplantation and on the y-axis is the mean tacrolimus trough of the group (ng/mL) corresponding to the day on the x-axis. The Dose Escalation Group is represented by the red line with corresponding standard deviation bars and the Dose Stable Group is

represented by the green line with corresponding standard deviation bars. P values at each time point were calculated using a two-way between-group ANOVA using contrasts to evaluate the two groups at each time point and is listed above each time point. B) Tacrolimus dosing is shown over the course of the first month. On the x-axis is the day after kidney transplantation and on the y-axis is the mean tacrolimus dosing of the group (mg/day) corresponding to the day on the x-axis. The Dose Escalation Group is represented by the red line with corresponding standard deviation bars and the Dose Stable Group is represented by the green line with corresponding standard deviation bars. P values at each time point were calculated using a two-way between-group ANOVA using contrasts to evaluate the two groups at each time point and is listed above each time point.

Gut Microbiota Early After Transplantation by Tacrolimus Dosing Groups

We evaluated whether the gut microbiota within the first week after kidney transplantation (N=19 fecal specimens) was associated with tacrolimus dosing groups by diversity assessment and by principal coordinate analysis. The Shannon diversity index in the Dose Escalation Group (N=5) was not significantly that that in the Dose Stable Group (N=14) (3.5±0.8 vs. 3.5±0.5, respectively,

P=0.78, Wilcoxon Rank Sum test). With respect to principal coordinate analysis, the gut microbiota in the Dose Escalation Group and the Dose Stable Group did not appear to have a separation (Figure 6).

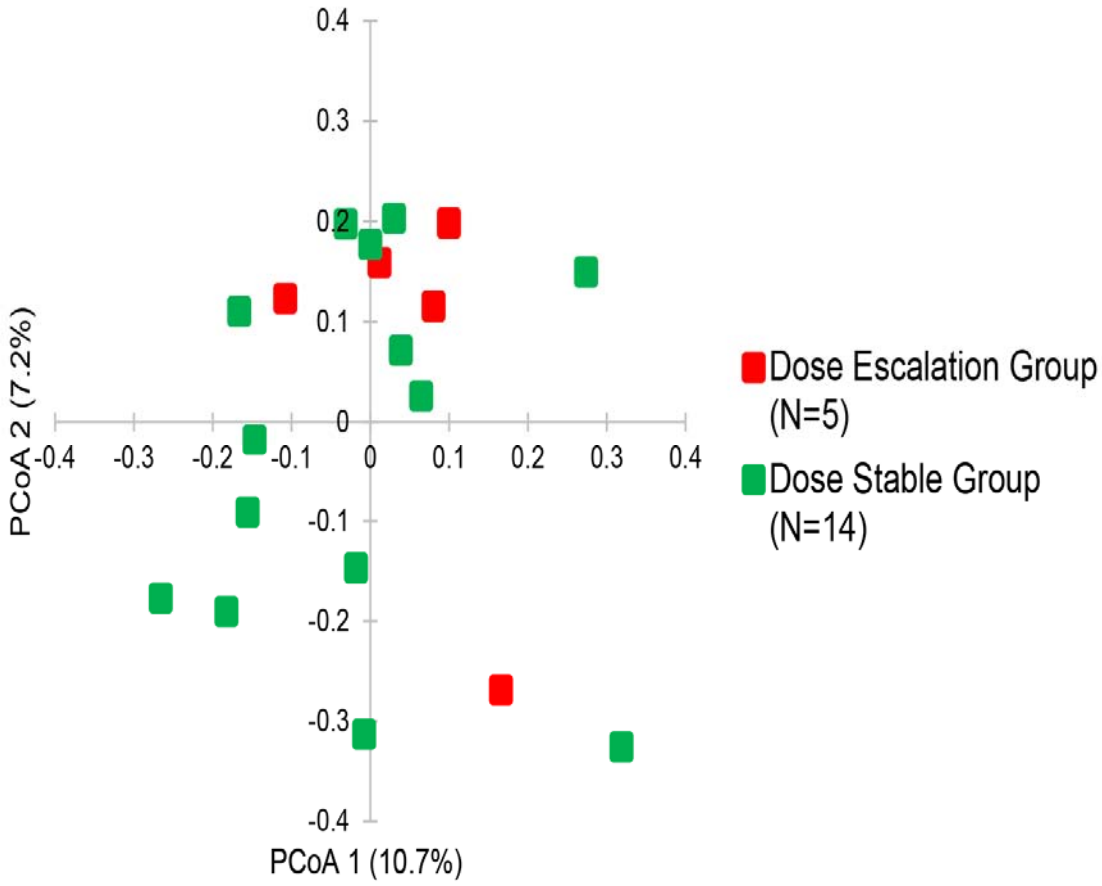

Figure 6. Differences in the Gut Microbiota Between the Tacrolimus Groups. The principal coordinate analyses of the week 1 fecal specimens from the 19 kidney graft recipients are shown. The first two axes of the principal coordinate analysis are represented with principal coordinate axis 1 on the y-axis (10.7% variability) and principal coordinate axis 2 on the x-axis (7.2% variability). The individual red points represent the 5 post-transplantation week 1 fecal specimens from the 5 subjects in the Dose Escalation Group and the individual

green points represent the 14 post-transplantation week 1 fecal specimens from the 14 subjects in the Dose Stable Group.

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In the same week 1 post-transplant fecal specimens (N=19), we evaluated whether there was a significantly different taxa at the phylum, order, genus, and species (evaluation of taxa >2% within each level) between the two tacrolimus groups (Table 6). At the genus level, the Dose Escalation Group had a significantly higher fecal *Faecalibacterium* abundance than the Dose Stable Group (11.8% vs. 0.8%, P=0.002, Wilcoxon rank sum test) at week 1 post-transplant. At the species level, the Dose Escalation group had a significantly higher fecal *Faecalibacterium prausnitzii* abundance than the Dose Stable Group (11.8% vs. 0.8%, P=0.002, Wilcoxon rank sum test) at week 1 post-transplant. The fecal abundance of *Faecalibacterium prausnitzii* in the 19 kidney transplant recipients is represented on Figure 7.

Table 6: Comparison of the Most Common Taxa Between the Tacrolimus Groups

At each of the most common taxa at the phylum, order, genus, and species levels, the mean bacterial abundances in the post-transplantation week 1 fecal specimens from the 5 transplant recipients in the Dose Escalation Group were compared to the mean bacterial abundances in those from the 14 transplant recipients in the Dose Stable Group. Unadjusted p values were calculated using Wilcoxon rank sum tests and the adjusted p values were calculated using the Benjamini-Hochberg correction for multiple hypotheses at each taxonomic level. Fecal *Faecalibacterium prausnitzii* abundance at week 1 post-transplantation was significantly higher in the Dose Escalation Group than in the Dose Stable Group (mean 11.8% vs. 0.8%, respectively, uncorrected P=0.002, Wilcoxon rank sum test, P<0.05 after Benjamini-Hochberg correction for multiple hypotheses).

Phylum	Dose Escalation Group (N=5)	Dose Stable Group (N=14)	P value
Firmicutes	0.866	0.906	0.89
Actinobacteria	0.039	0.036	0.96
Bacteroidetes	0.016	0.021	0.52
Order			
Clostridiales	0.611	0.610	0.99
Erysipelotrichales	0.173	0.165	0.89
Lactobacillales	0.012	0.087	0.71
Bifidobacteriales	0.036	0.032	0.71
Bacteroidales	0.016	0.021	0.52
Genus			
Clostridium	0.103	0.187	0.11
Eubacterium	0.149	0.137	0.82
Blautia	0.065	0.062	0.82

Faecalibacterium	**0.118**	**0.008**	**0.002**
Streptococcus	0.010	0.064	0.85
Ruminococcus 1	0.032	0.042	0.56
Ruminococcus 2	0.147	0.085	0.30
Bifidobacterium	0.036	0.032	0.71
Coprococcus	0.025	0.040	0.61
unclassified *Clostridiales*	0.022	0.034	0.49
Species			
Eubacterium dolichum	0.149	0.121	0.99
unclassified *Clostridium*	0.057	0.090	0.69
Faecalibacterium prausnitzii	**0.118**	**0.008**	**0.002**
Blautia producta	0.016	0.020	0.43
unclassified *Blautia*	0.016	0.032	0.75
Bifidobacterium breve	0.019	0.009	0.34
Streptococcus thermophilus	0.008	0.025	0.89
Ruminococcus bromii	0.103	0.042	0.14
Ruminococcus gnavus	0.011	0.012	0.96
unclassified *Coprococcus*	0.020	0.038	0.46
unclassified *Ruminococcus*	0.043	0.043	0.96
Streptococcus lutetiensis	0.0001	0.030	0.49
unclassified Clostridiales	0.022	0.034	0.49

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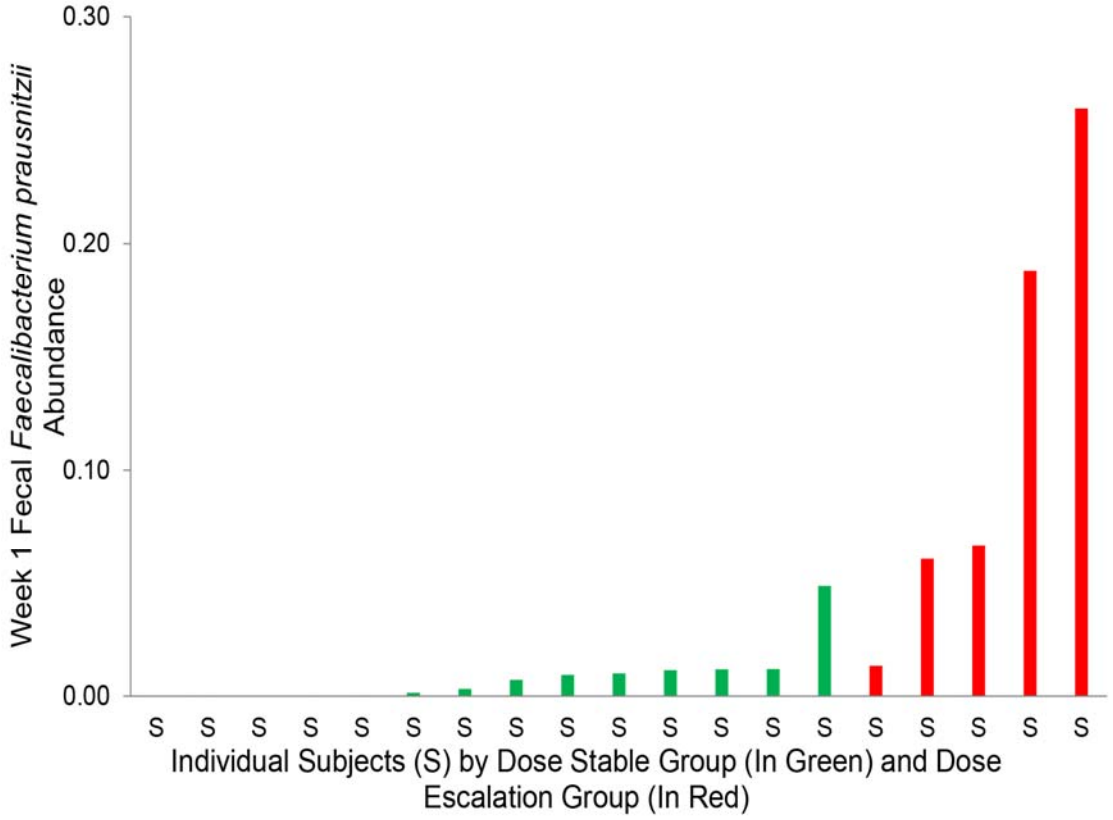

Figure 7. Fecal *Faecalibacterium prausnitzii* Abundance by Tacrolimus Dosing Groups. Each individual subject is represented on the x-axis and the week 1 fecal *Faecalibacterium prausnitzii* abundance is represented on the y-axis. Individual subjects in the Dose Stable Group are represented in green (the 14 subjects on the left) and individuals subjects in the Dose Escalation Group are represented in red (the 5 subjects on the right). The Dose Escalation Group had a significantly higher *Faecalibacterium prausnitzii* proportion than the Dose Stable Group (11.8% vs. 0.8%, P=0.002, Wilcoxon Rank-Sum test).

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14 of the 19 transplant recipients had available fecal specimens for microbial profiling at or after day 14 of transplantation. The fecal abundance of *Faecalibacterium prausnitzii* continued to be elevated in the Dose Escalation Group than in the Dose Stable Group (20.0% vs. 5.5%, respectively, P=0.07, Wilcoxon Rank Sum test) but this was only a trend towards significance.

Faecalibacterium prausnitzii's Correlation with Tacrolimus Dosing

We evaluated whether there was a linear relationship between the post-transplant week 1 fecal *Faecalibacterium prausntizii* abundance and tacrolimus dosing at 1 month. In this univariate linear regression with log-transformed *Faecalibacterium prausnitzii* abundance at 1 week post-transplantation, we found a significant coefficient±SE (1.7±0.6, P=0.01). We also evaluated whether several other transplant characteristics were associated with tacrolimus dosing. We also found that Age and Week 1 hemoglobin concentrations were associated with 1 month tacrolimus dosing (Table 7) (Age: Coefficent±SE -0.14±0.05, P=0.02; Hemoglobin: Coefficent±SE -1.4±0.6, P=0.03). The correlations of the log-transformed week 1 fecal *Faecalibacterium prausnitzii* abundance and 1 month tacrolimus dosing, of Age and 1 month tacrolimus dosing, and week 1 hemoglobin concentration and 1 month tacrolimus dosing are represented in Figure 8.

Table 7: Characteristics Associated with Tacrolimus Dosing at 1 Month

Linear regressions were performed for each of the listed characteristics and the coefficient, standard error (SE), and P values are listed. Characteristics that were associated with tacrolimus dosing at 1 month (P value < 0.10) were computed in a multivariable linear regression and the coefficient, SE, and P values are listed for each of these characteristics.

	Univariate Analysis			Multivariable Analysis		
Characteristic	Coefficient	SE	P-value	Coefficient	SE	P-value
Age (years) (continuous)	**-0.14**	**0.05**	**0.02**	**-0.09**	**0.05**	**0.07**
Transplant Weight (kg) (continuous)	0.05	0.06	0.37			
Male Gender (dichotomous)	2.5	1.5	0.11			
African American (dichotomous)	-1.5	2.1	0.49			
Deceased Donor Transplant (dichotomous)	0.14	1.6	0.93			
Steroid Maintenance (dichotomous)	-2.0	1.6	0.21			
Baseline ALT[1] (IU/L) (continuous)	0.03	0.09	0.77			
Baseline Albumin (mg/dL) (continuous)	0.5	1.5	0.76			
Hgb[2] 1 Week Post Tx[3] (mg/dL) (continuous)	**-1.4**	**0.6**	**0.03**	-0.9	0.5	0.10
Log *F.Prausnitzii* 1 Week Post Tx[3] (continuous)	**1.7**	**0.6**	**0.01**	**1.0**	**0.6**	**0.08**

[1] ALT: Alanine aminotransferase

[2] Hgb: hemoglobin

[3] Post Tx: post-transplantation

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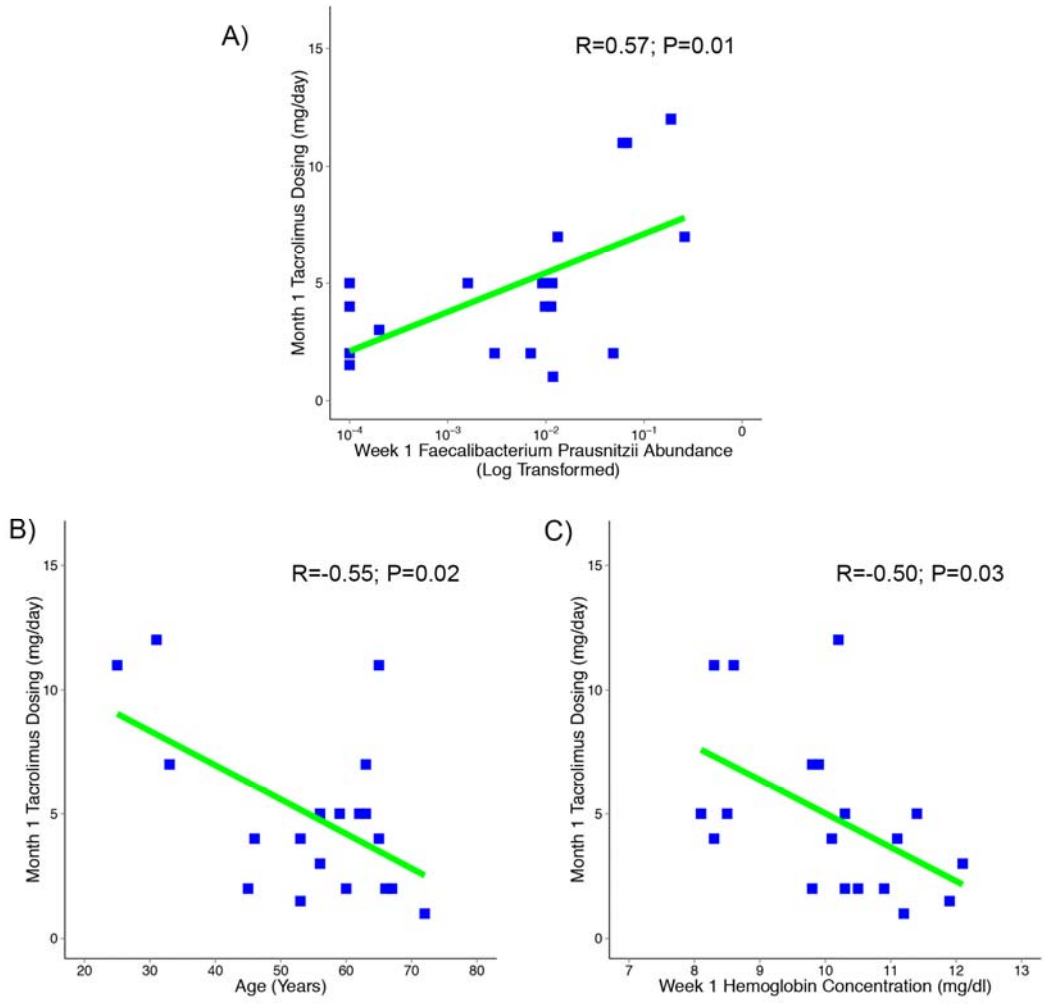

Figure 8. Correlations with Tacrolimus Dosing at 1 Month. A) Fecal *Faecalibacterium prausnitzii* abundance at 1 week post-transplantation is associated with tacrolimus dosing at 1 month. On the x-axis is the log transformed fecal week 1 *Faecalibacterium prausnitzii* abundance and on the y-

axis is the corresponding subject's tacrolimus dosing at 1 month (mg/day). There was a positive correlation between the log-transformed fecal week 1 *Faecalibacterium prausnitzii* abundance and tacrolimus dosing at 1 month (Pearson R=0.57, P=0.01). B) Age at transplantation is negatively associated with tacrolimus dosing at 1 month. On the x-axis is the age at transplantation and on the y-axis is the corresponding subject's tacrolimus dosing at 1 month (mg/day). There was a negative correlation between age and tacrolimus dosing at 1 month (Pearson R=-0.55, P=0.02). C) Post-transplantation week 1 hemoglobin concentration is negatively associated with tacrolimus dosing at 1 month. On the x-axis is the week 1 hemoglobin concentration and on the y-axis is the corresponding subject's tacrolimus dosing at 1 month (mg/day). There was a negative correlation between week 1 hemoglobin concentration and tacrolimus dosing at 1 month (Pearson R=-0.50, P=0.03).

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With factors associated with a P <0.10 in univariate linear regression, we performed a multivariable linear regression for 1 month tacrolimus dosing as the dependent variable. There continued to be a trend towards significance that the fecal *Faecalibacterium prausnitzii* abundance at week 1 post-transplantation was significantly correlated with future 1 month tacrolimus dosing (Coefficient±SE, 1.0±0.6, P=0.08) (Table 7).

CHAPTER 5

Discussion and Implications[5]

In this pilot study, we describe one of the first characterization of the changes in the gut microbiota after kidney transplantation and the relationship between the gut microbiota and the following post-transplant complications: diarrhea, *Enterococcus* urinary tract infections, and acute rejection. We also describe, for the first time, an association between the gut microbiota and tacrolimus dosing requirements in kidney transplantation.

Little is known about the changes in the gut microbiota after kidney transplantation. Kidney transplantation involves high dose immunosuppressive therapies and preoperative and prophylactic antibiotics and such therapies have unknown effects on the gut microbiota. In a cynomolgus monkey study, the effects of alemtuzumab were examined on the gut microbiota and the study revealed profound changes in the Lactobacillales, Enterobacteriales, and Clostridiales [35]. In a study of the rectal microbiota after kidney transplantation, Fricke et al. evaluated 60 kidney transplant recipients and described significant changes in various genera from the Firmicutes phylum from pre-transplantation to one month post-transplantation [21]. In the current study, we report a significant increase in the fecal Proteobacteria two weeks post kidney

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transplantation. Proteobacteria encompasses a group of gram negative bacteria that are known to cause pathogenic infections [36]. A recent study by Taur et al. revealed that Proteobacteria domination in the gut (as defined by 30% relative abundance) was associated with a 5 fold increase risk of Proteobacteria bacteremia [8]. While this study did not assess a similar type of risk, an increase in fecal Proteobacteria abundance may provide a potential explanation for the increased rate of infectious bacterial complications in kidney transplant recipients.

We also describe an association between the gut microbiota and post-transplant diarrhea in this series of kidney transplant recipients. Diarrhea is a common post-transplant complication with an incidence of approximately 20% [37] although the incidence is probably larger as the Bunnapradist et al. study was retrospective. Common strategies to treat the diarrhea include changes in the immunosuppressive regimen, particularly decreases in mycophenolate mofetil. Studies, however, have shown that reduction in mycophenolate mofetil has been associated with acute rejection and graft loss [38, 39]. In this study, we report that a lower abundance of *Bacteroides, Ruminococcus, Coprococcus,* and *Dorea* is associated with post-transplant diarrhea. *Bacteroides* and *Ruminococcus* are common commensal bacteria found in the gut and are thought to have a role in degradation of nondigestible carbohydrates and host carbohydrates [40, 41]. It is plausible that a lack of such bacteria or a decrease in such bacteria is associated with a gut dysbiosis that leads to diarrhea. While this pilot study did not provide a

mechanism, it nevertheless suggests potential gut microbiota-related treatment strategies as alternatives to reduction in immunosuppressive therapies like mycophenolate mofetil.

Recent studies have suggested that the gut microbiota may play a role in infectious bacterial complications beyond *Clostridium difficile.* In a study by Ubeda et al., *Enterococccus* abundance in the stool preceded development of *Enterococcus* bacteremia in 2 allogeneic bone marrow transplant recipients [7]. A follow up study of 94 allogeneic bone marrow transplant recipients reported that *Enterococcus* domination in the stool (> 30% relative abundance) was associated with a 9 fold increased risk for *Enterococcus* bacteremia [8]. In the current study, we report that *Enterococcus* abundance in the stool was elevated in the subset of kidney transplant recipients that developed *Enterococcus* UTI. While our study needs further validation, it supports the association of the gut microbiota and bacterial infectious complications and thus provides the basis for newer therapies (e.g. probiotics or fecal transplantation) to decrease the risk and/or treat complicated infectious complications.

In this study, we also report a preliminary gut microbial signature associated with acute rejection in kidney transplantation. We have found that the gut microbiota associated with acute rejection is characterized by a lower abundance of Bacteroidetes, Clostridiales, and Bacteroidales and a higher abundance of Lactobacillales. Several other studies have also found a gut microbial signature

with rejection processes in other organ transplantations. In a study of 19 small bowel transplant recipients, an increase in Proteobacteria and a decrease in Firmcutes was associated with acute rejection [11]. In a study of 16 allogeneic bone marrow transplant recipients, microbial chaos in fecal specimens was identified as a risk factor for development of graft-versus-host disease [12]. Taken together with the small bowel transplant and allogeneic bone marrow transplant studies, our preliminary results suggest the possibility that the gut microbiota, which has been attributed to have a role in shaping the immune system, may also play a role in shaping transplant immunity.

In addition to the gut microbiota's association with post-transplant complications, we also report a novel association between the gut microbiota and tacrolimus dosing requirements in kidney transplantation. Prior studies have examined clinical and genetic factors such as race and CYP3A5 polymorphisms as associated with tacrolimus dosing requirements [42, 43]. This is, to the best of our knowledge, one of the first studies to describe fecal *Faecalibacterium prausnitzii* abundance as associated with tacrolimus dosing requirements. We report the fecal abundance of *Faecalibacterium prausnitzii* at week 1 post-transplantation is associated with future 1 month tacrolimus dosing requirements. *Faecalibacterium prausnitzii* is a gram positive bacterium that has been reported to produce high amounts of butyrate [44, 45]. Butyrate is implicated in the maintenance of colonic mucosal health [46] and as such, disease processes like inflammatory bowel diseases have been reported to have a decrease in fecal

Faecalibacterium prausnitzii abundance [47]. The mechanism by which *Faecalibacterium prausnitzii* affects tacrolimus metabolism is unknown but may be postulated to be affecting the health of the intestinal microbiota and thus the functions of CYP3A4 and P glycoprotein. While this pilot study needs further validation, it does support a role for the gut microbiota in tacrolimus metabolism and may allow us to better understand the variability in tacrolimus trough levels in the setting of gastrointestinal disturbances like diarrhea.

Several limitations exist in the current study that are important to note. The sample size of the pilot study is small and most of the reported statistical associations are based on univariate analyses. The findings associated with acute rejection and *Enterococcus* UTI may be confounded by differences in antibiotic use. In terms of the association with tacrolimus dosing, we did not evaluate CYP3A5 polymorphisms, which is an important known contributor for tacrolimus metabolism. We also did not evaluate diet and this may have impacted gut microbial composition.

Despite these limitations, our pilot study reports gut microbial signatures associated with the post-transplant complications of diarrhea, *Enterococcus* UTI, and acute rejection and with tacrolimus dosing requirements. Our study lays the foundation for potential future studies utilizing gut microbiota modulation strategies like probiotics to prevent and/or treat post-transplant complications in kidney transplantation.

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